Estefânia Curralo Cruz Pereira Teixeira

O Papel do Transportador da Glutamina ASCT2 na Terapia Antineoplásica

The Role of The Glutamine Transporter ASCT2 in Antineoplastic Therapy

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Estefânia Gusmão Cruz Pereira Teixeira

NOME

ESTEFÂNIA CURRALO CRUZ PEREIRA TEIXEIRA

NÚMERO DE ESTUDANTE

201403369

E-MAIL

estefmacunha@gmail.com

DESIGNAÇÃO DA ÁREA DO PROJECTO

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THE ROLE OF THE GLUTAMINE TRANSPORTER ANT2 IN ANTINEOPLASTIC THERAPY

ORIENTADOR

MARIA DE Fátima MOREIRA MARTEL

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À minha família,
À minha orientadora,
Aos meus amigos

Title: The role of the glutamine transporter ASCT2 in antineoplastic therapy

(O papel do transportador da glutamina ASCT2 na terapia antineoplásica)

(Review article)

Authors: Estefânia Teixeira¹ and Fátima Martel^{2,*}

¹ Faculty of Medicine, University of Porto, Porto, Portugal.

² Department of Biomedicine – Unit of Biochemistry, Faculty of Medicine, University of Porto, Porto, Portugal and Instituto de Investigação e Inovação em Saúde (i3S), University of Porto, Porto, Portugal.

***Corresponding author.** Department of Biomedicine – Unit of Biochemistry, Faculty of Medicine, University of Porto, Al Prof. Hernâni Monteiro, 4200-319 Porto, Portugal.
Phone: 351 22 0426654; email-address: fmartel@med.up.pt

List of abbreviations:

AKG, α -ketoglutarate;

ASCT2, Alanine, Serine, Cysteine Transporter 2;

CCR, Colorectal carcinoma;

ccRCC, Clear cell renal cell carcinoma;

Gln, glutamine;

GS, glutamine synthetase;

GLUD, glutamate dehydrogenase;

GLS, glutaminase iso-enzyme;

GLS2, glutaminase 2 isoenzyme;

GSH, glutathione;

HCC, Hepatocellular carcinoma;

HNSCC, Head and Neck squamous cell carcinoma;

LAT-1, large amino acid transporter;

mTOR, mammalian target of rapamycin;

NSCLC, Non-small cell lung cancer;

ROS, reactive oxygen species;

SNAT, sodium-coupled neutral amino acid transporter;

SLC1A5, solute-linked carrier family A1 member 5

Abstract

Introduction: Cancer cells are metabolically reprogrammed, to support their high proliferative ratio. Cancer cell glucose metabolism plays a very important role in tumor prognosis but, currently, the role of glutamine metabolism in cancer cells has been receiving more attention. In order to supply their high glutamine needs, cancer cells developed an increased expression of membrane transporters that mediate the cellular uptake of this amino acid. ASCT2 (Alanine, Serine, Cysteine Transporter 2) is a Na⁺-dependent transmembrane transporter overexpressed in cancer cells and considered to be the primary transporter for glutamine in these cells.

Areas covered: The possibility of inhibiting ASCT2 for antineoplastic therapy is currently under investigation, and inhibition of ASCT2 appears to prevent tumor proliferation by interfering with glutamine entry into the cell. In this article, we will present the pharmacological tools currently known to act on ASCT2, which have been attracting a lot of attention in antineoplastic therapy research. We will also address the impact of ASCT2 inhibition on the prognosis of some cancers.

Expert opinion: ASCT2 inhibition seems to be a promising antineoplastic strategy. In addition, combining other anti-tumor therapies with ASCT2 inhibitors appears to be a very effective strategy to treat several cancers. However, more research is needed in this area.

Key-words: Metabolic Reprogramming, Glutamine uptake; ASCT2; Antineoplastic therapy

1. Introduction

Cancer is presently a highly prevalent pathology and, despite improvements in diagnostic and medical treatment, including the discovery of new therapies, the mortality associated with this condition is still very high. Therefore, it is absolutely necessary to find new antitumor drugs with new mechanisms of action, in order to improve cancer prognosis.

Cancer cells are metabolically reprogrammed, which is one of cancer hallmarks, in order to support their high proliferative ratio [1, 2]. This is essential to provide the energy needs and biosynthetic precursors and for maintenance of redox homeostasis of tumor cells [1]. Indeed, metabolic reprogramming is a mechanism of adaptation of cancer cells, allowing them to develop and evolve, competing with other cells for glucose and other nutrients [1, 2]. Of importance, metabolic reprogramming is associated with a poor prognosis [1, 2]. Thus, interference with metabolic reprogramming mechanisms of cancer cells constitutes a new hotspot for the development of new antineoplastic therapies.

It is known that glucose metabolism plays a very important role in tumor prognosis but, currently, the role of glutamine metabolism in cancer cells has been receiving more attention and is being investigated [3]. Glutamine is a non-essential amino acid with a very important role in cancer: it is not only a source for energy production and nucleotide/protein biosynthesis, but possesses also other effects in cancer cells (see below) that established this nutrient as playing an important role in cancer cells, contributing to malignancy [4-6]. Thus, knowledge/modulation of glutamine metabolism can be useful, both in the diagnosis, monitoring and cancer therapy [5].

In order to supply their high glutamine needs, cancer cells develop mechanisms that allow them to take up high amounts of this amino acid, namely by increasing the expression of membrane transporters that mediate the cellular uptake of glutamine [7]. ASCT2 (Alanine, Serine, Cysteine Transporter 2; encoded by SLC1A5 gene), a Na⁺-dependent transmembrane transporter involved in the cellular uptake of neutral amino acids such as glutamine, is a primary transporter of glutamine in cancer cells [3]. The expression of ASCT2 in cancer cells is greater than in non-malignant cells, which suggests that ASCT2 has a role in tumorigenesis and in the prognosis of malignant neoplasia [3, 8-13]. In agreement with this important role of ASCT2 in glutamine uptake by cancer cells, silencing ASCT2 has been shown to interfere with cancer cells proliferation and survival, thus improving prognosis [3]. Furthermore, silencing ASCT2 appears to improve response to other antineoplastic therapies [3]. Therefore, ASCT2 is currently attracting a lot of attention in oncology research, and the possibility of using it as a therapeutic target has been investigated [3, 8-13].

In this article, we will describe the pharmacological tools developed to act on ASCT2, which has been attracting a lot of attention in antineoplastic investigation. We will also address the impact of silencing/inhibition of this glutamine transporter on the prognosis of some cancers.

2. Glutamine metabolism and its functions in cancer cells

Glutamine is a very important amino acid in our body, being the main amino acid flowing in the bloodstream [14], and being transported across the plasma membrane by different transporters [15]. This nutrient has been receiving a growing attention in cancer research, due to its role in cancer cells and on cancer initiation and

progression. Indeed, cancer cells have a high glutamine demand (they use 10-100 times more glutamine than any other amino acid) and become glutamine “addicted”, as glutamine withdrawal can cause cell death [16]. This amino acid performs numerous important functions in cancer cells. It is not only an important energy source (because of the Warburg effect, there is less oxidation of pyruvate in the mitochondria, by increasing the conversion of glucose to lactate, and malignant cells use other energy sources, such as glutamine [2, 11, 17, 18]) but it is also involved in redox homeostasis, cell signaling, apoptosis inhibition and in autophagy [4-6]. In addition, glutamine is a source of carbon and nitrogen, allowing molecules such as amino acids, proteins, nucleotides and fatty acids, to be synthesized [19], as next described.

Glutaminolysis, which is increased in cancer cells, involves a set of reactions beginning with glutamine (Figure 1) [20]. Glutamine enters into the cell and, within the mitochondria, it is hydrolyzed, being converted into ammonia and glutamate, by glutaminases (GLS/GLS2) [19]. Silencing or inhibition of GLS (whose expression is increased in cancer cells) improves the prognosis of some cancers [5], including breast cancer and B lymphoma (Wang et al described an inhibitor molecule targeting glutaminase, which inhibits the progression of these cancers) [21] and glioblastoma (according to Cheng et al, glutaminase silencing inhibits glioblastoma growth) [22]. Despite targeting of GLS has been explored [2], inhibition of these enzymes appears to be less effective than inhibition of glutamine uptake, because it does not completely inhibit the function of glutamine outside the mitochondria [3].

In turn, glutamate may be converted to glutathione (an antioxidant neutralizing reactive oxygen species, ROS). From glutamate, it is possible to produce serine, which, in turn, allows glycine and cysteine to be synthesized; the combination of glutamate,

glycine and cysteine allows the synthesis of glutathione [23, 24], which is catalyzed by glutamate cysteine ligase [19].

Glutamate can also be converted to α -ketoglutarate, an intermediate of the TCA cycle [19, 20, 25], that can be converted into malate and citrate [26, 27]. Glutamate conversion to α -ketoglutarate is catalyzed by either aminotransferases (without ammonia production) or glutamate dehydrogenase (producing ammonia) [19]. Malate can be expelled from the mitochondria, being converted to pyruvate and lactate (NADPH is produced during these reactions) [26, 27]. Citrate, in turn, can also be expelled from the mitochondria and it permits both lipid and α -ketoglutarate (in association with NADPH production) synthesis [26, 27].

Glutamine metabolism is important for redox balance in cancer cells [19, 20] through glutathione and NADPH synthesis [28]. Consequently, inhibition of glutamine metabolism is related with higher ROS levels, increasing cancer cells apoptosis [3].

Cancer cells biosynthesis is enhanced to support their increased proliferation [19, 24, 27], and glutamine is also a source of nitrogen, allowing amino acids (alanine, serine, proline, aspartate, among others [24]) to be synthesized through its conversion to glutamate and by transamination reactions [19, 27]. In addition to amino acids, glutamine also allows nucleotides, proteins and lipids to be synthesized [19, 20, 29]. Consequently, in addition to its role in energy production, glutamine is also involved in biosynthesis, allowing other important molecules to be synthesized, being considered both a carbon source and a nitrogen donor (Figure 1) [19].

All of these functions of glutamine are very important for cancer progress. Consequently, interference with its metabolism may constitute a new therapeutic target in cancer.

3. Glutamine cellular uptake: ASCT2 as a primary transporter of glutamine in cancer cells

Glutamine is a nutrient that has been receiving crescent attention for its role in antineoplastic therapy. As shown in the previous section, in cancer cells glutamine is essential for tumor development and glutaminolysis is increased [7, 15, 30]. This amino acid can be endogenously synthesized by cancer cells (glutamine synthetase catalyzes a reaction between glutamate and ammonia, synthesizing glutamine) [19, 20, 25]. Additionally, glutamine can be taken up from the extracellular space [4]. In the case of cancer cells, with an increased metabolism and proliferation ratio, there is a greater demand for this nutrient [4]. Thus, the inlet of glutamine from the extracellular space becomes essential in tumor cells [4], and there is an overexpression of transporters for this amino acid in cancer cells [7].

Amino acid transporters are grouped in transport systems (e.g., system A, ASC, N and L), depending on the substrates for which they have greater specificity and on their Na⁺-dependence or independence, among other differentiating characteristics [31].

In normal cells, glutamine transporters expression varies depending on the tissue [32-37]. For instance, SNAT1 is a glutamine transporter that is expressed in the neocortex, hippocampus and neuroepithelium and it provides glutamine to neurons, contributing to neuronal glutamate synthesis [37]. On the other hand, SNAT3 is expressed in astrocytes (which produce glutamine), allowing them to release glutamine [36]. Thus, both SNAT1 and SNAT3 are involved in brain glutamine transport and in glutamine-glutamate cycling: SNAT3 allows the release of glutamine by astrocytes and SNAT1 allows neurons to take up glutamine [36, 37]. SNAT3 is also expressed in hepatic and renal tissues, allowing periportal cells and proximal renal tubule cells to

obtain glutamine and liver perivenous cells to release glutamine [32, 34, 35]. Additionally, SNAT2 is widely expressed in our body, playing very important roles [33]. It is associated with glutamine uptake and mTOR pathway signaling (contributing to cell growth and differentiation) [33, 38, 39].

As previously mentioned, although glutamine transporters exist in normal cells [32-37], in some cancers, their expression is increased [7]. ASCT2 (Alanine, Serine, Cysteine Transporter 2) is a Na⁺-dependent transmembrane transporter, transporting glutamine and other neutral amino acids across the plasma membrane [40]. These other neutral amino acids include serine, cysteine, valine, threonine and alanine [11, 40, 41]. ASCT2 is considered an obligatory exchanger of neutral amino acids and it is associated with both glutamine uptake and glutamine efflux [8, 42]. ASCT2 is encoded by SLC1A5 gene (solute-linked carrier family A1 member 5) and it is considered a primary transporter for glutamine uptake in cancer cells [40]. ASCT2 belongs to the SLC1A family (solute carrier 1A) [8]. This family of transporters includes ASCT1 and ASCT2 (neutral amino acid transporters) and the EAAT1-5 transporters (excitatory amino acid transporters, which are associated with glutamate transport) [8].

ASCT2 (which is overexpressed in cancer cells) accounts for the inlet of glutamine into the cell, favoring tumorigenesis and tumor progression with consequent metastasis [43], and is associated with a poor prognosis [3]. Consequently, inhibition of ASCT2 appears to prevent tumor proliferation by interfering with glutamine flow into the cell [43-47] and, so, ASCT2 is a hotspot to develop new antineoplastic strategies. For this reason, we will focus on ASCT2 (SLC1A5), being a possible target for the development of new antineoplastic therapies, due to its notable role in glutamine uptake by cancer cells.

However, other transporters are also involved in glutamine transport by cancer cells. One of these transporters is LAT1 [48]. LAT1 is a transporter responsible for leucine uptake into the cell [3]. LAT1 allows an exchange of glutamine with leucine, allowing leucine to enter into the intracellular space and glutamine, in turn, to pass into the extracellular space (Figure 1) [3, 25, 48]. LAT1 and CD98 constitute a heterodimeric complex, but only LAT1 plays a role in amino acid transport across the plasma membrane, as CD98 does not interfere with the transport function of this complex [48-50]. According to Napolitano et al, glutamine transport by LAT1 is mainly in the intracellular-to-extracellular direction [48]. LAT1-mediated leucine uptake into the intracellular space allows activation of mTOR, being associated with tumor proliferation and, so, indirectly, glutamine allows mTOR activation [3, 6, 51]. Since LAT1 action depends on the intracellular concentration of glutamine (because it acts as an exchanger), there is an association between LAT1 and ASCT2 activity [3]. Both ASCT2 and LAT1 expression are independent prognostic factors in different cancers, and according to El Ansari et al, the combined expression of ASCT2 and LAT1 seems to influence cancer prognostic [52].

SNAT2 is another transporter involved in glutamine uptake by cancer cells [53]. It is overexpressed in some cancers, such as liver and prostate cancers [33, 54]. Finally, ASCT1 is overexpressed in neuroblastoma tumor cells and targeting ASCT1 may be a good strategy, but more research is needed [55]. ASCT1 is upregulated both after ASCT2 silencing and LAT inhibition [8, 56, 57].

Nevertheless, although other glutamine transporters may be therapeutic targets for some cancers [33, 53-55], ASCT2 is crucial for its primary and essential role in glutamine transport in several different cancers and the therapeutic role of ASCT2 inhibition in some cancers has been well documented, as shown below [3, 9-13, 58, 59].

4. ASCT2 regulation in cancer cells

Tumor cells overexpress ASCT2. However, the mechanisms that regulate this increased expression are yet not completely understood, and more research is needed. Nevertheless, the following mechanisms have been described to regulate ASCT2.

4.1. *Glutamine*. Glutamine regulates the expression of ASCT2 [8]. According to Bungard et al, glutamine deprivation declined both ASCT2 expression, as well as tumor growth in an hepatoma (HepG2) cell line [60]. On the other hand, adding glutamine and, consequently, increasing glutamine availability, increased ASCT2 promoter activity and ASCT2 expression, favoring tumor growth [60]. Similarly, Dolinska et al verified that in C6 glioma cells, glutamine deprivation was associated with lower ASCT2-mediated glutamine uptake [61]. Glutamine appears to regulate ASCT2 expression in cancer cells either by interfering with its transcription or at post-transcriptional level [8].

4.2. *C-MYC*. The MYC family of transcription factors is associated, in cancer, with nutrient uptake, metabolism and proliferation. MYC activates the expression of some genes related to the acquisition and metabolism of glutamine, namely ASCT2 [62-64].

4.3. *Rb tumor suppressor*. Retinoblastoma (Rb) tumor suppressor family can also regulate glutamine uptake [65]. The entry of glutamine into the cell can be negatively regulated by Rb tumor suppressor, whose deletion enhances glutamine uptake by ASCT2 and GLS1 upregulation (via an E2F-dependent manner) [65].

4.4. *MicroRNA-137 (miR-137)*. This microRNA acts on SLC1A5 (ASCT2) mRNA, promoting its degradation or inhibiting its translation, and consequently decreasing

ASCT2 levels [66, 67], glutamine uptake and metabolism. Interestingly, MYC is associated with microRNAs downregulation [63].

4.5. RNF5. Another regulator of ASCT2 is RNF5 [68]. Paclitaxel is a stress factor for breast cancer, promoting ubiquitination, RNF5 association and a reduction of ASCT2 levels [68]. Accordingly, RNF5 deletion is associated with greater expression of ASCT2, which is related with a worse prognosis and less response to paclitaxel in breast cancer [68].

4.6. Leptin. A study using *in vitro* human intestinal cells (Caco-2 cell line) evaluated the impact of leptin on the uptake of glucose and aminoacids by these cells [69]. In relation to glutamine, this study demonstrated that, after the addition of leptin, uptake of glutamine rapidly decreased, together with a decrease in membrane ASCT2 protein expression [69]. Thus, leptin appears to inhibit glutamine uptake by regulating ASCT2 expression [69].

4.7. Insulin. A study using rat adipocytes showed that both insulin and cell swelling stimulate ASCT2-mediated glutamine uptake [70]. In addition, insulin not only increased glutamine uptake directly, but also indirectly, through increased cell swelling [70]. The mechanism by which insulin increased ASCT2-mediated glutamine transport involves activation of the ERK/MAPK cascade pathway, suggesting that activation of the ERK pathway contributes to the regulation of ASCT2 by insulin [70].

5. ASCT2 silencing/inhibition strategies

Currently, glutamine metabolism is seen as a target for cancer therapies development [3]. Glutamine has an essential role, both in the development and

progression of the tumor [1, 2]. This amino acid is particularly important for cancer cells, compared to normal cells [4]. It is associated with metabolic reprogramming, allowing cells to adapt to their demands [3]. By interfering with glutamine metabolism, it is possible to inhibit one of the mechanisms of tumor adaptation. Therefore, inhibiting glutamine metabolism seems to be a good therapeutic strategy [3, 9].

Several strategies to inhibit glutamine metabolism have been investigated. One of the strategies consists in inhibiting transmembrane transporters involved in glutamine cellular uptake. In this context, ASCT2 is considered a primary glutamine transporter and ASCT2 inhibitors have been developed [8], using distinct strategies: neutral amino acid analogues, which act as competitive inhibitors [8]; monoclonal antibodies targeting ASCT2 [9, 12, 13]; non-competitive ASCT2 inhibitors [71]; and strategies causing ASCT2 downregulation [10, 59]. However, only a few ASCT2 inhibitors exist presently, and these are:

5.1. Benzyl-serine/benzyl-cysteine derivatives: they are competitive inhibitors of ASCT2 [72]. They bind to ASCT2, competing for the binding site with neutral amino acids that are transported by it [72]. Consequently, these drugs prevent the transport of neutral amino acids through ASCT2 [72]. However, benzyl-serine reduce, significantly, both glutamine and leucine uptake in melanoma cells [46] and breast cancer cells (MDA-MB-231, MCF-7 and HCC1806 cells) [73]. Therefore, benzyl-serine derivatives are not specific ASCT2 inhibitors, as they block also other amino acid transporters, such as LAT1 [73].

5.2. Phenyl-glycine analogs: These compounds are not ASCT2-specific inhibitors, inhibiting also other amino acid transporters such as ASCT1 [74]. They were used in neurological studies for targeting both ASCT1 and ASCT2 [74].

5.3. L- γ -glutamyl-p-nitroanilide (GPNA): It is an analogue of glutamine [8, 75]. GPNA inhibits several transporters related to glutamine metabolism, such as ASCT2, LAT1, SNAT1 and SNAT2 [75, 76]. So, it is not specific for ASCT2 [3].

5.4. Monoclonal antibodies (mAb): Monoclonal antibodies are being created in order to recognize ASCT2, interfering with its function or promoting its internalization [9, 13]. Hara et al., developed a monoclonal antibody that recognizes ASCT2, trying to understand if resorting to this therapeutic strategy would improve KRAS-mutated cancers prognosis [9]. They verified that it would be a good strategy for KRAS-mutated cancers treatment, improving its prognosis and being promisor because cancers with this mutation have few effective pharmacological treatments [9]. Ab 3-8 mAb binds to ASCT2, promoting its internalization in SW1116 and HCT116 colon cancer cell lines harboring KRAS mutation [9]. Consequently, Ab 3-8 mAb reduces glutamine uptake, which results in an anti-tumor effect, by promoting ASCT2 internalization [9]. This monoclonal antibody was also found to bind ASCT2 in colon cancer cells without a KRAS mutation (HT29 cells) [9]. However, contrary to what happens in mutated cancer cells, Ab3-8 mAb does not cause ASCT2 internalization in cells not harboring KRAS mutation (HT29 cells) [9]. The reason for non-internalization of ASCT2 in these non-mutated cancer cells is unknown [9]. Additionally, Suzuki et al also developed mAbs recognizing ASCT2 [13]. They isolated KM4008, KM4012 and KM4018 mAb, and found that they bind to cells expressing ASCT2, mainly recognizing EL2 (an extracellular domain of ASCT2) [13]. KM8094 mAb was found to suppress gastric cancer progression [12]. KM8094 appears to decrease tumor growth by inhibiting glutamine uptake, increasing oxidative stress and inducing antibody-dependent cellular cytotoxicity in gastric tumor cells [12]. So, monoclonal antibodies

recognizing glutamine transporters appear to have a therapeutic role in cancer, slowing tumor growth and proliferation [9, 13]. Monoclonal antibodies have some advantages, being selective and causing other effects such as antibody-dependent cellular cytotoxicity [12, 13].

5.5. microRNA-137 (miR-137): this compound suppresses tumor development by interfering with ASCT2 (SLC1A5) [66]. According to Dong, Xiao et al., decreasing miR-137 transcription increases ASCT2 expression and, consequently, glutamine uptake and its metabolism [66]. Thus, there is an inverse association between miR-137 and ASCT2 [66].

5.6. 1, 2, 3-dithiazoles: According to a study carried out on proteoliposomes, these compounds appear to inhibit ASCT2 [71]. It is a non-competitive inhibition with a covalent interaction between these compounds and cysteine thiol groups [71]. This non-competitive inhibition is advantageous because it is associated with a longer duration of the inhibition [71].

5.7. Others: In addition to these strategies in inhibiting/silencing ASCT2, other drugs already known and with other known functions, also seem to interfere with ASCT2-mediate glutamine transport. These include (a) topotecan, an inhibitor of DNA topoisomerase I (Topo I) which prevents both the replication of DNA and RNA synthesis, causing the death of malignant cells [59]. It is used to treat non-small cell lung cancer and ovarian cancer as first-line therapy [59]. Despite its anti-tumor effect acting as a Topo I inhibitor, according to a study that evaluated the impact of this compound on gastric cancer cells metabolism, topotecan also downregulates ASCT2, reducing the entry of glutamine into the cell, decreasing proliferation and increasing oxidative stress and apoptosis in gastric cancer cells [59]; (b) resveratrol, which downregulates ASCT2 in hepatocellular carcinoma cells

[10]; and (c) δ -tocotrienol, which inhibits not only ASCT2, but also LAT1 and the mTOR pathway in non-small cell lung cancer cells [58].

5.8. V-9302: This compound was initially presented as an ASCT2 inhibitor, decreasing glutamine uptake with a higher potency than GPNA [45]. Currently, it is known that this molecule does not act by inhibiting ASCT2, but rather SNAT2, another glutamine transporter, and LAT1 [77]. Combining V-0302 with an ASCT2 inhibitor more drastically decreases glutamine uptake in some cancers, such as head and neck squamous cell carcinoma [3]. Nevertheless, caution is needed when combining an ASCT2 inhibitor with V-9302 because non-malignant cells also use glutamine, triggering adverse effects [3].

So, it is necessary to develop new strategies to inhibit ASCT2, since most of the existing inhibitory drugs are not sufficiently potent or selective [8].

6. Impact of ASCT2 silencing/ inhibition in different cancers

As next described, ASCT2 is associated with the development and progression of several cancers, namely gastric cancer, colorectal carcinoma, breast cancer, prostate cancer, non-small cell lung cancer, head and neck squamous cell carcinoma and liver cancer [3, 41, 78-82] (Tables 1 and 2). It has been reported that, by inhibiting this transporter in cancer cells, it is possible to improve outcomes. So, this can be a therapeutic strategy, mainly if associated with other therapies, by improving the response to them [3].

6.1. Head and Neck squamous cell carcinoma (HNSCC)

Studies carried out have demonstrated an association between glutamine and the development and progression of HNSCC [3, 83, 84]. According to Zhang et al, ASCT2 silencing reduced intracellular glutamine levels and significantly decreased the survival and growth of HNSCC tumor cells (SCC15 and FaDu cell lines), both *in vitro* and *in vivo* (human xenograft that was transfected to mice) [3] (Tables 1 and 2). Moreover, tumor cells in which ASCT2 was silenced responded more effectively to cetuximab [3]. Cetuximab (an anti EGFR monoclonal antibody approved for HNSCC treatment) acts on EGFR, which is expressed at high levels in HNSCC [3, 85, 86]. Interestingly, ASCT2 forms a complex with EGFR and AP1G1 [3, 85, 86]. Thus, cetuximab, by acting on EGFR (causing EGFR endocytosis), also targets ASCT2, decreasing glutamine transport into the cell [3, 85, 86].

Zhang et al. used three different strategies to inhibit ASCT2: ASCT2 silencing with shRNA, miR-137 and shRNA combined with V-9302 [3]. All strategies decreased, significantly, glutamine uptake [3]. However, combining ASCT2 silencing with V-9302 (a SNAT2 inhibitor), further reduced the entry of glutamine into the cell [3] (Table 1). It was suggested that ASCT2 is not the unique transporter accounting for glutamine uptake in HNSCC and that SNAT2 (which is inhibited by V-9302) is also involved in this process and, probably, it is induced when ASCT2 is silenced in order to compensate the entry of glutamine into the cancer cell [3, 15, 53].

Thus, based on the actual knowledge, glutamine cellular uptake and its metabolism appear to be essential for the development and progression of HNSCC [3]. Moreover, inhibiting ASCT2 appears to significantly improve the prognosis, being an effective strategy for HNSCC treatment [3]. Combined therapy with ASCT2-inhibitors and cetuximab appears to be a promising mechanism, improving prognosis [3].

6.2. Colorectal carcinoma (CCR)

Suzuki et al developed monoclonal antibodies recognizing ASCT2 (KM4008, KM4012 and KM4018 mAbs) [13]. They found that these antibodies inhibited the proliferation of colorectal cancer cells (WiDr cells), *in vitro*, by blocking glutamine uptake [13] (Table 1). Therefore, ASCT2 inhibition appears to have an anti-tumor effect on colorectal carcinoma [13].

CCR is associated with several mutations, including KRAS-mutation [9]. KRAS mutation occurs in several cancers (CCR, non-small-cell lung cancer and pancreatic ductal adenocarcinoma) [87-90]. KRAS (codified by an oncogene and, when mutated, it contributes to cancer development) is a GTPase which interferes with MAPK signaling pathway, contributing to cancer development, by propagating the signal from the extracellular environment to the nucleus, increasing the expression of proteins involved in tumorigenesis and cell proliferation [91]. It is necessary to create new treatments for tumors with this mutation, since there are a limited number of effective drugs [9]. A very recent work investigated whether ASCT2 inhibition could interfere with the outcome of KRAS-mutated tumors [9]. It was verified that, in CCR cells with KRAS-mutation, Ab3-8 mAb (a monoclonal antibody that targets human ASCT2) reduced the entry of glutamine into the cells and tumor development [9]. On the other hand, in CCR cells without KRAS mutation, Ab3-8 mAb was not effective in decreasing tumor development [9] (Table 1). Consequently, interfering with the entry of glutamine into the intracellular space seems to be a good strategy to treat KRAS-mutated CCR [9].

6.3. Leukaemia

The role of ASCT2 in hematopoiesis and in the development of leukemia is being investigated [11]. According to Ni et al., silencing of ASCT2 has a slight impact

on hematopoiesis, in the absence of a stress factor, but, in contrast, it decreases the development and progression of leukemia [11]. According to these authors, the role of ASCT2 in tumorigenesis is not entirely justified for its association with glutamine transport, and other amino acids that are transported by ASCT2 are also important [11]. Moreover, although ASCT2 inhibition had a different impact on malignant and non-malignant cells, mainly interfering with the malignant ones, it was concluded that caution is necessary, when combining ASCT2 inhibition with chemotherapy that damages DNA [11]. This is because in the presence of a stress factor (such as fluorouracil exposition), both in *in vitro* and *in vivo* models, glutamine starts to play an important role in hematopoiesis, even in non-malignant cells, and, consequently, combining these two therapies can be associated with adverse consequences [11] (Tables 1 and 2).

6.4. Gastric cancer

A new monoclonal antibody recognizing ASCT2 -KM8094- appears to improve gastric cancer prognostic, causing cytotoxicity and suppressing tumor growth both *in vitro* and *in vivo* (by using human xenografts transfected to mice) [12]. Moreover, docetaxel (an antineoplastic therapy used in gastric cancer) enhanced this effect [12]. KM8094 was found to decrease the entry of glutamine into the cell and intracellular glutathione levels, resulting in an increase in oxidative stress levels [12] (Tables 1 and 2).

Similar results were obtained with the ASCT2 inhibitor GPNA or ASCT2 knockdown [59]. These treatments reduced glutamine uptake, decreasing glutathione production, increasing ROS level and significantly suppressing tumor growth, both *in*

vivo and *in vitro* [59]. ASCT2 knockdown also induced apoptosis via the mitochondrial pathway [59]

Topotecan is a Topo I inhibitor, preventing DNA replication and RNA synthesis and being indicated to treat some cancers [59]. However, topotecan appears to perform anti-tumor effects on gastric cancer cells both by acting as a Topo I inhibitor and by interfering with cancer metabolism [59]. More specifically, it was found to cause downregulation of ASCT2, and inhibition of gastric cancer growth induced by topotecan was found to be partially ASCT2 inhibition-mediated [59] (Table 1).

6.5. Hepatocellular carcinoma (HCC)

HCC is associated with a poor prognosis [92]. Consequently, developing new therapeutic strategies is necessary. ASCT2 expression is increased in HCC cells and ASCT2 upregulation is directly related to tumor size and prognosis [92]. So, ASCT2-based therapies may be of interest for this type of cancer.

One of the most effective chemotherapeutic agents for HCC treatment is cisplatin [10]. However, some people with HCC relapse due to resistance to cisplatin [10]. Interestingly, the dietary polyphenol resveratrol was found to decrease ASCT2 expression, potentiating cisplatin-induced cytotoxicity and improving prognosis [10] (Table 1).

6.6. Non-small cell lung cancer (NSCLC)

ASCT2 expression is increased and directly related to tumor size, disease stage, lymphatic and vascular invasion, metastasis and prognosis in patients with pulmonary adenocarcinoma [82]. So, ASCT2-based therapies may be also interesting for this type of cancer. In support of this conclusion, δ -tocotrienol was recently found to inhibit the

glutamine transporters ASCT2 and LAT1, which prevents glutamine uptake into cancer cells, inhibiting the proliferation of malignant cells and increasing apoptosis, associated with mTOR pathway downregulation [58] (Table 1).

6.7. Clear cell renal cell carcinoma (ccRCC)

An increased expression of ASCT2 (SLC1A5) is related to a more advanced TNM (tumor, node, metastases) stage, higher Fuhrman degree and worst outcomes, in patients with ccRCC [81]. So, ASCT2 is an independent prognostic factor in ccRCC [81].

6.8. Other cancer types

Besides the cancer types mentioned above, targeting glutamine metabolism improves the prognosis of other cancers, including epithelial ovarian cancer, breast cancer and tongue cancer [79, 93, 94].

7. Conclusions

Metabolic reprogramming is one of the hallmarks of cancer and favors tumor progression, being a mechanism for malignant cells adaptation [1, 2]. One of the significant metabolic alterations verified in cancer cells corresponds to glutamine metabolism, which is currently a focus of interest, in the context of finding new therapies to improve the outcomes of patients with malignant neoplasms [3].

In order to compensate for the greatest energy, carbon and nitrogen demands, an increase in the expression of some transmembrane transporters, including transporters for the nutrient glutamine, is observed in most cancer cells [3]. ASCT2 is a primary transporter of glutamine, being upregulated in malignant cells [3]. So, the possibility of

inhibiting this transporter, and consequently decreasing the entry of glutamine into the cell and its subsequent metabolism, has been investigated, and strategies have been developed targeting ASCT2 [3, 13, 45, 75-77]. It has been verified that the inhibition of glutamine cellular uptake, by inhibition/silencing of ASCT2, appears to improve the prognosis of several cancers, namely gastric cancer, breast cancer, prostate cancer, non-small cell lung cancer, head and neck squamous cell carcinoma, liver cancer, among others [3, 41, 78-82]. Thus, inhibiting glutamine uptake and its metabolism, through ASCT2 inhibition/silencing, appears to be a promising strategy in anti-neoplastic therapy [3, 9-13, 58, 59]. However, more research is needed in this area.

8. Expert opinion

Cancer cells are metabolically reprogrammed, in order to support their high proliferative ratio [1, 2]. This is fundamental to provide the energy needs and biosynthetic precursors and to decrease the oxidative stress in tumor cells [1]. Consequently, there is an overexpression of some transporters, such as ASCT2, in cancer cells [7].

ASCT2 is associated with the growth and progression of different tumors, namely gastric cancer, colorectal carcinoma, breast cancer, prostate cancer, non-small cell lung cancer, head and neck squamous cell carcinoma and liver cancer [3, 41, 78-82]. Due to the essential role of ASCT2 in cancer development [3, 8-13], compounds have been developed to inhibit this transporter, which prevents glutamine (a crucial nutrient for cancer cells) uptake [8].

ASCT2 inhibitors have been developed via different strategies: neutral amino acid analogues, acting as competitive inhibitors [8]; monoclonal antibodies targeting

ASCT2 [9, 12, 13]; non-competitive ASCT2 inhibitors [71]; and other compounds causing ASCT2 downregulation [10, 59]. However, several limitations are presently known for research in this area, and these must be overcome in order that the full impact of ASCT2 in cancer therapy may be ascertained.

First, the ASCT2 inhibitors currently known are not sufficiently potent or selective for this transporter, blocking not only ASCT2, but also other amino acid transporters [8]. These characteristics embarrass the assessment of ASCT2 inhibition effects on cancer because the outcomes obtained do not result from an effective or selective inhibition of this transporter [8]. So, developing therapeutic strategies to treat cancer targeting ASCT2 has been hampered by the lack of effective and selective inhibitors [8].

Second, the development of this therapeutic strategy is troubled by the fact that different membrane transporters carry the same amino acids (in the specific case of glutamine, it is transported not only by ASCT2, but also by other transporters such as LAT1 [8] and ASCT1 [8]). Moreover, and related to this fact, some reports concluded that ablation of ASCT2 resulted in an increased expression of other glutamine transporters in some cancers, namely SNAT1 and SNAT2, which are also able to mediate glutamine cellular uptake [56], and that this can be sufficient to supply the tumor needs, compensating for the silencing of ASCT2 [15, 53]. So, cancer cells which have the ability to increase the expression of other glutamine transporters, are less vulnerable to ASCT2 inhibition [56].

Third, in addition to its role in cancer, ASCT2 is also expressed in non-malignant tissues in our body: brain, lung, intestine, kidney and skeletal muscle [8, 95-

97]. So, inhibition of ASCT2 might result in unwanted negative effects in non-cancer cells.

Fourth, the role attributed to glutamine and, consequently, to ASCT2, appears to be different, depending on the tumor type [4, 98]. This may be due, not only to the different origins of the tissues undergoing malignancy, but also to the tumor microenvironment [4, 98].

Despite these limitations, silencing / inhibition of ASCT2 appears to improve the outcome of different cancers by itself [3, 9, 11-13]. So, we believe that targeting ASCT2 may have a hopeful effect on the prognosis of different cancers, by interfering with the uptake and consequent metabolism of glutamine [1, 4, 7, 8, 28, 77]. Additionally, the combination of other anti-tumor therapies with ASCT2 inhibitors appears to be a very effective strategy to treat some cancers, improving cancer cells response to treatment [3, 12]. However, more research is needed in this area, and of particular interest, the development of more effective and selective drugs to inhibit ASCT2 is a crucial topic [8].

Article Highlights

- Glutamine has been receiving a growing attention, due to its role in cancer cells and on cancer initiation and progression
- Glutamine is not only a source for energy production and nucleotide/protein biosynthesis, but possesses also other effects in cancer cells
- ASCT2 is a primary transporter of glutamine in cancer cells
- In cancer cells, there is a greater demand for glutamine and, so, ASCT2 is overexpressed
- Several strategies to inhibit glutamine metabolism in cancer cells by targeting ASCT2 have been developed
- Silencing/inhibition of ASCT2 appears to be a promising strategy to treat different types of cancer

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Tables

Table 1. *In vitro* effects of ASCT2 silencing/inhibition on different tumor cell lines.

Tumor	Strategies used to ASCT2 silencing/inhibition	<i>In vitro</i> results	References
Head and Neck squamous cell carcinoma (HNSCC)	ASCT2 shRNA/ miR137 transfection	<u>SCC15 and FaDu HNSCC cell lines:</u> <ul style="list-style-type: none"> • Reduces ASCT2 expression • Diminishes glutamine uptake • Decreases levels of glutathione (even after the addition of H₂O₂, which stimulates glutamine uptake) • Increases levels of reactive oxygen species • Suppresses tumor growth/proliferation • Sensitizes cells to H₂O₂-induced apoptosis (PARP cleavage) • Increases levels of autophagy markers • Inhibits mTOR pathway activation 	[3]

	Combination of ASCT2 shRNA with V-9302 (SNAT2 inhibition)	<u>SCC15 and FaDu HNSCC cell lines:</u> <ul style="list-style-type: none"> Decreases glutamine uptake, reduces tumor growth, decreases glutathione levels, increases apoptosis, decreases mTOR pathway activation Sensitizes cells to cetuximab-induced apoptosis Compared with ASCT2 silencing, the combined strategy with ASCT2 silencing and V-9302 further reduces the cellular entry of glutamine 	
Colorectal carcinoma CCR	Ab3-8 mAb (monoclonal antibody that recognizes human ASCT2)	<u>KRAS-mutated CRC cell lines (SW1116 and HCT116 CRC cell lines)</u> <ul style="list-style-type: none"> Diminishes the expression of ASCT2 in membrane surface (ASCT2 internalization) Markedly decreases glutamine uptake Inhibits the expression of AKT, p-ERK and Ki67 <u>ASCT2-knocked out HEK293 and</u>	[9]

		<p><u>SW1116 cancer cells:</u></p> <ul style="list-style-type: none"> • No reaction <p><u>KRAS-wild type cells (CRC HT29 and HeLa uterus malignant cells)</u></p> <ul style="list-style-type: none"> • No effect on ASCT2 • Has no impact on tumor growth and proliferation <p><u>Non-malignant breast cell lines (HME1 and MCF10A):</u></p> <ul style="list-style-type: none"> • Weak effects 	
	KM4008, KM4012 and KM4018 mAbs	<p><u>WiDr CRC cell line:</u></p> <ul style="list-style-type: none"> • Inhibits glutamine uptake • Inhibits cellular proliferation 	[13]
Leukaemia	SLC1A5 (ASCT2) <i>knockout</i> (deletion)	<p><u>SLC1A5 (ASCT2) knockout non-malignant hematopoietic mice cells</u></p> <ul style="list-style-type: none"> • In the presence of a stress factor (such as fluorouracil exposition), hematopoiesis is significantly impaired (indicating that ASCT2 is 	[11]

		<p>important for hematopoiesis under stress conditions)</p> <ul style="list-style-type: none"> • Glutamine uptake decreases, but it is not completely blocked (suggesting that there are other glutamine transporters) • They are more sensitive to reduced glutamine levels (increasing apoptosis) • In a glutamine-free medium, these cells die more than the cells expressing ASCT2, suggesting that there are other important amino acids being transported by ASCT2 <p><u>Leukemic (oncogene MLL-AF9/PTEN deficiency) SLC1A5 knockout mice cells</u></p> <ul style="list-style-type: none"> • Decreases tumor growth/proliferation • Increases apoptosis 	
	GPNA	<u>Human acute myeloid leukaemia cells</u>	

		<ul style="list-style-type: none"> Decreases cells survival Enhances apoptosis Decreases the number of colonies of leukaemic cells <p><u>Normal bone marrow cells</u></p> <ul style="list-style-type: none"> No significant effects in non-malignant cells 	
Gastric cancer	KM8094 mAb	<p><u>HSC-40A, MKN28, HSC-39, SNU-16, 60As6 and HSC-60 gastric cancer cell lines</u></p> <ul style="list-style-type: none"> Inhibits tumor growth Increases the number of gastric cancer cells in G1-phase (cell-cycle) and reduces the number of cells at S and G2/M phases (cell-cycle) Increases apoptosis and oxidative stress (due to lower glutathione levels) Induces antibody-dependent cellular cytotoxicity 	[12]

		<u>MKN1 gastric cancer cell line</u> <ul style="list-style-type: none"> No significant effect 	
	Topotecan	<u>BGC-823 and MGC-803 gastric cancer cell lines</u> <ul style="list-style-type: none"> Induces ASCT2 down-regulation , inhibiting glutamine metabolism 	[59]
Hepatocellular carcinoma	Resveratrol	<u>C3A and SMCC7721 hepatoma cell lines</u> <ul style="list-style-type: none"> Inhibits cell growth Increases the toxicity caused by cisplatin (inducing apoptosis) When combined with cisplatin, increases cell apoptosis (more than cisplatin alone) Inhibits glutamine metabolism, decreasing glutathione levels The combination of resveratrol and cisplatin increases ROS levels more than the increase in ROS levels observed using each strategy alone Causes DNA damage Downregulates ASCT2 expression 	[10]

		<u>Non-malignant liver cell lines:</u> <ul style="list-style-type: none"> • Does not increase the cisplatin effects 	
Non-small cell lung cancer NSCLC	δ -tocotrienol	<u>NSCLC cancer cell lines (A549 and H1299)</u> <ul style="list-style-type: none"> • Decreases glutamate and glutathione levels • Decreases leucine and essential amino acids cellular levels • Reduces metabolites associated with cellular proliferation • Inhibits glutamine uptake, glutamate and glutathione levels, some essential aminoacids uptake, cellular proliferation and increases apoptosis • Inhibits ASCT2, LAT-1 and the mTOR pathway 	[58]

Table 2. *In vivo* effects of ASCT2 silencing/inhibition on several tumor types.

Tumor	Strategies used to ASCT2 silencing/inhibition	<i>In vivo</i> animal results	References
Head and Neck squamous cell carcinoma (HNSCC)	ASCT2 shRNA/ miR137 transfection	Mice; human xenograft (SCC15 and FaDu HNSCC cells) transfected to animals	[3]
	Combination of ASCT2 shRNA with V-9302 (SNAT2 inhibition)	<ul style="list-style-type: none"> • Both cetuximab and ASCT2 silencing alone suppress tumor growth • ASCT2 silencing (ASCT2 silencing alone and ASCT2 silencing combined with V-9302) sensitizes HNSCC to cetuximab • The association of both cetuximab and ASCT2 silencing suppresses HNSCC xenografts more significantly 	

Colorectal carcinoma (CCR)	Ab3-8 mAb	<p>Mice, human xenograft (SW1116, HCT116 and HT29 colon cancer cells) transfected to animals</p> <ul style="list-style-type: none"> • Decrease in the tumor growth of CRC cells with KRAS mutation (SW1116 and HCT116) • No effect on tumor growth of non-KRAS mutated cancer cells (HT29 cells) 	[9]
Leukaemia	ASCT2/SLC1A5 knockout (deletion)	<p>SLC1A5 (ASCT2) knockout mice</p> <ul style="list-style-type: none"> • Hematopoiesis is only slightly affected, without differences both in red blood cells and platelets • After fluorouracil (a stress factor that causes myelotoxicity) exposure, hematopoiesis is impaired and SLC1A5 knockout mice died faster <p>Leukemic cells (MLL-AF9 oncogene/PTEN deficiency) with SLC1A5 deletion inoculated into mice</p> <ul style="list-style-type: none"> • Leukaemogenesis is impaired in 	[11]

		<p>mice with SLC1A5 knockout cells</p> <ul style="list-style-type: none"> • Increases mice survival, with less infiltration of malignant cells in the liver/ lung • Supresses tumor growth • Mice present a slower evolution of the disease, without evolve to more severe states <p><u>Mice with leukemic (MLL-AF9 oncogene) SLC1A5 knockout cells:</u></p> <ul style="list-style-type: none"> • Exhibit a less severe acute myeloid leukemia <p><u>Mice with leukemic (PTEN-deficiency) SLC1A5 knockout cells:</u></p> <ul style="list-style-type: none"> • Exhibit less severe myeloproliferative neoplasm, not progressing to acute leukemia <p>Leukemic cells (MLL-AF9/PTEN deficiency) without SLC1A5 deletion inoculated into mice</p>	
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		<ul style="list-style-type: none"> • Decreases mice survival, increasing the infiltration of malignant cells in the liver/ lung • Enhances tumor cells proliferation • Associated with more severe states of the disease <p><u>Mice with leukemic (MLL-AF9) cells without SLC1A5 deletion:</u></p> <ul style="list-style-type: none"> • Exhibits more severe acute myeloid leukemia <p><u>Mice with leukemic (PTEN-deficiency) SLC1A5 knockout cells:</u></p> <ul style="list-style-type: none"> • Exhibit more severe myeloproliferative neoplasm, progressing to acute leukemia 	
	GPNA	<p>Mice, human xenograft transfected to animals</p> <ul style="list-style-type: none"> • Slower disease progression, with less hepatomegaly and splenomegaly and less infiltration of leukaemic cells in other tissues 	

Gastric cancer	<p>KM8094 mAb</p> <p>(anti- human ASCT2)</p>	<p>Mice, human xenograft (HSC-40A, MKN28, HSC-39, SNU-16, 60As6, HSC-60, MKN1 gastric cancer cell lines) transfected to animals</p> <ul style="list-style-type: none"> • Inhibition of tumor growth (many cell lines of gastric cancer: HSC-40A, MKN28, HSC-39, SNU-16, 60As6 and HSC-60) • No effect in tumor growth (MKN1 gastric cancer cell line) • Combining KM8094 with docetaxel improves outcomes 	[12]
	<p>Topotecan</p>	<p>Mice, human xenograft - BGC-823 and MGC-803 gastric cancer cell lines - transfected to animals</p> <ul style="list-style-type: none"> • Inhibits tumor growth 	[59]

Figures

Figure 1. Overview of glutamine metabolism in cancer cells. Glutamine is converted into ammonia and glutamate, by glutaminases (GLS/GLS2) [19]. In turn, glutamate may be converted to glutathione: from glutamate, it is possible to produce serine, which, in turn, allows glycine and cysteine to be synthesized; the combination of glutamate, glycine and cysteine allows the synthesis of glutathione [23, 24]. Glutamate can also be converted to α -ketoglutarate, an intermediate of the TCA cycle [19, 20, 25]. Malate can be expelled from the mitochondria, being converted to pyruvate and lactate (NAPDH is produced during these reactions) [26, 27]. Citrate can also be expelled from the mitochondria and it permits both lipid and α -ketoglutarate (in association with NADPH

production) synthesis [26, 27]. Glutamine is a source of nitrogen, allowing amino acids to be synthesized (alanine, serine, proline, aspartate, among others [24]) through its conversion to glutamate and by transamination reactions [19, 27]. In addition to amino acids, glutamine also allows nucleotides, proteins and lipids to be synthesized [19, 20, 29]. Taken from [24].

Abbreviations: ACC, Acetyl-CoA carboxylase; AKG, α -ketoglutarate; AS, asparagine synthetase; ALT, alanine transaminase; Ac-CoA, acetyl-coenzyme A; AST, aspartate transaminase; CS, citrate synthase; ACO, aconitase; G6P, glucose-6-phosphate; FH, fumarate hydratase; GCS, gamma-glutamylcysteine synthetase; GFAT, glutamine:fructose-6-phosphate aminotransferase; F6P, fructose-6-phosphate; Gln, glutamine; GLS, glutaminase iso-enzyme; GLS2, glutaminase 2 isoenzyme; Glc, glucose; Gln, glutamine; GLUD, glutamate dehydrogenase; GS, glutamine synthetase; IDH-1, cytosolic isocitrate dehydrogenase; GSH, glutathione; HIF-1, hypoxia-inducible factor-1; IDH-2, mitochondrial isocitrate dehydrogenase; KGDH, ketoglutarate dehydrogenase; LRH-1, nuclear receptor liver receptor homolog 1; LDH, lactate dehydrogenase; ME, malic enzyme; MDH, malate dehydrogenase; NF- κ B, nuclear factor-kappa B; OAA, oxaloacetate; ODC, mTOR, mammalian target of rapamycin; OXPHOS, oxidative phosphorylation; ornithine decarboxylase; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; PKM2, pyruvate kinase M2 isoform; ROS, reactive oxygen species; PPP, pentose phosphate pathway; SDH, succinate dehydrogenase; SCS, succinyl-Coenzyme A-synthetase; STAT1, signal transducer and activator of transcription 1.

Figure 2. Mechanisms to inhibit ASCT2. Several ASCT2 inhibitors have been developed. Nevertheless, most of the existing inhibitory drugs are not sufficiently potent or selective [8]. GPNA inhibits several transporters related to glutamine metabolism, such as ASCT2, LAT1, SNAT1 and SNAT2 [75, 76]; V-9302 does not act by inhibiting ASCT2, but rather SNAT2, another glutamine transporter [77]; monoclonal antibodies recognizing ASCT2 appear to have a therapeutic role in cancer, slowing tumor growth and proliferation [9, 13]; δ -tocotrienol inhibits not only ASCT2, but also LAT1 and the mTOR pathway [58]; miRNA-137 acts on ASCT2 mRNA, promoting its degradation and inhibiting its translation [66, 67].

Expert Opinion on Therapeutic Targets: Author Guidelines

1. Overview

Expert Opinion on Therapeutic Targets is the premier journal dedicated to in-depth reports and reviews of the latest developments in the field of drug target discovery and validation. It is distinguished from other publications by its high quality authorship and expertly drafted articles, which are structured to incorporate the author's own expertise on the subject.

The following document details the requirements for article submissions. Please refer to the **Submission Checklist** at the end of this document, and ensure that all criteria are met before submission. This will ensure the timely publication of your article.

1.1 Audience

The audience consists of scientists, managers and decision makers in the pharmaceutical industry, academic researchers working in the field of molecular medicine and others closely involved in R&D. Reviews are intended to be concise updates on the field, both providing interest for the specialist reader as well as a clear introduction for those with less familiarity.

1.2 Peer-review

All articles are subject to double-blind, independent peer-review. When all comments have been submitted to the Editorial Office, they will be collated and forwarded to the author, along with any Editorial recommendations. Comments remain confidential and are shared only with the corresponding author or submitting party. For a detailed description of the journal's peer review process, authors are referred to the [journal's website](#).

2. Manuscript content

Every article must contain:

2.1 Title (max. 50 words)

All article types should have a concise, informative title that contains no brand names (except in Technology Evaluations). Meeting Highlights titles should have the meeting name, date and location as the title.

2.2 Authors' names and addresses

Including address, academic qualifications and job titles of all authors, as well as telephone number, fax number and email address of the author for correspondence on a **separate cover sheet** as the peer reviewers will be blinded to the authors' identity. Please note that only the address of the first author of the article will appear on Medline/PubMed, not necessarily the corresponding author.

2.3 Abstract (max. 200 words)

The aim of the abstract is to draw in the interested reader and provide an accurate reflection of the content of the paper. We therefore request the following structure is followed for full-length review articles:

Introduction Authors are required to describe the significance of the topic under discussion.

Areas covered Authors are required to describe the research discussed and the literature search undertaken.

Expert opinion Authors are required to summarise briefly their Expert Opinion section.

For shorter article types, such as Editorials, the above sections are not required, but the abstract must describe the nature and objective of the paper. For Original Research, authors should refer to the specific guidelines.

References must not be included in the abstract.

2.4 Keywords

A brief list of keywords, in alphabetical order, is required to assist indexers in cross-referencing. The keywords will encompass the therapeutic area, mechanism(s) of action, key compounds and so on.

2.5 Body of the article

Depending on what type of article you are preparing, please refer to the relevant section from the list below:

Guide Table.

Article type	Length (not including tables, figures and references)	Number of figures	Number of tables	Length of Expert opinion section	Number of references
EDITORIAL	1000 – 1500	2	2	200 – 500	< 15
KEY PAPER EVALUATION	1000 – 1500	2	1	200 – 500	< 10
MEETING HIGHLIGHT	1000 – 1500	2	2	200 – 500	< 20
REVIEWS	4000 – 6000	5	5	500 – 1000	100
META-OPINION	2000 - 2500	2	2	500 – 1000	20 - 50

• Editorial:

The author should discuss the various therapeutic strategies which have been, and are being, explored, providing the reader with an overview of the research field.

• Key Paper Evaluation:

Introduction - The paper under discussion must be introduced and referenced as Reference [1]. The scientific and/or commercial rationale behind the paper is presented, giving some perspective on the information disclosed, placing it in context with previous research in the same area and indicating the relative importance of this new work. Authors may highlight other contemporary papers, which have relevance to the main paper; these may support or conflict the results. It is essential that a critical stand is taken when writing.

Results from the paper - Comment on the extent and quality of the trials, how elegantly they were performed. Quote the number of patients, criteria for selection, doses used, route of administration, adverse effects and so on, as appropriate.

Significance of the results - Comment on the claims made in the author's discussion section. Do the results look promising for having an effect on that field of pharmacotherapy? How is this paper going to change treatment practice in the field? Or is the paper the evidence for a significance theory?

Expert opinion should also contain: Your opinion of the developments; is the paper going to affect future research? Is this treatment likely to become standard practice? If not, indicate why you think the paper is nevertheless of interest. Give your opinion on the developments that you have discussed in the article. Comparative assessment is encouraged. When evaluating the paper, the authors should place emphasis on the therapeutic significance and possibly compare this to other therapies in the same area.

• Meeting Highlights:

- Should include summary of important presentations by particular speakers, with particular reference to novel drugs and therapies in development.

- The Expert opinion and conclusion section also allows the summation of the meeting together with your opinion and discussion of the overall event with reference to some of the more exciting research areas.

- **Meta-opinion:**

The aim of the Meta-opinion is to comment on the variety of opinions given in recent papers in the field and to suggest a consensus on the direction of the field, based on these and the author's own opinion.

Introduction - provide basic background information on the area under review and the papers considered.

Current evidence – provide commentary on the studies/papers under discussion, highlighting key points made by the authors of those papers and summarising the opinions discussed in the context of the current status of the field.

Expert opinion – see section 2.7.

- **Review article:**

Introduction - Incorporating basic background information on the area under review.

Body - Body of the review paper covering the subject under review.

Expert opinion - Should also compare and contrast the approach/drug reviewed in the article with the range of alternative approaches/drugs.

2.6 Conclusion

The conclusion for all articles should contain an analysis/summary of the data presented in the article. Please note that this section is meant to be distinct from, and appear before the 'Expert opinion' section.

2.7 Expert opinion

To distinguish the articles published in the *Expert Opinion* series, authors **must** provide an additional section entitled 'Expert opinion'. This section affords authors the opportunity to provide their interpretation of the data presented in the article and discuss the developments that are likely to be important in the future, and the avenues of research likely to become exciting as further studies yield more detailed results. Authors should answer the following:

- What are the key findings and weaknesses in the research done in this field so far?
- What potential does this research hold? What is the ultimate goal in this field?
- What research or knowledge is needed to achieve this goal and what is the biggest challenge in this goal being achieved?
- Where do you see the field going in the coming years? What is going to happen?
- What advantages does this target have for novel drug design?
- Is there any particular area of the research you are finding of interest at present?

Please note that 'opinions' are encouraged in the Expert opinion section, and as such, referees are asked to keep this in mind when peer-reviewing the manuscript.

Also, please refer to Section 2.5 for article-specific advice on how to frame the Expert opinion.

2.8 Article highlights box

Please provide, in the form of a bulleted list (five or six points), statements covering the key aspects of the paper.

2.9 Annotated bibliography

Important references should be highlighted with a one/two star system and brief annotations should be given (see Section 4 of these guidelines for examples and for a more detailed description of our referencing style).

3. House style

3.1 File formatting

Keep all formatting to a minimum. Do not assign 'styles' to headings, extracts or paragraphs. Make sure that the 'normal' style is used throughout the text. Turn off the automatic hyphenation feature.

3.2 Spacing and headings

Please use double line spacing throughout the manuscript. Headings, sub-headings and title paragraphs should be used to divide the text. Please use numbers (Arabic numerals) to indicate a hierarchy of headings/sub-headings (i.e., 1., 1.1, 2., 2.1, 2.1.1, 2.1.2 and so on).

3.3 Abbreviations and units

Abbreviations should be defined on their first appearance both in the abstract and in the text; commonly used abbreviations need not be defined. Authors are encouraged to submit a list of abbreviations used to the Editorial Office alongside the manuscript. Use SI units or quote SI equivalents where possible. To indicate atom positions in a molecule, use the convention C-1, C-2 and so on.

3.4 Spelling

Manuscripts may be submitted in either US or UK English and will be published using the version of English used in the manuscript submitted to the Editor. All accepted manuscripts will be copyedited in house upon acceptance, but authors are advised to check their work for English spelling and grammar prior to submission. **The Editorial Office can arrange an English language edit prior to submission if required - please contact the editorial office for details of available services and costs.**

3.5 Companies and drug brand names

- Companies are treated as single entities requiring a verb in the third person singular (e.g., GSK is developing an AII antagonist).
- Drug brand names should not appear in paper titles. In the body of the review, the generic name should be used in preference to brand names. Drug brand names are to be used only if absolutely necessary. In such a case, when referring to a lead compound (or compounds claimed in patents) for the first time, please ensure that the ® or ™ symbols are used as required, and that the name of the relevant company is also stated. Generic names always take a lower case first letter unless they are beginning a new sentence.

4. References

Articles should principally review recent primary literature and scientific meeting reports, rather than patents, although relevant patent information may be included where appropriate.

Websites of interest may also be referenced. Occasional 'historic' papers may be cited.

Ensure that all key work relevant to the topic under discussion is cited in the text and listed in the bibliography. Reference to unpublished data should be kept to a minimum and authors must obtain a signed letter of permission from cited persons to use unpublished results or personal communications in the manuscript.

4.1 Numbering

References MUST be numbered consecutively, using Arabic numerals in square brackets, in the order in which they are first mentioned in the text. The reference list should appear in the *same sequence* as the numbers in the text.

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Papers or patents of particular interest should be identified using one or two asterisk symbols (* = of interest, ** = of considerable interest), and annotated with a brief sentence explaining why the reference is considered to be of interest.

4.3 Bibliography

References can be formatted using EndNote or Reference Manager according to the style of *Current Medical Research and Opinion*. References use plain, unformatted text, as in the following examples:

Journals:

1. Weissman P, Goldstein BJ, Rosenstock J, et al. Effects of rosiglitazone added to submaximal doses of metformin compared with dose escalation of metformin in type 2 diabetes: the EMPIRE Study. *Curr Med Res Opin* 2004;21:2029-35

Books:

2. Gottman J. Time Series Analysis. Cambridge: CUP, 1981

Working party reports and similar:

3. Clinical Disputes Forum Working Party. Pre-action protocol for the resolution of clinical disputes. London: Clinical Disputes Forum, 1998

Pre-publication articles assigned DOI numbers:

4. de Lau LM, Koudstaal PJ, Hofman A, Breteler MM. Subjective complaints precede Parkinson disease: the Rotterdam study. Arch Neurol 2006; published online 9 January 2006, doi:10.1001/archneur.63.3.noc50312

Internet articles and website information:

5. Suicidality in adults being treated with antidepressant medications. FDA Public Health Advisory. Washington, DC: FDA/Center for Drug Evaluation and Research, 2005. Available at: www.fda.gov/cder/drug/advisory/SSRI200507.htm [Last accessed 3 January 2006]

Patents:

6. Basf AG. Means and methods for preventing and treating caries. WO2006027265 (2006)

Use the following formats for patent numbers issued by the World, US and European patent offices, respectively: WO0113324; US6803189; EP1549318

Note, for citations with four or fewer authors/assignees, cite all names; for citations with more than four authors, cite three author names plus et al.

Full reference details must be provided in the bibliography (for example, for journal citations, author surnames and initials, article title, journal name, year, volume, page range). Failure to do so may lead to a delay in publication or a return of the paper by the Editor to the author.

5. Tables

Number tables consecutively in the order of their first citation in the text. Place explanatory matter in the footnotes, not in the header. Define in the footnotes all abbreviations that are used in the table. Be sure each table is cited in the text. If you are using data from another published or unpublished source, please obtain permission and acknowledge the source(s).

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Do include illustrations (figures/diagrams/structures) as appropriate. Please ensure that the following recommendations are adhered to as closely as possible:

- Provide electronic copies if possible; otherwise, please ensure that camera-ready copy is of the highest resolution available.
- Please submit figures as eps, illustrator, jpeg, ISISdraw or ChemDraw format.
- Figures should employ CMYK rather than RGB colour scheme.
- Small figures should be 300 dpi and large figures should be 72 dpi.
- Figures and structures should be in separate files to the text. It is unnecessary to incorporate the figures into the body of the manuscript. If there are several figures, please submit these individually, rather than as one file (preferably the original source files). We cannot improve resolution beyond that of the file submitted.
- If these formats are not possible, figures can be submitted in PowerPoint or Word as a last resort.
- Figures should be numbered consecutively according to the order in which they have been first cited in the text. Define in the legend all abbreviations that are used in the figure.
- If a figure has been previously published, acknowledge the original source and please submit written permission from the copyright holder to reproduce the material.

The Editorial Office can arrange for figure re-drawing and medical illustration - please contact us for details of available services and costs.

Publication in colour will be charged at a rate of \$575 per paper.

6.1 Chemical structures

Please submit structures drawn in ISISDraw or Chemdraw and ensure that you submit these in their original, editable format. Also, use the following conventions:

- Always indicate stereochemistry where necessary – use the wedge and hash bond convention for chiral centres and mark *cis/trans* bonds as such.
- Draw small peptides (up to five amino acids) in full; use amino acid abbreviations (Gly, Val, Leu and so on) for larger peptides.
- Refer to each structure with a number in the text and submit a separate file (i.e., not pasted throughout the text) containing these numbered structures in the original chemical drawing package that you used.
- Where structures do not appear as part of a figure, number each structure (using Arabic numerals only) and cite the compound number in the body of the article. Note that structures within figures can also be cited in this way.

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For a detailed description of all the journal's editorial policies, including plagiarism, authorship, patient privacy, and redundant publication, authors are referred to the editorial policy document on the [journal's website](#).

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Please submit manuscripts in Microsoft Word (double spaced, Times New Roman, 12 pt) using the Manuscript Central online submission system (<http://mc.manuscriptcentral.com/eott>). As the peer reviewers will be blinded to the identity of the authors, contributors MUST provide a copy of the manuscript containing no author names or affiliations. These details should be provided on a separate cover sheet designated as a file 'not for review'. Pages must be numbered. The disclosure form should also be uploaded and designated 'not for review'.

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- Once the manuscript has been received in-house, it will be peer-reviewed. A further 2 weeks is then allowed for any revisions (suggested by the Referees/Editor) to be made.
- The final version is then typeset and edited. Page proofs are sent to the author for final approval before the journal is printed.

Note: If a manuscript requires company authorisation before submission, please remember to take this into account when working towards these deadlines.

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Commissioning Editor:

Julia Galway-Witham
Commissioning Editor
Expert Opinion
Informa Healthcare
5 Howick Place
London,
SW1P 1WG
United Kingdom

Tel: +44 (20) 755 19972

Fax: +44 (0)207 017 7667

E-mail: julia.galway-witham@informa.com

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13. Submission Checklist

Before submission of your manuscript, please ensure that the following criteria are met:

General

- Number of figures: no more than 5
- Number of tables: no more than 5
- Submit a list of abbreviations used in the manuscript
- Submit confirmation of figure permissions with submission
- Disclosure statement MUST be submitted WITH manuscript
- Submit manuscript online at <http://mc.manuscriptcentral.com/eott>
Include: i) covering letter, ii) completed Disclosure Form, iii) main document (containing no author details) (in MS Word, double spaced, Times New Roman, 12 pt) and iv) any separately attached image files.
- Pages must be numbered
- Author details must be provided on separate cover sheet
- Paper will be published in UK or US English depending on how it is submitted
- All figures and tables cited in the text are supplied. No figures or tables appear without being cited in the text
- Tables are in cellular format, appearing at the end of the manuscript (following the references)
- All figures are supplied as editable, separate files with the legends appearing on a fresh page at the end of the manuscript.

Sections

- Abstract
- Keywords
- Expert Opinion
- All article-specific requirements listed in the Guide Table are met

References

- Number of references: no more than 100
- Reference annotations present
- Journal reference style is adhered to as closely as possible
- References are listed numerically, and not in alphabetical order
- Abstract/titles do not contain references
- Check references match the citations in the text to avoid later problems

Please be aware that failure to meet the above criteria may delay the publication of your manuscript.