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Review

The amino acid transporter SLC6A14 in cancer and its potential use in chemotherapy

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ABSTRACT

Tumor cells have an increased demand for glucose and amino acids to support their rapid growth, and also exhibit alterations in biochemical pathways that metabolize these nutrients. Transport across the plasma membrane is essential to feed glucose and amino acids into these tumor cell-selective metabolic pathways. Transfer of amino acids across biological membranes occurs via a multitude of transporters; tumor cells must upregulate one or more of these transporters to satisfy their increased demand for amino acids. Among the amino acid transporters, SLC6A14 stands out with specific functional features uniquely suited for the biological needs of the tumor cells. This transporter is indeed upregulated in tumors of epithelial origin, including colon cancer, cervical cancer, breast cancer, and pancreatic cancer. Since normal cells express this transporter only at low levels, blockade of this transporter should lead to amino acid starvation selectively in tumor cells, thus having little effect on normal cells. This offers a novel, yet logical, strategy for the treatment of cancers that are associated with upregulation of SLC6A14. In addition, a variety of amino acid-based prodrugs are recognized as substrates by SLC6A14, thus raising the possibility that anticancer drugs can be delivered into tumor cells selectively via this transporter in the form of amino acid prodrugs. This strategy allows exposure of SLC6A14-positive tumor cells to chemotherapy with minimal off-target effects. In conclusion, the amino acid transporter SLC6A14 holds great potential not only as a direct drug target for cancer therapy but also for tumor cell-selective delivery of anticancer drugs.

1. Introduction

Amino acids are obligatory for cellular functions and survival. While some of these amino acids can be synthesized in mammalian cells from a variety of metabolic precursors, others must be obtained from extracellular sources due to the lack of metabolic pathways for their synthesis de novo. As such, the former are called “non-essential” amino acids and the latter “essential” amino acids. Mammalian cells express a

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multitude of transporters in their plasma membrane for the uptake of both non-essential as well as essential amino acids. Most of these amino acid transporters have been identified at the molecular level. In human, there are approximately 400 transporters for various nutrients, metabolites, and drugs that are classified into 52 gene families; each of the transporters is called a SoLute Carrier (SLC) transporter [1, 2]. The SLC gene families are organized based on the similarity in the amino acid sequence (i.e., primary structure) of the transporter proteins and not on the chemical and structural features of the transported substrates, transport mechanism (e.g., uniporter, coupled transporter, exchanger, facilitated transporter, active transporter) and coupling ions (e.g., H^+, Na^+, K^+, Cl^-, anions). Therefore, amino acid transporters are found in different SLC gene families. To date, there are 11 SLC gene families with one or more amino acid transporters as members: SLC1, SLC6, SLC7, SLC15, SLC16, SLC25, SLC36, SLC38, and SLC43 [3–5]. Of these, only 7 are associated with amino acid transporters that are expressed in the plasma membrane and hence responsible for the uptake of amino acids from the extracellular medium whereas the remaining 4 are associated with amino acid transporters expressed in intracellular membranes such as the mitochondrial membrane, lysosomal membrane or synaptic vesicular membrane. In addition, there are four genes that do not encode amino acid transporters but encode proteins that serve as the chaperones for proper trafficking of selective amino acid transporters to the plasma membrane; two of these genes are classified as SLC transporters even though these do not function as transporters per se (SLC3A1 and SLC3A2) and the other two (collectrin and angiotensin converting enzyme 2 or ACE2) are not included in the SLC gene families for reasons not known [6,7].

2. Amino acid nutrition in cancer and the relevance of amino acid transporters to tumor growth

Amino acids are obligatory for normal cells as well as tumor cells; however, tumor cells must have an increased demand for these nutrients compared to normal cells due to their rapid growth and proliferation. This is true for non-essential amino acids as well as essential amino acids. Even though mammalian cells have the ability to synthesize non-essential amino acids, the rate of synthesis does not match the increased demand in tumor cells, highlighting the obligatory nature of plasma membrane transporters not only for essential amino acids but also for non-essential amino acids to support tumor cell growth and proliferation. In addition, tumor cell metabolism is very different from normal cell metabolism, and the tumor-specific metabolic pathways such as “glutaminolysis” are receiving increasing attention in recent years [8–10]. However, the fact that accelerated influx of amino acids into cells is the primary driver of many of these tumor-specific metabolic pathways has not received much attention. This situation is changing however, as evident from the recent high-profile articles on the role of certain specific amino acid transporters in cancer; these include the interaction between CD44 and cystine/glutamate exchanger SLC7A11 [11, 12], and the functional coupling between the amino acid transporters SLC1A5 and SLC7A5 [13].

Glutamine, though a non-essential amino acid, plays a critical role in tumor cells. Tumor cells are addicted to glutamine (“glutamine addiction”); they cannot grow in the absence of an exogenous supply of this amino acid [8–10]. DNA synthesis is obligatory for cell proliferation, which relies upon de novo synthesis of nucleotides; glutamine supplies nitrogen for the synthesis of purines and pyrimidines. Glutamine nutrition is also coupled to mTOR signaling, which integrates signals from growth factors, energy status, and amino acid nutritional status, and coordinates these signals with cell growth and cell cycle progression, and also to antioxidant machinery [14–16]. When cells have excess amounts of certain specific amino acids (e.g., leucine and glutamine), the kinase activity of mTOR is stimulated, initiating a signaling cascade and thereby regulating protein synthesis and cell growth/survival. One of the proteins whose synthesis is regulated by the mTOR pathway is HIF-1α that is closely linked to tumor growth and progression. Thus, in addition to its well-recognized role in protein synthesis, glutamine is essential for a variety of other metabolic pathways that support tumor cell growth and proliferation.

It was believed for a long time that the increased need for glutamine in tumor cells is solely because of its role in protein synthesis and as a nitrogen source for the synthesis of purine and pyrimidine bases. Recent studies have shown that the role of glutamine in tumor cells goes well beyond these synthetic processes [8–10]. A significant fraction of lactate generated in tumor cells arises from a metabolic pathway involving glutamine. This pathway, known as glutaminolysis, involves the entry of glutamine into citric acid cycle in the form of α-ketoglutarate following the actions of glutaminase and transaminases. In the citric acid cycle, α-ketoglutarate is converted into malate within the mitochondria. Malate then reaches cytoplasm via the malate-aspartate shuttle to serve as a substrate for the $NADP^+$-dependent malate dehydrogenase (also known as malic enzyme) to be converted into pyruvate and then into lactate. Thus, glutamine metabolism in glutaminolysis serves two important purposes: generation of ATP through the truncated part of the citric acid cycle and production of NADPH necessary for anabolic processes such as the synthesis of fatty acids and cholesterol. Arginine is unique in that it serves as a substrate for nitric oxide synthases (NOSs). NOS activity is increased in cancer, and increased entry of arginine might be obligatory to support this activity. Asparagine becomes an essential amino acid for certain specific types of tumor. This is particularly obvious in acute lymphoblastic leukemia and other non-ALL hematologic malignancies based on the efficacy of treatment with asparaginase [17,18]. Cysteine is essential for the synthesis of glutathione. Even though glutathione is composed of three different amino acids (glutamate, cysteine, and glycine), cysteine is the rate-limiting amino acid. Therefore, uptake of cysteine is an important determinant of glutathione status in cells. Since cysteine is present in blood predominantly in the oxidized form (cystine), uptake of cysteine in mammalian cells occurs mostly in the form of cystine rather than cysteine. Glutathione serves an important role in tumor cells by serving as an antioxidant. It also regulates cell cycle by various

mechanisms [19]. A decrease in the cellular levels of glutathione leads to cell cycle arrest not only through inactivation of various kinases but also through oxidative stress with consequent activation of DNA damage response processes. Recently, we reviewed the literature on the expression of amino acid transporters in cancer and its relevance to glutaminolysis [20].

3. Glutamine transporters

Glutamine transporters are obligatory to sustain "Glutamine addiction" in tumor cells. There are multiple transporters for glutamine uptake in mammalian cells; this includes SLC1A5, SLC6A14, SLC6A19, SLC7A5, SLC7A6, SLC7A7, SLC7A8, SLC38A1, SLC38A2, SLC38A3, SLC38A4, and SLC38A5 [3–5]. The mode of transport, coupling ions, and alternative names of these transporters are given in Table 1. Of these, SLC6A19, SLC7A6, SLC7A7, and SLC7A8 are expressed almost exclusively in absorptive epithelial cells such as the intestinal enterocytes and kidney proximal tubular cells. On the basis of transport mechanism, the plasma membrane glutamine transporters can be divided into six distinct groups. Group A (SLC7A5 and SLC7A8) involves the influx of neutral amino acids into the cells in exchange of efflux of neutral amino acids out of the cells without the participation of coupling to movement of any ion. When a given amino acid enters the cells from the extraceullular medium via these transporters, some other amino acid exits the cells; this exchange is obligatory, meaning that influx is always coupled to efflux and that the transport process is electroneutral without affecting the membrane potential. Group B (SLC7A6 and SLC7A7) involves the obligatory exchange of Na^+-coupled movement of neutral amino acids in one direction with the movement of cationic amino acids in the opposite direction. Again, the transport process is electroneutral. However, because of the presence of an inwardly directed Na^+ gradient across the plasma membrane of mammalian cells, under physiological conditions, the transporters in this group mediate the influx of neutral amino acids into cells coupled to the efflux of cationic amino acids out of the cells. Group C (SLC1A5) also involves an obligatory exchange mechanism consisting of Na^+-coupled movement of neutral amino acids in one direction linked to Na^+-coupled movement of neutral amino acids in the opposite direction. The transport process is again electroneutral. Group D (SLC6A19, SLC38A1, SLC38A2, and SLC38A4) consists of transporters which mediate the influx of neutral amino acids in a Na^+-coupled manner; the process is therefore electrogenic, meaning that the transfer process is associated with depolarization of the membrane potential. As such, the inside-negative membrane potential provides an additional driving force for these transporters. Group E (SLC38A3 and SLC38A5) consists of transporters, which mediate Na^+-coupled movement of neutral amino acids in one direction linked to the exchange of H^+ in the opposite direction. This renders the transport process electroneutral. Group F (SLC6A14) is responsible for the influx of neutral and cationic amino acids into cells coupled to cotransport of Na^+ as well as Cl^- in the same direction. The transport process is electrogenic irrespective of whether the transported amino acid is neutral or cationic in nature. As such, this transporter is driven by three different driving forces, namely the inward-directed Na^+ gradient, the inward-directed Cl^- gradient, and the inside-negative membrane potential. In theory, all of these transporters are capable of moving their amino acid substrates into the cell or out of the cell, the direction of the movement solely dictated by the net transmembrane gradients of the amino acids and the coupling ions. For example, SLC38A3 and SLC38A5 mediate the influx of glutamine in some cells but efflux of glutamine in certain other cells depending on the intracellular concentrations of glutamine. On the other hand, SLC6A14, SLC6A19, SLC38A1, SLC38A2, and SLC38A4 always mediate the influx of their amino acid substrates into cells because of the direction of transmembrane gradients of the ions and the membrane potential involved in the transport process.

Table 1 – Glutamine transporters in mammalian cells.

HUGO Name	Alternative name	Mode of transport	Coupling ion
SLC1A5[a]	ASCT2	Exchange	Na^+
SLC6A14[b]	$ATB^{0,+}$	Symport	Na^+, Cl^-
SLC6A19[c]	B^0AT1	Symport	Na^+
SLC7A5[d]	LAT1	Exchange	None
SLC7A6[e]	y^+LAT2	Exchange	Na^+
SLC7A7[e]	y^+LAT1	Exchange	Na^+
SLC7A8[d]	LAT2	Exchange	None
SLC38A1[c]	SNAT1/ATA1	Symport	Na^+
SLC38A2[c]	SNAT2/ATA2	Symport	Na^+
SLC38A3[f]	SNAT3/SN1	Symport	Na^+, H^+
SLC38A4[c]	SNAT4/ATA3	Symport	Na^+
SLC38A5[f]	SNAT5/SN2	Symport	Na^+, H^+

[a] Na^+-amino acid/Na^+-amino acid exchange.
[b] Na^+/Cl^-/amino acid symport.
[c] Na^+/amino acid symport.
[d] Neutral amino acid/neutral amino acid exchange.
[e] Na^+-neutral amino acid/cationic amino acid exchange.
[f] Na^+-amino acid/H^+ exchange.

4. What is so special about SLC6A14 that makes it ideal for tumor cells?

We hypothesized that tumor cells must upregulate one or more of the plasma membrane amino acid transporters to satisfy their "glutamine addiction." But the amino acid transporters that are responsible for the uptake into cells differ widely in their substrate selectivity and concentrative capacity. How do then the tumor cells manage to satisfy their increased demand for amino acids? They must upregulate one or more amino acid transporters depending on their molecular signature in terms of metabolic profile. Based on what we knew of the functional features of all glutamine transporters, SLC6A14 seemed the most likely candidate for potential relevance to cancer. Unlike SLC6A14, the other amino acid transporters possess only some, but not all, functional features that are essential to promote tumor growth (Table 2). The most important of these features include broad substrate selectivity encompassing all essential amino acids as well as glutamine, high

Table 2 – Functional features of SLC6A14 that distinguish this transporter from other glutamine transporters and make it ideal to support tumor growth.

Feature	Glutamine Transporter						
	SLC6A14	SLC1A5	SLC38A1	SLC38A2	SLC38A3	SLC38A5	SLC7A5
Transport of glutamine	+	+	+	+	+	+	+
Transport of all essential amino acids	+	–	–	–	–	–	–
Transport of mTOR activator leucine	+	–	–	–	–	–	+
Transport of serine, a necessary amino acid for tumor growth	+	+	+	+	–	+	+
Transport or arginine, an essential amino acid in tumor cells	+	–	–	–	–	–	–
Component of antioxidant machinery	+	–	–	–	–	–	–
Energy from Na^+ gradient	+	+	+	+	+	+	–
Energy from Cl^- gradient	+	–	–	–	–	–	–
Energy from membrane potential	+	–	+	+	–	–	–
Uniport of amino acids into cells	+	–	+	+	+	+	–

concentrative capability due to coupling to three different energy sources (Na^+ gradient, Cl^- gradient, and membrane potential), and coupling to mTOR signaling. Our studies have shown that SLC6A14 is indeed upregulated in several cancers of epithelial origin such as colon cancer [21], cervical cancer [22], certain subtypes of breast cancer [23], and pancreatic cancer [24].

Previous studies have identified three other amino acid transporters that are upregulated in cancer; these are SLC1A5 [13,25,26], SLC7A5 [13,27], and SLC7A11 [11,12,28,29]. It is important to note that all these three transporters are obligatory exchangers, meaning that influx of certain selective amino acids into cells is always coupled to efflux of certain other amino acids. Since tumor cells need all amino acids to support their rapid growth, whether the exchange process mediated by these three transporters is the best for the tumor cells is debatable. This is not the case with SLC6A14, which essentially functions as a unidirectional transporter for almost all amino acids. The only amino acids that are not transported by SLC6A14 are glutamate and aspartate, which are non-essential. Theoretically, the energetics of SLC6A14 indicates that the transporter has the capacity to concentrate its amino acid substrates inside the cells more than 1000-fold compared to the extracellular medium. This is in stark contrast to the nature of the obligatory exchangers. Furthermore, the transport process mediated by SLC6A14 may be functionally coupled to the function of the other exchangers (Fig. 1). Concentrative entry of amino acids (e.g., glutamine) into tumor cells via SLC6A14 would provide a sufficient intracellular supply of amino acids to serve as the exchangeable substrates for SLC1A5, SLC7A5, and SLC7A11, thus optimizing the biochemical pathways in support of the rapidly proliferating tumor cells.

5. SLC6A14 in colon cancer, cervical cancer, pancreatic cancer, and estrogen receptor-positive breast cancer

The upregulation of SLC6A14 was first demonstrated in colorectal cancer using paired normal and cancer tissue specimens from patients [21]. Normal colon expresses minimal SLC6A14 mRNA. The expression consistently shows a several-fold increase in cancer over the expression in the corresponding normal tissue in all patients. Patients with colorectal cancer show approximately 20-fold increase in SLC6A14 mRNA levels in tumor tissue over normal tissue. The increased expression of SLC6A14 protein in cancer specimens has also been demonstrated convincingly by immunofluorescence methods [21]. SLC6A14 protein expression at metastatic sites has been examined in hepatic metastasis and lymph node metastasis of colonic primary tumors in two patients. Normal liver tissue far removed from the metastasis expresses very low levels of SLC6A14, but the expression is robust in the liver metastasis itself. The upregulation of the transporter also occurs in lymph node metastases. Another interesting finding with regard to the relevance of SLC6A14 to colorectal cancer is that the transporter is upregulated markedly in patients with ulcerative colitis [30,31]. Since it is well known that chronic inflammation in the colon, as occurs in ulcerative colitis, increases the risk for colorectal cancer, the observed upregulation of SLC6A14 in ulcerative colitis may have pathological significance. Inflammation is associated with increased expression of the inducible isoform of nitric oxide

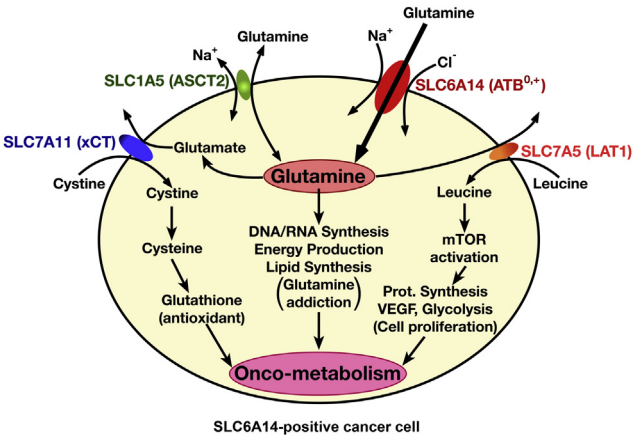

Fig. 1 – Relevance of SLC6A14 to other amino acid transporters and to tumor growth.

synthase (iNOS) resulting in increased levels of nitric oxide production. Since SLC6A14 has the unique ability to mediate the concentrative entry of arginine into cells in a Na^+/Cl^--coupled manner, the coordinated upregulation of iNOS and SLC6A14 may indicate a functional coupling between the two proteins, with the transporter supplying the substrate directly to the enzyme.

To determine if the upregulation of SLC6A14 observed in colon cancer in humans is also true in animal models of colon cancer, we analyzed the expression levels of this transporter in intestinal and colon tissues from wild type mice and $Apc^{Min/+}$ mice. $Apc^{Min/+}$ mice are widely used as an animal model for colon cancer even though this mouse model is associated with adenomas in small intestine as well as in colon [32,33]. We found marked upregulation of SLC6A14 in tumor tissues collected from $Apc^{Min/+}$ mice compared to corresponding tissues from wild type mice. Interestingly, even in the intestinal and colon tissues where there were no tumors, the expression of SLC6A14 was higher in $Apc^{Min/+}$ mice. Homozygous Apc^{Min} mutation is embryonically lethal in mice, and tumor sites in heterozygous mice ($Apc^{Min/+}$) exhibit loss of heterozygosity. Therefore, our findings indicate a dose-dependent relationship between the Apc^{Min} mutation and upregulation of SLC6A14. Recently we examined the expression of SLC6A14 in colon cell lines. These studies showed that the transporter is expressed at low or below detectable levels in normal cell lines (NCM460 and CCD841). In contrast, in cancer cell lines, five out nine cell lines expressed the transporter. The SLC6A14-positive colon cancer cell lines included SW620, KM12C, KM12L4, HCT116, and LS174T.

The upregulation of SLC6A14 is not unique to colon cancer. We demonstrated this phenomenon also in several cases of cervical cancer [22]. We also found that the transporter is upregulated in breast cancer, but interestingly only if the cancer is ER-positive [23]. The transporter is not expressed in ER-negative breast cancer. This phenomenon is true also in breast cancer cell lines [23]. We have established that *SLC6A14* is a direct target for estrogen/ER, thus providing the molecular basis for the upregulation of the gene only in ER-positive breast cancer [34]. Interestingly, the transporter is upregulated in the HCC1937 cell line, which does not possess ER but harbors a mutated inactive BRCA1. This suggests that BRCA1 may suppress the expression of the transporter and, more importantly, inactivation of BRCA1 may induce the transporter even in the absence of ER. It has been shown recently that wild type BRCA1 causes heterochromatin-mediated silencing of specific genes [35], and we hypothesize that *SLC6A14* may be one of these genes. Most recently we discovered that SLC6A14 is also upregulated markedly in pancreatic cancer, both in primary tumor specimens and in pancreatic cancer cell lines [24].

6. "Starve the tumor cells to death" – a novel therapeutic approach with a logical basis

Rapid growth and proliferation are the hallmarks of tumor cells. This is achieved by facilitation of cell cycle and resistance to apoptosis. Enhanced cell proliferation places increased demand for nutrients to serve as the building blocks for the synthesis of macromolecules (DNA, RNA, proteins, and lipids) and as the carbon source for generation of metabolic energy in tumor cells. These nutrients include glucose, amino acids, fatty acids, vitamins, and micronutrients such as trace elements. Most of these nutrients are hydrophilic and do not permeate the plasma membrane easily in mammalian cells. Uptake of hydrophilic nutrients into cells requires specific transporters in the plasma membrane. Tumor cells employ various mechanisms to satisfy their increased demand for nutrients. Vascularization in solid tumors enhances the blood flow, thus increasing the availability of blood-borne nutrients to tumor cells. Entry of nutrients into tumor cells is enhanced by upregulation of specific transporters in the plasma membrane. Since the ability of tumor cells to support their increased demand for nutrients is obligatory for their growth, the pathways involved in this process have potential as drug targets for the treatment of cancer. The underlying principle is simple and logical. If tumor cells cannot obtain essential nutrients, they cannot proliferate fast enough to sustain their growth. It should be possible to starve tumor cells to death by interfering with the availability of essential nutrients and their entry into cells. The feasibility of this approach is exemplified by the significant success in recent years in cancer therapy with drugs targeting vasculogenesis, which compromises the availability of essential nutrients to tumor cells. The entry of nutrients offers an equally promising drug target for cancer treatment, but this area has not received much attention. If the nutrient transporters which are specifically induced in tumor cells compared to normal cells are identified, compounds with the ability to inhibit the cellular signaling pathways responsible for their induction or to block the function of the induced transporters would have potential as chemotherapeutic agents. Since tumor cells induce these transporters specifically for their unique metabolic needs, normal cells are expected to be relatively resistant to the therapeutic actions of such compounds, thus reducing undesirable side effects.

7. SLC6A14 as a potential target for cancer therapy

Our studies have shown that SLC6A14 is upregulated markedly in several cancers of epithelial origin: colon cancer, cervical cancer, pancreatic cancer, and certain subtypes of breast cancer. The transporter drives glutamine addiction in these tumor cells and support amino acid nutrition, thus playing a critical role in the growth and proliferation of these cells. In contrast, normal cells express the transporter at low levels. This provides an ideal condition to target the transporter as a means to induce amino acid starvation selectively in tumor cells without having any significant effect on normal cells, thus interfering with the growth and proliferation of only tumor cells. Normal cells also need amino acids for their proliferation and survival, but their need is satisfied by some other amino acid transporters rather than by SLC6A14 based on the low level of the expression of this transporter. Therefore, blockade of SLC6A14 would have the desired tumor cell

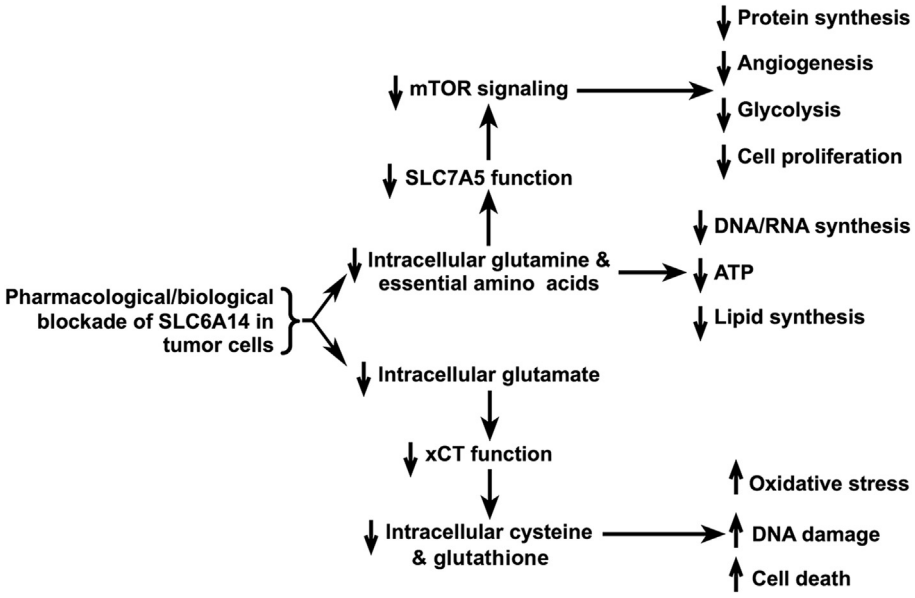

Fig. 2 – Potential consequences of SLC6A14 blockade in tumor cells.

selectivity and avoid undesirable off-target effects. In theory, selective blockade of the transporter, either with small molecules or monoclonal antibodies, should be able to achieve this goal (Fig. 2). Since the tumor cells rely primarily on SLC6A14 for their amino acid supply, blocking its function would lead to decreased intracellular levels of all essential amino acids as well as glutamine. This would decrease protein, lipid, and nucleotide synthesis, thereby interfering with cell proliferation. Amino acid starvation would also dampen mTOR signaling, thus blocking angiogenesis and key metabolic pathways. The function of the obligatory exchanger SLC7A5 would also be suppressed under these conditions because of the decreased intracellular levels of amino acids necessary for the exchange with extracellular amino acids. With the same logical basis, blockade of SLC6A14 would also interfere with the function of SLC7A11, a transporter critical for the antioxidant machinery of the cell. The function of this transporter and its ability to promote the synthesis of glutathione depend on the availability of glutamate inside the cell that is obligatory for exchange with the extracellular cystine. The first step in the intracellular metabolism of glutamine is its conversion to glutamate. Therefore, blockade of SLC6A14 would result in decreased levels of glutamine, and hence glutamate, inside the tumor cells, thereby decreasing the entry of cystine. This would result in decreased synthesis of glutathione with consequent induction of oxidative stress, which would be detrimental to the survival of the tumor cells.

8. Discovery of α-methyltryptophan as a blocker of SLC6A14 and its potential as an anticancer agent

Recently while studying the interaction of several tryptophan derivatives with SLC6A14, we identified α-methyl-DL-tryptophan (α-MT) as a blocker of this transporter [23]. This allowed us to use this blocker as a pharmacological tool to interfere with the function of SLC6A14. We examined the potential of α-MT to induce amino acid deprivation in SLC6A14-positive colon cancer cells and evaluate the consequences. The mTOR pathway plays a critical role in nutrient signaling in mammalian cells. Amino acid starvation leads to specific changes in this pathway. We sought evidence for amino acid starvation in SLC6A14-positive colon cancer cell lines when exposed to α-MT (2.5 mM). With a 24 h exposure to α-MT, the genes coding for asparagine synthetase (ASNS) and the endoplasmic reticulum stress signal molecule CHOP were induced in SLC6A14-positive HCT116 and LS174T cells (cancer cell lines) but not in SLC6A14-negative CCD841 cells (a normal cell line). ASNS and CHOP are important sensors of amino acid nutrition in mammalian cells and their expression is upregulated during amino acid deprivation [36,37]. These data show that α-MT induces amino acid starvation specifically in SLC6A14-positive cancer cells but has no effect on normal cells. We also found decreased phosphorylation of S6 protein and S6 kinase in the mTOR pathway in HCT116 and LS174T cells but not in CCD841 cells when exposed to α-MLT for 24 h. The phosphorylation pattern observed in the two cancer cell lines is a hallmark of changes in mTOR pathway in response to amino acid deprivation. Autophagy is another cellular process that is induced when cells undergo amino acid deprivation [38,39]. To survive in the absence of exogenous essential amino acids during amino acid deprivation, cells engage in autophagic degradation of intracellular macromolecules and organelles as a source of these amino acids. Therefore, we investigated the process of autophagy in LS174T cells with and without exposure to α-MT for 24 h. For this, we analyzed the distribution of LC-3 protein. During autophagy, LC-3 gets concentrated in autophagosomes; thus cells undergoing autophagy exhibit punctate fluorescent signals for

LC-3 when detected by immunofluorescence. Confocal analysis of LS174T cells, when not exposed to α-MT, did not show punctate fluorescent signals for LC-3 whereas the same cells when exposed to α-MT showed clear evidence of such signals. The normal colon cell line CCD841 did not undergo autophagy in the absence or presence of α-MT. When amino acid starvation continues for a long time, pro-survival autophagy turns into pro-death autophagy because cells cannot survive with continued autophagosomal degradation of cellular proteins and organelles. We tested this by monitoring apoptosis in CCD841, HCT116, and LS174T cells with or without α-MT treatment for 72 h. Apoptosis was quantified by FACS analysis. Treatment with α-MT increased the percent of apoptotic cells in HCT116 and LS174T cells but not in CCD841 cells, showing that α-MT induces apoptosis specifically in SLC6A14-positive cancer cell lines without having any detrimental effect in normal cells.

9. Efficacy of α-MT *in vivo*

For *in vivo* studies, the ER-positive ZR75.1 cells were xenografted into nude mice as a model of ER-positive breast cancer. As a negative control for detection of any off-target effects, we used the ER-negative breast cancer cell line MB231. Both cells readily formed tumors in nude mice. α-MT in drinking water (2 mg/ml) reduced the growth of ZR75.1 cells, but did not affect the growth of MB231 cells [34]. The plasma concentration of α-MT in mice after two weeks of treatment was 8.5 ± 0.5 μM. α-MT was not detectable in control mice. It was interesting to note that for about two weeks, the growth of the tumor with ZR75.1 cells remained unaffected by α-MT; however, subsequently the tumor not only stopped to grow but also regressed as a result of treatment with α-MT. We performed similar studies with LS174T cells (a SLC6A14-positive colon cancer cell line). These cells grow into tumors in mouse xenografts, but administration of α-MT in drinking water (2 mg/ml) blocked the growth almost completely.

10. Design and development of selective small molecule blockers of SLC6A14

α-MT is a simple derivative of the amino acid tryptophan; as such this compound may not be selective for SLC6A14. In fact, studies in our laboratory have shown that this derivative does interact with SLC7A5 (unpublished data). Therefore, it may be necessary to identity new blockers that are selective for SLC6A14 using a logical structure-based approach. Even though all amino acid transporters exhibit significant similarities in function in that they all transport amino acids, the three-dimensional structure of the substrate-binding pocket is not likely to be the same for all of these transporters as the transporters differ markedly in amino acid selectivity and coupling to ions. There are also significant differences in the primary sequence among these transporters evident from the fact that they are placed in different gene families rather than in a single family. Unfortunately, there is no information available in the literature on the crystal structure of any of the mammalian amino acid transporters. However, the x-ray crystal structure of LeuT, a bacterial amino acid transporter belonging to the SLC6 gene family as does SLC6A14, is known [40, 41]. Interestingly, while LeuT recognizes leucine as the transportable substrate, tryptophan serves as a blocker of this transporter. The structures of the binding-pocket of LeuT in leucine-bound form and tryptophan-bound form have been elucidated. Therefore, it is feasible to model the structure of SLC6A14 based on the structure of LeuT and theoretically deduce the structural requirements necessary for binding to the substrate-binding pocket of SLC6A14. This might prove to be a powerful strategy in successful design and development of small molecules that selectively block SLC6A14 with little or not interaction with other amino acid transporters for potential use in cancer therapy.

11. SLC6A14 as a delivery system for anticancer drugs in the form of amino acid-based prodrugs

We have studied extensively the potential of SLC6A14 as a delivery system for drugs and prodrugs [42]. Aspartate and glutamate are not substrates for SLC6A14, but asparagine and glutamine are. This suggests that if the side chain carboxyl group in aspartate and glutamate is modified such that the resultant side chain is not anionic, SLC6A14 may recognize such derivatives as substrates. We tested the feasibility of this approach by examining the interaction of various β-carboxyl derivatives of aspartate and γ-carboxyl derivatives of glutamate with SLC6A14 [43]. These studies showed that various esters of aspartate and glutamate are indeed substrates for SLC6A14. We then tested the utility of this approach for the delivery of prodrugs by synthesizing a derivative in which a drug was coupled to glutamate as an ester of the γ-carboxyl group of the amino acid [43]. We used acyclovir, an antiviral drug, for this purpose. Acyclovir itself is not a substrate for SLC6A14. However, the γ-glutamyl ester of acyclovir (γ-Glu-Acv), is recognized as a substrate by the transporter. These findings provide supporting evidence for the idea that therapeutic agents can be coupled to the side chain of aspartate or glutamate in the form of esters to enable the delivery of these drugs into SLC6A14-expressing cells. Subsequent studies have shown that conjugation of the drugs can also be done at the α-carboxyl group of neutral amino acids without affecting the ability of SLC6A14 to recognize the modified amino acids as substrates. Acyclovir has poor oral bioavailability. However, when this drug is coupled to valine as an ester of the α-carboxyl group, the oral bioavailability of the resultant valacyclovir is much greater than that of the parent drug due to its recognition as a substrate by the intestinal peptide transporter [44,45]. We asked whether SLC6A14 would also recognize valacyclovir as a substrate. Our studies showed that the transporter does indeed transport valacyclovir [43]. This was surprising because valacyclovir does not possess any free carboxyl group as this group is esterified with acyclovir. Apparently, the free carboxyl group is not obligatory for recognition by SLC6A14. Subsequently we showed that valganciclovir, the α-carboxyl ester derivative of valine with the antiviral drug ganciclovir, is also an excellent substrate for SLC6A14 [46]. These studies provide the proof-of-concept for

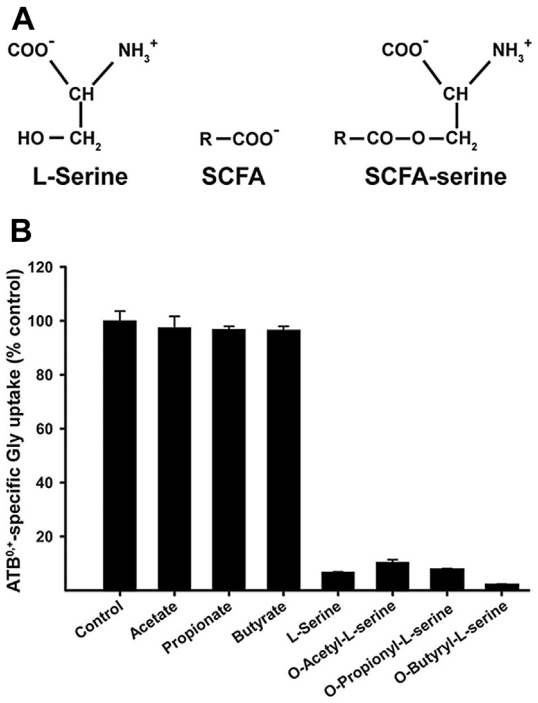

Fig. 3 – SLC6A14-mediated transport of amino acid-based prodrugs of HDAC inhibitors: mammalian cell-based assay. Human SLC6A14 was expressed heterologously in the human retinal pigment epithelial cell line HRPE and the activity of the expressed transporter was monitored by the uptake of glycine in the presence of NaCl. A, Structures of short-chain fatty acids (SCFAs) esterified at the hydroxyl group of serine. B, differential influence of free SCFAs and serine-esters of SCFAs on SLC6A14 ($ATB^{0,+}$)-mediated glycine uptake.

the potential use of SLC6A14 for tumor-selective delivery of chemotherapeutic drugs for cancer treatment.

12. SLC6A14 as a potential delivery system for amino acid-based prodrugs of epigenetic modifiers

Identification of a mechanism for the tumor cell-specific delivery of small molecules with capability to modify epigenetic profile has immense clinical and therapeutic importance. Histone acetylation and DNA methylation represent two important epigenetic modifications that are critical to cancer biology. Several small molecule inhibitors of histone deacetylases and DNA methyltransferases are in clinical use for cancer therapy, highlighting the therapeutic relevance of these epigenetic modifications to cancer progression and development. However, none of the currently used histone deacetylase inhibitors (HDIs) or DNA methylation inhibitors (DMIs) has tumor cell-selectivity. Consequently, their use is fraught with significant undesirable, often times severely debilitating, side effects. The potential delivery of HDIs and DMIs in the form of amino acid-based prodrugs via an exceptionally energy-coupled amino acid transporter is a novel idea that has never been tested. SLC6A14 is ideally suited for this purpose.

13. Amino acid-based prodrugs of the HDAC inhibitors butyrate and propionate and their transport via SLC6A14

Butyrate and propionate are potent inhibitors of histone deacetylases HDAC1 and HDAC3 [47–49]. While normal cells express a Na^+-coupled transporter, known as SLC5A8, for the transport of these HDAC inhibitors, tumor cells silence this transporter, presumably to avoid their entry because inhibition of HDACs would cause cell death in tumor cells [50,51]. Based on our data that SLC6A14 is upregulated markedly in tumor cells and that it recognizes a variety of amino acid-based drugs and prodrugs as substrates, we hypothesized that the HDAC inhibitors butyrate and propionate, if coupled to the side chain hydroxyl group of the amino acid serine as an ester, will be accepted as substrates by SLC6A14. Therefore, we used *O*-butyrylserine and *O*-propionylserine to test our hypothesis; as these esters were not commercially available, we synthesized these esters. Structures of these esters are given in Fig. 3A. We first tested whether these serine esters of propionate and butyrate are able to compete with [^{3}H]-glycine for transport via cloned human SLC6A14 in a mammalian cell expression system. In this system, HRPE (human retinal pigment epithelial) cells, transfected with vector alone, show very little glycine uptake activity. When transfected with SLC6A14 cDNA, glycine uptake activity is increased more than 35-fold. As expected, the cDNA-mediated glycine uptake was not inhibited by free SCFA (acetate, propionate, and butyrate) (5 mM) (Fig. 3B). However, serine and the serine esters of acetate (which is commercially available), propionate, and butyrate inhibited the uptake markedly. The IC_{50} (i.e., concentration necessary to cause 50% inhibition) was 148 ± 28 μM for serine, 423 ± 72 μM for *O*-acetylserine, 332 ± 59 μM for *O*-propionylserine, and 118 ± 8 μM for *O*-butyrylserine. These data show that the serine-esters of the HDAC inhibitors butyrate and propionate are recognized by SLC6A14 with affinities comparable to that of serine.

The studies with mammalian cells indicated that serine-SCFA esters interact with SLC6A14 but they do not show that these esters are transported into the cells. It is possible that these esters bind to the substrate-binding site of SLC6A14 and block the transport of glycine without themselves entering into cells. Therefore, we used the *Xenopus laevis* oocyte expression system to assess directly the transport of these esters via SLC6A14. This method monitors transporter function by measuring substrate-induced currents under voltage-clamp conditions. SLC6A14 is electrogenic, and a compound would induce currents only if it is transported across the membrane via the transporter. With this effective technique, we could demonstrate that serine and the serine-SCFA esters induced marked currents in oocytes expressing SLC6A14 but not in water-injected oocytes (Fig. 4A). These currents were obligatorily dependent on Na^+ and Cl^- as expected because SLC6A14 is a Na^+/Cl^--coupled transporter.

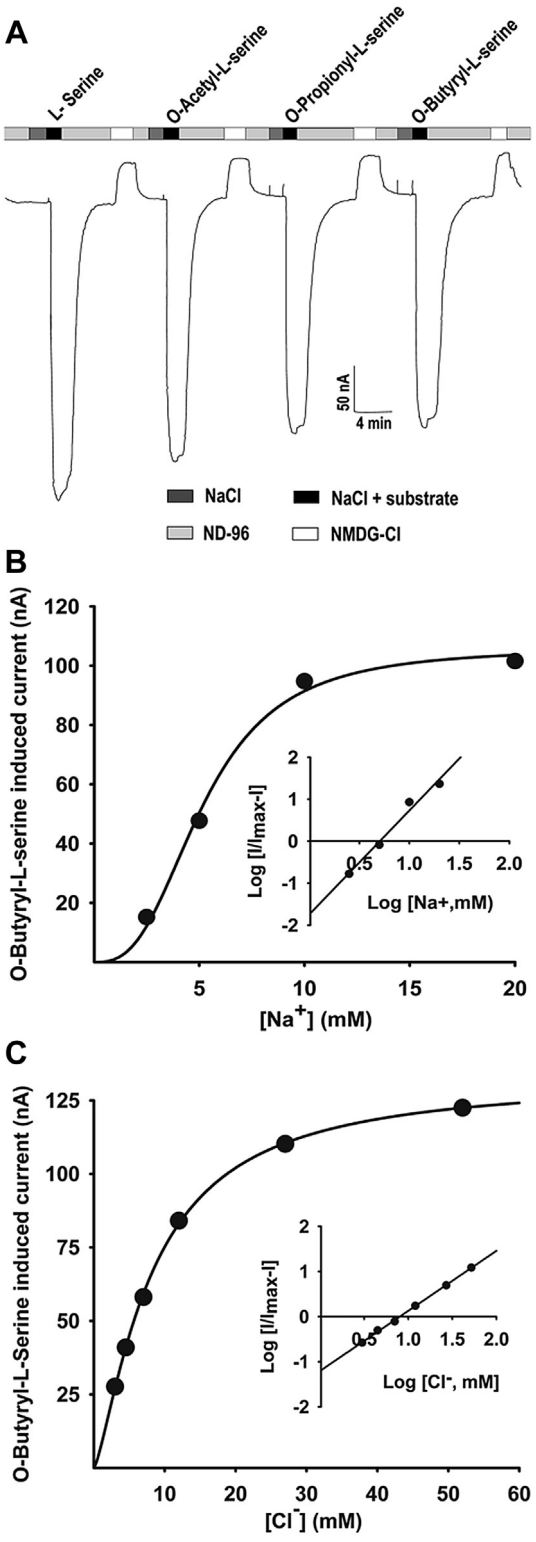

Fig. 4 – SLC6A14-mediated transport of amino acid-based prodrugs of HDAC inhibitors: *X. laevis* oocyte-based assay. Human SLC6A14 was expressed heterologously in *X. laevis* oocytes and the activity of the expressed transporter was monitored electrophysiologically in the form of inward currents in the presence of NaCl. A, Inward currents induced by serine, and serine esters of SCFAs (acetate, propionate and butyrate). ND-96 is the oocyte maintenance buffer that contains NaCl. NMDG-Cl is a Na^+-free buffer in which NaCl was replaced isoosmotically with N-methyl-D-glucamine (NMDG) chloride. B, Na^+-activation kinetics of O-butyryl-serine-induced currents. The inward currents were monitored in the presence of 5 mM O-butyryl-serine and increasing concentrations of Na^+. NMDG was used to replace Na^+ isoosmotically. Cl^- concentration remained constant at 100 mM. C, Cl^--activation kinetics of O-butyryl-serine-induced currents. The inward currents were monitored in the presence of 5 mM O-butyryl-serine and increasing concentrations of Cl^-. Gluconate was used to replace Cl^- isoosmotically. Na^+ concentration remained constant at 100 mM.

The currents induced by *O*-butyrylserine increased with increasing concentrations of Na^+ (Fig. 4B) and Cl^- (Fig. 4C). Analysis of the data according to Hill equation indicated a Na^+:Cl^-:substrate stoichiometry of 2:1:1, a value characteristic of SLC6A14. The Michaelis constant (K_m) was 175 ± 6 μM for serine, 369 ± 25 μM for *O*-acetylserine, 305 ± 31 μM for *O*-propionylserine, and 119 ± 2 μM for *O*-butyrylserine. These data demonstrate unequivocally that the serine-based esters of acetate, propionate, and butyrate are actually transportable, high-affinity substrates for SLC6A14. The almost universal downregulation of the monocarboxylate transporter SLC5A8 in cancers and the upregulation of SLC6A14 in many cancers provide a logical basis for the exploitation of SLC6A14 as the tumor-selective delivery system for certain specific HDAC inhibitors in the form of amino acid-based prodrugs (Fig. 5).

14. Amino acid-based prodrugs of DNA methyltransferase inhibitors as substrates for SLC6A14

The nucleosides 5′-azacytidine and 5′-azadeoxycytidine are effective inhibitors of DNA methyltransferases, which are currently used for the treatment of certain types of cancers. These epigenetic modifiers get access into mammalian cells via nucleoside transporters. There is no evidence in the literature to indicate that these transporters are upregulated in tumor cells. In fact, the transporters may be downregulated in certain tumors thus causing resistance to nucleoside-based chemotherapeutic agents [52–54]. This means that 5′-azacytidine and 5′-azadeoxycytidine enter into tumor cells as well as normal cells indiscriminately via the nucleoside transporters. In fact, some tumors might be exposed to these drugs to a lesser extent compared to normal cells if the tumors are associated with downregulation of these transporters. Tumor cell-selective delivery of these epigenetic modifiers would be greatly desirable to have targeted effect on tumor cells with minimal influence on normal cells (Fig. 5). This rationale applies not only to epigenetic modifiers but also to many other nucleoside-based chemotherapeutic agents (e.g., 5-fluorouracil, gemcitabine, cytarabine, etc). It is potentially feasible for these nucleoside drugs to be delivered into tumor cells selectively via SLC6A14 in the form of amino acid-based prodrugs. The overwhelming evidence for the ability of

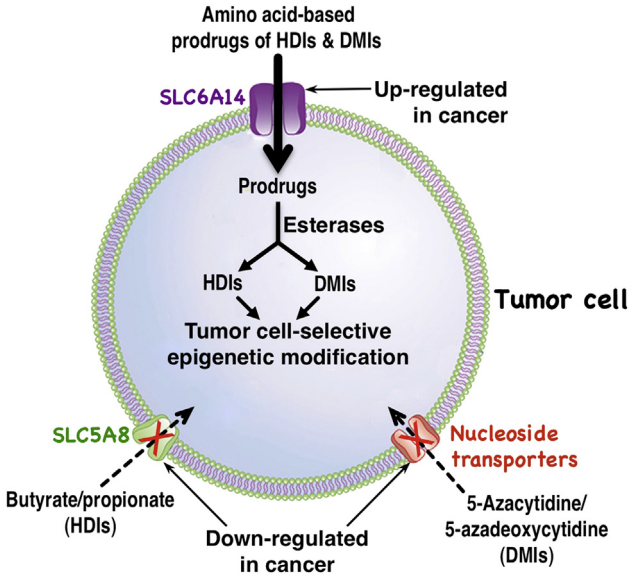

Fig. 5 – Rationale for the use of SLC6A14 for tumor cell-selective delivery of HDAC inhibitors (HDIs) and DNA methylation inhibitors (DMIs).

SLC6A14 to transport amino acid-based prodrugs of acyclovir and ganciclovir supports this idea.

15. Conclusion

Tumor cells have an increased demand for amino acids, both essential amino acids as well as non-essential amino acids, including glutamine. This is needed to support anabolic pathways for the synthesis of DNA, RNA, lactate, ATP, lipids, and antioxidants. Tumor cells meet this increased demand by upregulating one or more of plasma membrane transporters. SLC6A14 is one such transporter that is expressed at markedly high levels in certain specific cancers, particularly those of epithelial origin. Since tumor cells depend on this transporter for amino acid supply, blocking the transporter function should, in theory, starve the tumor cells of amino acid nutrition and hence interfere with their growth and proliferation. This offers a novel, hitherto untested, strategy for cancer therapy. In addition to being a direct target for chemotherapy, the transporter can also be exploited for the tumor cell-specific delivery of chemotherapeutic agents in the form of amino acid-based prodrugs. Nutrient deprivation represents a logical strategy to kill cancer cells; yet none of the currently available anticancer drug development programs has seized the opportunity to utilize this strategy. We have firmly established that blockade of SLC6A14 induces cell death in SLC6A14-positive tumor cells by four different mechanisms: (a) it prevents the entry of essential amino acids; (b) it targets the "glutamine addiction" behavior; (c) it inhibits mTOR; and (d) it induces oxidative stress. There is no evidence of off-target effects. This represents the first time a nutrient transporter is exploited as a drug target for cancer therapy, and ushers us into a new era of novel cancer therapeutics beyond kinase inhibitors and growth factor receptor blockers.

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