Review

Amino Acid Depletion Therapies: Starving Cancer Cells to Death

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Targeting tumor cell metabolism is an attractive form of therapy, as it may enhance treatment response in therapy resistant cancers as well as mitigate treatment-related toxicities by reducing the need for genotoxic agents. To meet their increased demand for biomass accumulation and energy production and to maintain redox homeostasis, tumor cells undergo profound changes in their metabolism. In addition to the diversion of glucose metabolism, this is achieved by upregulation of amino acid metabolism. Interfering with amino acid availability can be selectively lethal to tumor cells and has proven to be a cancer specific Achilles' heel. Here we review the biology behind such cancer specific amino acid dependencies and discuss how these vulnerabilities can be exploited to improve cancer therapies.

Introduction

Advances in chemotherapy and supportive care, but also the introduction of targeted- and immune-therapies, have led to increased survival of cancer patients over the past decades [World Health Organization, 2018 (https://www.who.int/news-room/fact-sheets/detail/cancer)]. However, a large number of cancer survivors, especially those treated at a young age, present with late effects, which severely impacts quality of life [1]. Therefore, next to improving cure rates, alternative therapies that are not associated with long-term toxicities require attention. Tumor cell metabolism may prove to be a vulnerability that can be targeted with minimal collateral damage [2,3].

To accommodate their enhanced proliferation, tumor cells increase their metabolic rate to provide sufficient cellular building blocks (proteins, DNA, RNA, and lipids), energy, and reducing agents [3]. This often involves the activation of prominent oncogenes, including Ras and c-Myc or loss of tumor suppressors such as PTEN and P53, driving changes in cellular metabolism to meet the increased demand for metabolites [2,4] (Figure 1). Their elevated uptake of nutrients is already put to use in routine diagnostics, where intratumor accumulation of radiolabeled nutrients such as glucose but also specific amino acids, can be visualized using positron emission tomography (PET) scans [5,6]. While the cancer cell-specific diversion of carbohydrate intermediates from oxidative phosphorylation towards several anabolic pathways, known as the Warburg effect, has been long recognized, attention for amino acid dependencies in cancer cells came decades later. Tumor cells often rely on an exogenous supply of amino acids. Surprisingly, this not only holds true for essential amino acids (EAA), the type that the body cannot synthesize, but also several nonessential amino acids (NEAA) appear to be rate limiting for the growth of tumor cells [7]. Here we discuss the biology behind the most prominent tumor-specific amino acid dependencies and their potential clinical applications.

Amino Acid Availability as a Therapeutic Target for Cancer Therapy

Next to forming the building blocks of proteins, amino acids provide many of the structural elements of a cell and are an important source of energy. It is therefore not surprising that cancer

Highlights

Targeting amino acid metabolism is an attractive form of therapy, as it can mitigate long-term treatment-related toxicities by reducing the need for genotoxic agents.

The effect of amino acid depletion therapies can be further enhanced when combined with either conventional therapies or by targeting tumor cell-specific survival mechanisms.

A number of novel amino acid depletion strategies is currently under clinical evaluation.

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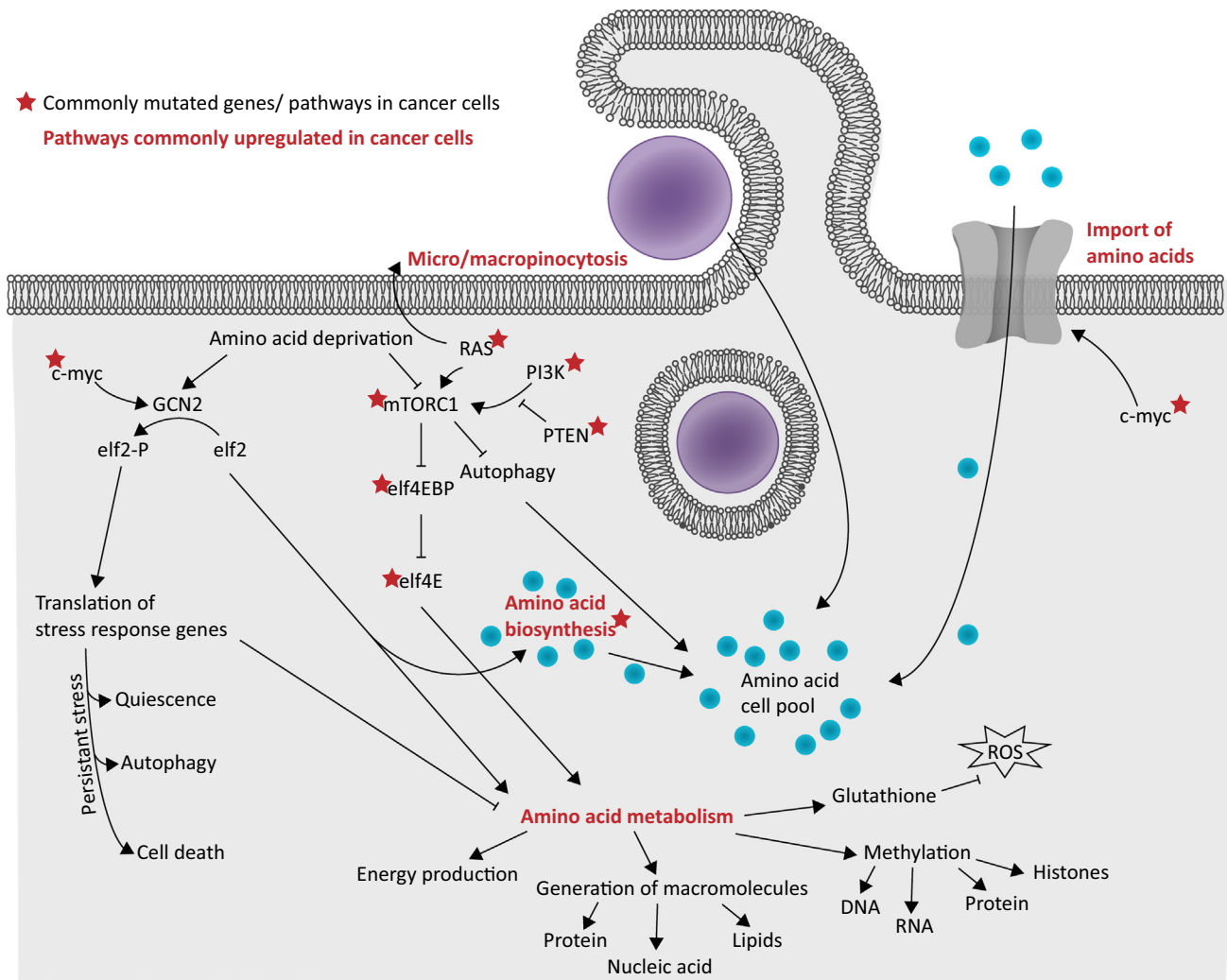
Figure 1. Commonly Deregulated Genes/Pathways Controlling Amino Acid Homeostasis and Metabolism in Cancer Cells. Import of amino acids from the bloodstream, amino acid biosynthesis, amino acids derived from autophagy, and micro/micropinocytosis contribute to the internal amino acid cell pool. These are used for energy production, macromolecular biosynthesis (proteins, nucleic acids, lipids, and glutathione), and methylation processes. The tight balance between amino acid uptake, biosynthesis, and catabolism is controlled by mammalian target of rapamycin complex 1 (mTORC1) and general control nonderepressible 2 (GCN2). mTORC1 facilitates amino acid metabolism in response to amino acid availability. In response to amino acid shortages, mTORC1 is inhibited while GCN2 is activated, resulting in the activation of the amino acid response pathway. This includes a global shutdown of translation, bringing cells into quiescence in order to be able to survive nutrient shortage. If these stress conditions persist, apoptosis is induced. To fulfill their enhanced demand for amino acids, cancer cells often upregulate amino acid homeostasis and metabolism (shown in red). This involves mutations in commonly mutated cancer genes, including *c-Myc*, *Ras*, *PI3K*, *PTEN*, and *mTOR*, but also genes directly implicated within pathways of amino acid homeostasis and metabolism (e.g., *Elf4EBP* and *elf4E*). Abbreviation: ROS, reactive oxygen species.

cells, although striving to maintain amino acid homeostasis (Box 1) by promoting amino acid synthesis or salvage (Figure 1), become more dependent on exogenous supply of amino acids. This increased demand for amino acids may even cause auxotrophy (i.e., the inability to sustain growth in the absence of a particular nutrient) for NEAAs [8]. The latter can be either acquired during tumor progression, or related to the cell of origin, such as the insufficient expression of asparagine synthetase (ASNS) in leukemic cells and their origin, immature lymphocytes (Box 2) [8]. Both the selective dependency and the potential to target a specific amino acid makes some more suitable as a therapeutic target than others [4]. The importance of amino acid

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Box 1. Sensors of Amino Acid Availability

Amino acids, best known as the structural units that make up our proteins, also serve as a resource for various other key cellular processes, including the generation of other macromolecules, hormones and neurotransmitters, energy production, and methylation.

To ensure sufficient supply of amino acids, both normal and transformed cells are able to sense and respond to conditions of limited nutrient availability, by coordinating amino acid uptake, biosynthesis, and catabolism (see Figure 1 in main text) [3]. The mammalian target of rapamycin complex 1 (mTORC1) and general control nonderepressible 2 (GCN2) are the two most prominent nodes in the pathways controlling the cellular response to low amino acid availability. mTORC1 activation in response to amino acid availability is primarily mediated by RAG GTPases, which in turn are activated by a number of amino acid sensors, including solute carriers that transport amino acids over the cell membrane [99]. Specific amino acids, including leucine, Arg, and Gln appear to be more relevant for mTORC1 signaling than others [99].

At the same time the cellular amino acid sensor GCN2 is activated by uncharged tRNAs or stalled ribosomes, suppressing global protein translation by phosphorylation of the eukaryotic initiation factor 2 (eIF2), effectively stalling CAP-dependent protein translation [100]. Simultaneously, CAP-independent translation of activating transcription factor 4 (ATF4) is increased. This stress induced transcription factor controls expression of a wide range of adaptive genes within the amino acid response (AAR) pathway. These include amino acid transporters, enzymes that promote *de novo* synthesis of amino acids, as well as activators of autophagy that together act to restore homeostasis. However, under persistent stress conditions, ATF4 will trigger a transcriptional program that favors the induction of apoptosis [101].

With nutrient sensing being crucial for tumor cell survival and proliferation, it is not surprising that essential sensors and effectors are frequently mutated or upregulated [3]. Upstream regulators such as PI3K and RAS, but also mTORC1 itself are often subject to overactivation or mutations [4,9,55]. Mutations in downstream effectors like 4E-BP1 and ELF4E have been found in certain tumors [102].

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metabolism is underscored by the fact that limiting the availability of these nutrients can be selectively lethal to tumor cells.

Targeting amino acid metabolism can be approached from different angles: inhibition of either amino acid transporters [3], amino acid biosynthesis, or by depletion of amino acids. Although upregulated expression of amino acid transporters is used by cancer cells to meet an increased demand, the functional redundancies between amino acid transporters make these rather


Box 2. Asparaginase, the Prime Example of Targeting Amino Acid Availability

Although tumor cell metabolism has long been recognized as a potential therapeutic target and antimetabolites such as methotrexate and 5-fluorouracil (5-FU) were successfully introduced as anticancer therapy decades ago [3,103], most of these compounds fail to discriminate between the tumor and rapidly dividing normal tissues, such as skin, gut epithelium, and bone marrow [104]. However, some metabolic therapies, for instance those involving the selective depletion of a particular amino acid, can be quite tumor cell specific. Unlike other cell types in the body, lymphocytes, including leukemic blasts, are selectively dependent on Asn. As a consequence, the introduction of the Asn depleting enzyme asparaginase (ASNase) in the treatment of pediatric acute lymphoblastic leukemia, has profoundly improved cure rates [105]. Upon injection, the bacterially derived ASNase hydrolyses Asn to aspartic acid and ammonia, and Gln into glutamic acid, effectively depleting Asn from the blood. While most cells express asparagine synthetase (ASNS), the enzyme that converts aspartate into Asn, expression in immature lymphocytes and their malignant counterparts, leukemic blasts, is insufficient, making these cells auxotrophic for Asn. As a result, ASNase treatment kills the leukemic cells. ASNase treatment, is associated with several acute, but mostly manageable toxicities [106], although in some cases severe pancreatitis, thrombosis, or allergic reactions develop. However, unlike genotoxic agents, late and long-term effects are mostly absent. Nowadays, ASNase is a key component of the multiagent therapy regimen that cures more than 90% of pediatric acute lymphoblastic leukemia (ALL) patients [105]. While ASNase is only one of the many drugs that are included in the treatment protocols, its added value becomes apparent from the fact that premature discontinuation immediately translates into an inferior disease-free survival [107]. Although initial trials with other tumor types showed disappointing results, more recently a better understanding of the intimate connection between ASNS expression and the response to therapy has rekindled the interest in this protein drug, which is now under clinical investigation for the treatment of lymphomas, acute myeloid leukemia (AML), natural killer (NK)/T cell lymphoma, pancreatic ductal adenocarcinoma, glioblastoma, ovarian carcinomas, breast cancer, gastric cancer, and liver cancer [13,76,108]. Moreover, upcoming amino acid targeting therapies will benefit greatly from the experience gained with the decades of using asparaginase therapy in clinical practice. These include the use of therapeutic drug monitoring, strategies to avoid immune response and related adverse effects, and management of acute toxicities.

unattractive therapeutic targets. By contrast, inhibition of enzymes involved in *de novo* and/or salvage pathways of amino acid synthesis as, for example, phosphoglycerate dehydrogenase (PHGDH), part of the serine (Ser) biosynthesis pathway [9] or glutaminase (GLS) [10], shows more promise. Amino acid depletion can also be achieved by the degradation of a specific amino acid in the bloodstream. When tumor cells are selectively dependent on exogenous supply of a specific amino acid, this will lead to amino acid starvation, cessation of growth, and ultimately, induction of apoptosis [11] (Figure 2). Here we will discuss different amino acids that are targets for amino acid depletion therapy.

Asparagine

To date, asparagine (Asn) is the most successful and best documented target for amino acid depletion therapy in the treatment of cancer (Box 2). Particularly in pediatric acute lymphoblastic leukemia (ALL), the bacterially derived enzyme ASNase has become an essential component of ALL treatment [12] and its therapeutic efficacy in some solid tumors is under clinical investigation.
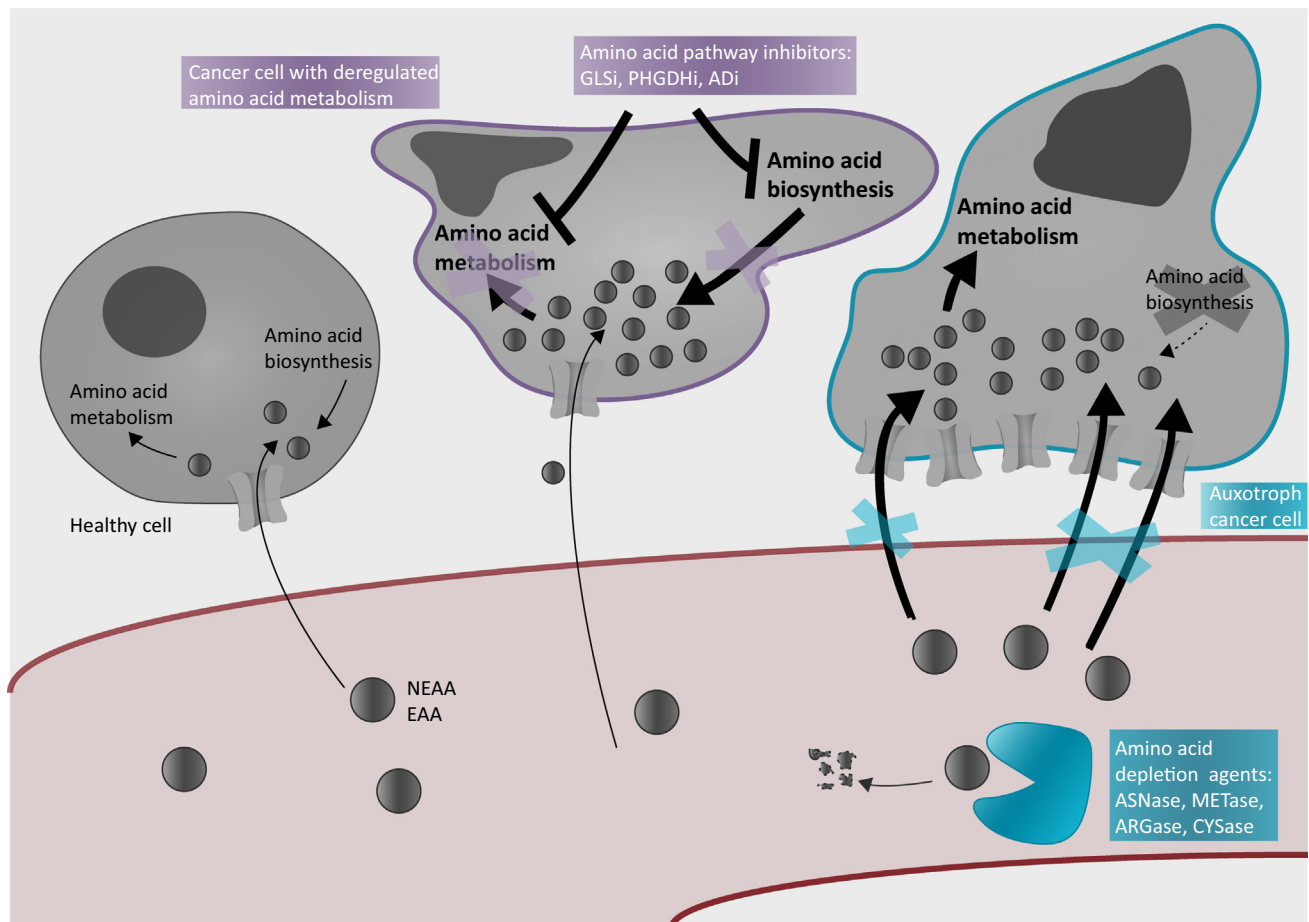
Trends in Endocrinology & Metabolism

Figure 2. Schematic Overview of Different Strategies to Target Amino Acid Metabolism. Cancer cells that are dependent on endogenous amino acid biosynthesis can be targeted by amino acid pathway inhibitors (purple). Cancer cells that are dependent on the exogenous supply of a specific amino acid, can be targeted by depletion of this specific amino acid (blue). Normal cells show a much lower demand for amino acids and can survive either amino acid biosynthesis inhibition or depletion of amino acids (gray). Abbreviations: ADI, arginine deiminase; EAA, essential amino acid; GLS, glutaminase; NEAA, nonessential amino acid; PHGDH, phosphoglycerate dehydrogenase.

For instance, breast cancer cells were shown to be dependent on Asn, although these tumors show high ASNS expression. Two independent studies show that Asn can stimulate *de novo* glutamine (Gln) biogenesis and high levels even promote epithelial to mesenchymal transition (EMT), a crucial event in the cascade of events that drive metastasis. Accordingly, ASNase-mediated limitation of Asn repressed both primary tumor growth as well as the development of metastasis, not only by depriving the tumor of Asn, but by proxy depleting Gln [13,14].
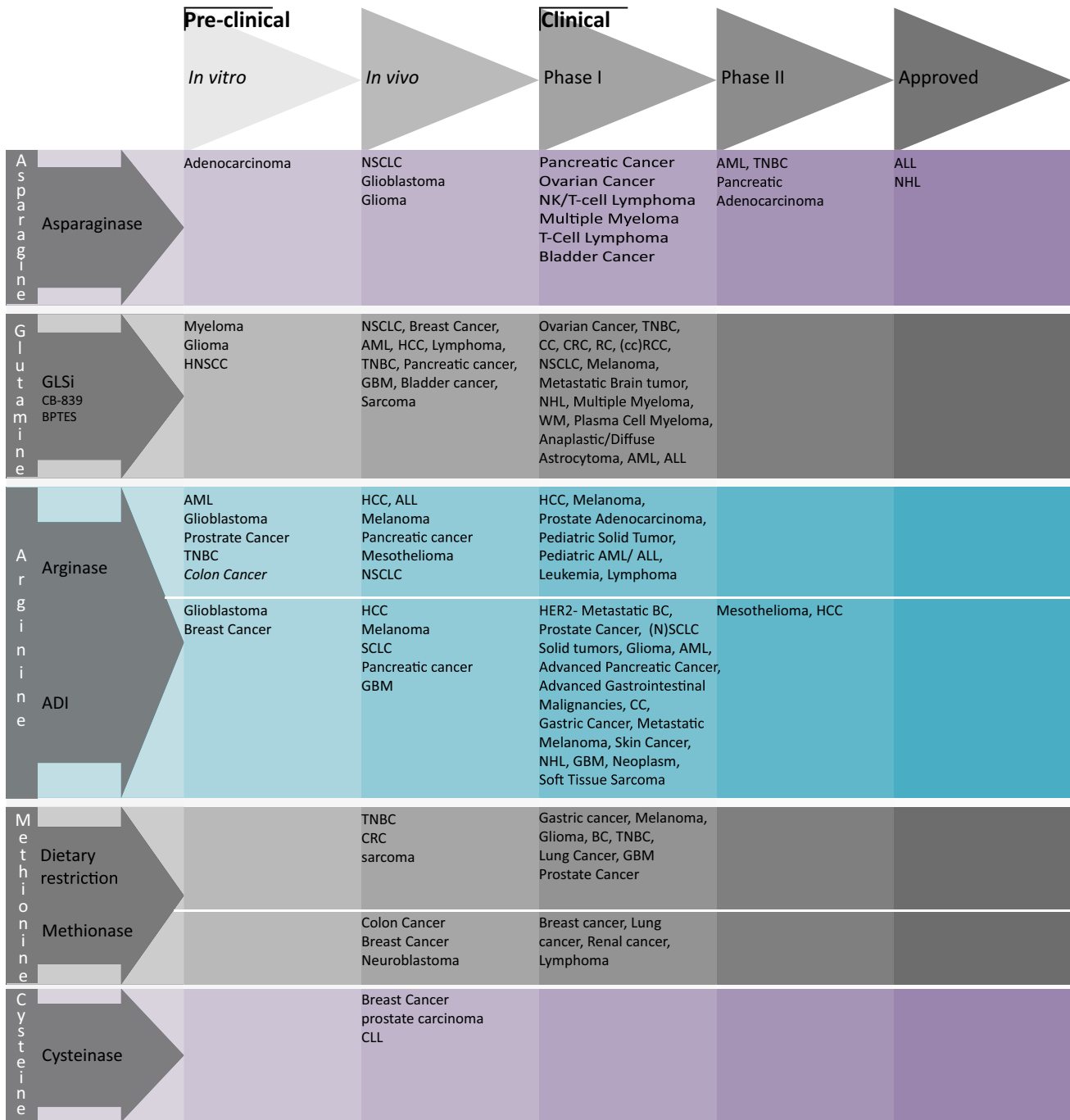
Glutamine

The finding that Gln availability can limit tumor cell proliferation may be surprising, given the fact that it is the most abundant amino acid in serum and, next to glucose, also the most consumed nutrient. In addition to its role as a proteogenic amino acid, Gln is the major source of α-ketoglutarate in the tricarboxylic acid (TCA) cycle. Furthermore, it is utilized in the biosynthesis of all NEAAs [7] and its intermediate glutamate functions as an exchange factor for the import of EAAs [15]. As a result of the high consumption, Gln is considered a conditionally EAA and, as discussed earlier, may be rate limiting to tumor growth. Gln metabolism is highly regulated. Both *de novo* biosynthesis of Gln and glutaminolysis are upregulated in several cancers, via common oncogenes/tumor suppressors including c-Myc and p53 [16]. Increased Gln synthesis is often due to upregulation of Gln synthetase (GS) [15], whereas enhanced glutaminolysis is caused by increased GLS activity and correlates with poor prognosis in glioblastoma, ovarian cancer, breast cancer, medulloblastoma, and lung cancer [16,17].

Tumors driven by c-Myc or KRAS are particularly dependent on exogenous Gln [18,19]. This not only validates Gln as a therapeutic target, but can also be exploited for diagnostic imaging where tumors are detected in PET scans as a result of accumulation of radiolabeled Gln [5].

The identification of Gln metabolism as a therapeutic target has led to the development of Gln mimetics. Despite impressive efficacy *in vitro*, their high toxicity has precluded further development. Pharmacological inhibition of GLS shows more promise. Particularly CB-839 has shown encouraging results as it inhibits tumor cell proliferation in different cancer types both *in vitro* and *in vivo* including triple-negative breast cancer (TNBC) [20], acute myeloid leukemia (AML) [21,22] and non-small cell lung cancer (NSCLC) [21]. CB-839, commercialized as Telaglenastat, has moved to Phase II clinical trials for hematological malignances and solid tumors [10,18,21] (Figure 3). As mentioned before, while Asn is the prime target of ASNase, this enzyme also exhibits GLS activity. With ongoing efforts to study the efficacy of ASNase for the treatment of solid tumors, the GLS effect may become more valuable, and rational design of the enzyme could enhance the GLS activity and thereby the efficacy of ASNase in these distinct tumor contexts.

Arginine

Arginine (Arg) is also a semi-essential amino acid: synthesized from Gln or proline, but with conditional dependence on dietary intake [7]. As a charged amino acid, Arg is essential for the stabilization of protein structure. In addition, it is consumed by the TCA cycle and is a precursor for compounds such as creatine, polyamines, and nitric oxide (NO) [7,23]. Polyamines and NO promote tumor cell proliferation and metastasis, the latter acting as a free radical causing DNA damage [23]. The enzymes involved in *de novo* synthesis of Arg, argininosuccinate synthetase (ASS1), and argininosuccinate lyase (ASL) are frequently deregulated in cancer cells. While elevated expression of these key enzymes is associated with poor survival in different cancer types including glioblastoma, ovarian cancer, and gastric cancer, in other tumors expression is suppressed, leading to Arg auxotrophy [2,23,24]. The enzymes involved in the Arg salvage pathway, using ornithine as a source for Arg, are also subject to deregulation [23,24].

		Pre-clinical		**Clinical**		
		In vitro	*In vivo*	Phase I	Phase II	Approved
Asparagine	Asparaginase	Adenocarcinoma	NSCLC Glioblastoma Glioma	Pancreatic Cancer Ovarian Cancer NK/T-cell Lymphoma Multiple Myeloma T-Cell Lymphoma Bladder Cancer	AML, TNBC Pancreatic Adenocarcinoma	ALL NHL
Glutamine	GLSi CB-839 BPTES	Myeloma Glioma HNSCC	NSCLC, Breast Cancer, AML, HCC, Lymphoma, TNBC, Pancreatic cancer, GBM, Bladder cancer, Sarcoma	Ovarian Cancer, TNBC, CC, CRC, RC, (cc)RCC, NSCLC, Melanoma, Metastatic Brain tumor, NHL, Multiple Myeloma, WM, Plasma Cell Myeloma, Anaplastic/Diffuse Astrocytoma, AML, ALL		
Arginine	Arginase	AML Glioblastoma Prostrate Cancer TNBC *Colon Cancer*	HCC, ALL Melanoma Pancreatic cancer Mesothelioma NSCLC	HCC, Melanoma, Prostate Adenocarcinoma, Pediatric Solid Tumor, Pediatric AML/ ALL, Leukemia, Lymphoma		
	ADI	Glioblastoma Breast Cancer	HCC Melanoma SCLC Pancreatic cancer GBM	HER2- Metastatic BC, Prostate Cancer, (N)SCLC Solid tumors, Glioma, AML, Advanced Pancreatic Cancer, Advanced Gastrointestinal Malignancies, CC, Gastric Cancer, Metastatic Melanoma, Skin Cancer, NHL, GBM, Neoplasm, Soft Tissue Sarcoma	Mesothelioma, HCC	
Methionine	Dietary restriction		TNBC CRC sarcoma	Gastric cancer, Melanoma, Glioma, BC, TNBC, Lung Cancer, GBM Prostate Cancer		
	Methionase		Colon Cancer Breast Cancer Neuroblastoma	Breast cancer, Lung cancer, Renal cancer, Lymphoma		
Cysteine	Cysteinase		Breast Cancer prostate carcinoma CLL			

Figure 3. Current Progress of Amino Acid Depletion Therapies in the Treatment of Cancer. Preclinical and clinical evidence for antitumor activity of the different amino acid therapies. Information on preclinical data was subtracted from Research Papers, whereas clinical information was subtracted from *https://www.clinicaltrials.gov/*. Abbreviations: ADI, arginine deiminase; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; BC, breast cancer; CC, colon cancer; CLL, chronic lymphocytic leukemia; CRC, colorectal cancer; GBM, glioblastoma multiforme; GLS, glutaminase; HCC, Hepatocellular carcinoma; HNSCC, head and neck squamous cell carcinoma; NHL, non-Hodgkin lymphoma; NK, natural killer; NSCLC, non-small cell lung carcinoma; RCC, renal cell carcinoma; TNBC, triple-negative breast cancer; WM, Waldenstrom macroglobulinemia.

In auxotrophic tumors, Arg depletion induces autophagy and apoptosis. By contrast, normal cells enter a state of quiescence and can survive long periods of starvation [25]. Enzymatic depletion of Arg by administration of the human arginase (ARGase) or the bacterial arginine deiminase (ADI) is being explored as anticancer therapy for pediatric patients with relapsed/refractory cancers [Register, E.U.C.T. 2020 (https://www.clinicaltrialsregister.eu/ctr-search/trial/2017-002762-44/NL)]. ARGase is part of the normal urea cycle and converts Arg to ornithine and urea. It is nonimmunogenic and shows no toxicity. However, the antitumor effects of ARGase have been rather disappointing, possibly as a result of a homeostatic feedback where the Arg salvage pathway is utilized to convert ornithine back into Arg, effectively relieving cells from starvation [11,24,26].

ADI converts Arg into citrulline and ammonia and selectively kills cancer cells, while at the same time suppresses angiogenesis [10,24]. Although citrulline can be used for *de novo* synthesis of Arg, in auxotrophic cells this appears to be insufficient [23]. ADI is now under clinical evaluation for different types of cancers [11] with encouraging results: the treatment is well tolerated and shows a therapeutic benefit in Phase I/II clinical trials (Figure 3).

Methionine

Apart from its role in protein synthesis, and as a precursor for cysteine (Cys) and polyamine synthesis, the EAA methionine (Met) is indispensable for the generation of S-adenosylmethionine (SAM), the sole methyl donor for methylation of DNA, histones, and other proteins. Met is catabolized in a series of reactions known as the Met cycle, which is often hyperactivated in tumor cells as a result of upregulation of Met adenosyltransferase 2A (MAT2A) [27]. In contrast to other amino acids, Met metabolism can play an active role in malignant transformation. Downstream of MAT2A, the methyltransferase nicotinamide N-methyltransferase (NNMT) catalyzes the conversion of SAM into S-adenosylhomocysteine (SAH). By effectively consuming all available SAM, this enzyme prevents DNA and histone methylation, affecting the epigenetic landscape of cancer cells [28].

The Met salvage pathway is the only source of Met apart from exogenous supply. This salvage route requires the activity of methylthioadenosine phosphorylase (MTAP) and Met synthase (MS) [11], enzymes that are often downregulated in malignant cells [29]. Furthermore, MTAP is frequently codeleted with the cell cycle regulator CDKN2A [6], rendering cells exclusively dependent on the import of Met from the extracellular environment [6,11]. The increased uptake of Met is used for diagnostic purposes, for instance by visualizing intratumor accumulation of radiolabeled Met using a PET scan for high grade gliomas, but also to predict the therapeutic response in multiple myeloma and brain tumors [6].

With Met being an EAA and given its central role in methylation, it is a prime candidate for therapeutic targeting, particularly in tumors that are driven by mutations in epigenetic modifiers such as TET and IDH proteins and methyl transferases. Indeed, a Met-free diet in tumor bearing mice hampered tumor growth of TNBC, colorectal cancer, sarcoma, glioma, and mixed-lineage leukemia (MLL)-rearranged leukemia, and suppressed metastasis formation [6,30,31]. Exploring its potential in humans, a study showed that dietary Met restriction is relatively well tolerated over a period of 8–17 weeks, with limited side effects, apart from weight loss [32].

Alternatively, promising antitumor effects of enzymatic depletion of Met have been observed using the bacterially derived enzyme L-methionine-gamma-lyase (METase) *in vitro* as well as *in vivo* in neuroblastoma, colorectal cancer, melanoma, and brain tumors [11,33]. METase converts Met to α-ketobutyrate, ammonia, and methanethiol, and showed limited toxicity in Phase I trials [11]. By supplementing homocysteine, vitamin B12, and folate to promote Met

synthesis using the salvage pathway in non-malignant cells, the toxicity of Met depletion can be mitigated in patients with tumors deficient in this route [32].

Serine and Cysteine

Although selective dependencies on other amino acids have been reported, the development of these potential tumor vulnerabilities into therapeutic strategies lags behind the previously discussed amino acids. Ser is a NEAA and next to its role in biosynthesis of proteins, phospholipids and glycine, it feeds into the folate cycle for the production of nucleotides [34]. Tumors, including those driven by the c-MYC oncogene [35], exploit the Ser biosynthesis route to become less dependent on exogenous supply by controlling expression of the enzymes involved [20,36]: PHGDH, phosphoserine aminotransferase 1 (PSAT1), and phosphoserine phosphatase (PSPH). However, this dependency makes them vulnerable to inhibitors of this route. For example: PHGDH inhibitors suppress cancer cell proliferation *in vitro* and in patient-derived xenograft (PDX) models [20,36]. Conversely, other cancers become auxotrophic for Ser during tumor progression [20], facilitating Ser limitation as a possible therapeutic approach. This is particularly the case in TP53 deficient tumors, as they lack the ability to mount an appropriate prosurvival response after Ser depletion [37]. Initial preclinical studies show that combinatorial dietary restriction of Ser and glycine suppresses tumor cell proliferation in mouse models of intestinal cancer and lymphoma [38], as well as in a PDX colon cancer model [37], although information on toxicity profiling and efficacy is currently not available.

As one of the few sulfur containing amino acids and one of the building blocks of glutathione, Cys is crucial for redox homeostasis [39]. While Cys can be produced from Met via homocysteine, this is often insufficient to satisfy the demand, leading to Cys auxotrophy and susceptibility to Cys depletion therapy [40]. In various tumor types, including gastrointestinal cancer, certain lymphomas, leukemias, breast cancers, and hepatocellular carcinoma, transcriptional silencing of enzymes involved in Cys synthesis lead to Cys auxotrophy [40]. The first preclinical tests of enzymatic Cys depletion using Cyst(e)inase (CYSase) showed decreased tumor cell proliferation and increased survival of mice transplanted with PDX derived from prostate cancer [40] or chronic lymphocytic leukemia (CLL) [41], potentially involving ferroptosis as an inducer of cell death [42].
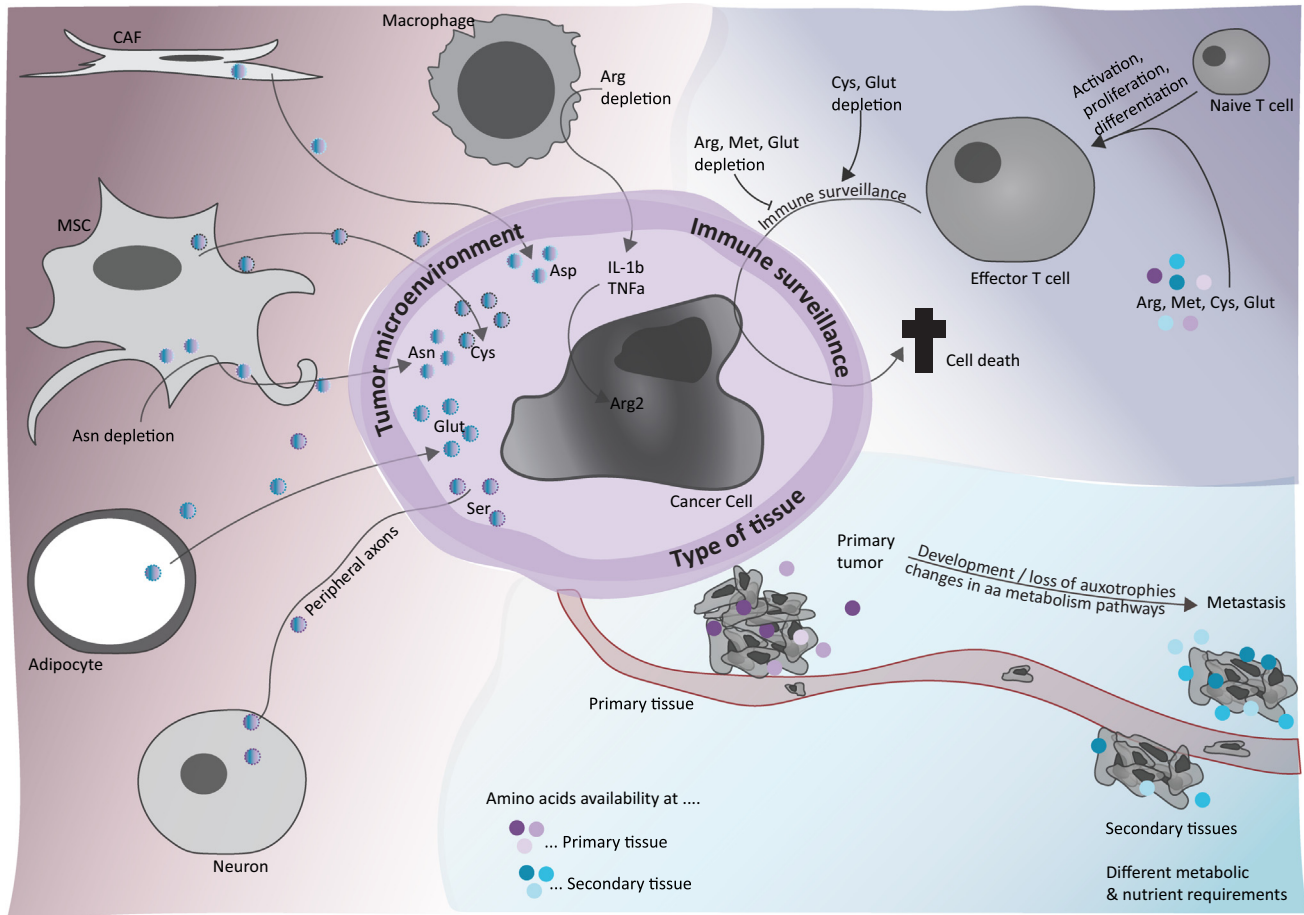
The Effects of Amino Acid Interventions Are Highly Context Dependent

Amino Acid Supplementation as an Antitumor Strategy

In spite of the broad applicability of amino acid depletion therapies in the treatment of cancer, in some cases high abundance of a specific amino acid may have antitumor effects. This can be either intrinsic to the tumor cells or involve extrinsic mechanisms such as stimulation of an antitumor immune response. Dietary Gln supplementation for example, was shown to block melanoma tumor growth and prolong survival in a transgenic mouse model by affecting epigenetic reprogramming [43], whereas the supplementation of histidine (His) increased the sensitivity of leukemic xenografts to methotrexate (MTX) [44]. Hereby His flux drains the cellular pool of tetrahydrofolate, the enzymatic cofactor required for nucleotide biosynthesis, which is also targeted by MTX [44].

Location of the Tumor

The efficacy of therapeutic interventions using amino acid availability is determined not only by cancer cells themselves, but also by external factors, including the location of the tumor and their impact on immune surveillance (Figure 4) [4]. Hence, it might not come as a surprise that the same tumor cells can show different metabolic and nutrient requirements at their primary or metastatic site. For instance, breast cancer cells were shown to upregulate Ser biosynthesis to support

Figure 4. The Effects of Amino Acid Interventions are Highly Context Dependent. The efficacy of therapeutic interventions using amino acid availability is determined by different external factors including the type of tissue, the tumor microenvironment, and immune surveillance. Tumor cells originating from the same cancer have different metabolic and nutrient requirements at their primary or metastatic sites. Furthermore, cells from the tumor microenvironment can supply cancer cells with depleted amino acids. To effectively fight cancer cells, T cells need to get activated, proliferate, and differentiate, which consumes high amounts of amino acids. As a consequence, immune surveillance is strongly dependent on amino acid availability. Abbreviations: AA, amino acids; CAF, cancer associated fibroblast; MSC, mesenchymal stem cells.

mammalian target of rapamycin complex 1 (mTORC1) growth signaling when metastasized to the lung, but not at their primary location [45], while Cys uptake via the transporter xCT appears to be more important for mammary metastases as compared with the primary tumor [46]. Moreover, the limited availability of Ser and Gly in the brain forces metastasized cells in this tissue to upregulate Ser biosynthesis making these, but not the extracranial growing tumors, sensitive to PHGDH inhibitors [47].

Immune Surveillance

When it comes to antitumor immunity, amino acid depletion predominantly suppresses the anti-tumor activity of immune cells, including T cells. The clonal expansion and maturation process that is needed to generate effector T cells that target cancer cells, requires increased metabolic needs for glucose and amino acids. Cancer cells are able to suppress immune cell function by outcompeting immune cells for specific amino acids, and obviously, amino acid depletion strategies may antagonize an immune response [48].

The role of Arg, Cys, Gln, and Met in T cell function has been relatively well studied. Arg is one of the most essential amino acids for T cell proliferation, activation, and effector function and supplementation of Arg might thus be clinically beneficial for Arg non-auxotrophic tumors. Indeed, Arg supplementation promotes immune surveillance by supporting T cell mediated antitumor activity and even synergizes with (chemo-) immunotherapy in different cancer models [48,49]. Furthermore, inhibition of the arginase Arg1 was shown to induce antitumor immunity by inhibition of myeloid cell mediated suppression of T cell proliferation [48]. Also Met is critical for T cell survival and Met starvation causes alterations in histone methylation that impair T cell function [50]. Dietary supplementation of Met can restore those epigenetic alterations and increase T cell immunity in tumor bearing mice and patients with colon cancer [50]. Cys and Gln are essential for T cell expansion and activation, respectively [48]. However, in other reports amino acid depletion was shown to have immunostimulatory effects. Single treatment with CYSase was shown to induce antitumor T cell responses and its cytotoxicity against tumor cells could be further increased by T cell mediated release of interferon γ induced by immunotherapy [programmed death-ligand 1 (PD-L1) blockade] [48]. Gln blockade induced nutrient depletion in tumor cells and T cells and was shown to evoke different molecular responses in both cell types. T cells upregulate their oxidative metabolism resulting in long-lived and highly activated T cells, whereas tumor cells fail to do so and eventually die [51]. Given the diversity in effects of amino acid availability on immune surveillance, it will be important to investigate the efficacy of amino acid depletion therapies in immunocompetent models.

In summary, before amino acid depletion can be clinically applied, not only do metabolic dependencies of a particular cancer type need to be investigated, but also extrinsic factors need to be considered, such as their location and metabolic crosstalk with other cell types including immune cells (Figure 4). Also, the effects of amino acid depletion on the efficacy of other treatments will have to be carefully investigated and scheduling and dosing will need to be optimized.

Future Perspectives and Challenges for Amino Acid Depletion Therapies
Synergy of Amino Acid Depletion with Other Therapies
Amino acid starvation may enhance the efficacy of conventional chemotherapy: the induction of cell cycle arrest in normal cells [52] may protect these cells from the DNA damage inflicted by chemotherapeutics while synergizing with these drugs in killing tumor cells.

Synergistic effects in combination with amino acid depletion have been reported with both chemotherapeutics and targeted therapies (Table 1). While the mechanism underlying these synergies often remains to be elucidated, for some combinations the mode of (inter)action is clearer: fluorouracil (5-FU), a pyrimidine analogue, and Met depletion converge on the folate cycle, both acting to inhibit thymidylate synthase (TS) function. Moreover, Met restriction leads to downregulation of O6-alkylguanine-DNA alkyltransferase (AGT), an enzyme that eliminates alkyl groups from DNA [53], thereby enhancing the effect of alkylating agents. Interestingly, although amino acid depletion, unlike classical chemotherapeutic agents, does not act primarily by provoking DNA damage, amino acid depletion can lead to nucleotide imbalances that could affect mutational signatures. A positive effect from such changes can be the generation of neo-antigens on the tumor cells as targets for immunotherapy [54].

Alternatively, strategies that enhance the effect of amino acid depletion may help to reduce the need for conventional chemotherapeutics, although the design and use of sensitizers is mostly in a preclinical phase. An obvious approach is to counteract cell-intrinsic mechanisms of therapy resistance. The most straightforward mechanism for auxotrophic tumor cells to acquire resistance, is by upregulating enzymes responsible for cellular production of the depleted amino

Table 1. Drug Combinations with Amino Acid Depletion Therapies

Depletion of	Drug combination	Refs
Glutamine	Venetoclax	[21]
	FLT3 tyrosine kinase inhibitor	[75]
	Notch inhibition	[76]
	Glucose metabolism	[77,78]
	Modulators of the integrated stress response	[64]
	mTORC1	[55,65]
	Metformin	[66]
Asparagine	Doxorubicin	[79]
	BH3 mimetics	[80]
	KRAS pathway inhibition	[56]
	Chloroquine	[57]
	Targeting Gln metabolism	[60]
	GCN2 inhibition	[81]
	GLS inhibition	[60,62]
	ZBTB1	[72]
	Wnt/STOP signaling	[73]
	Metformin	[67]
Arginine	Cytarabine	[26]
	Histone-deacetylase inhibitor (SAHA)	[82]
	Chloroquine	[58]
	Targeting Gln metabolism	[61,63]
Methionine	Fluorouracil	[83]
	Doxorubicin	[84]
	Vincristine	[84]
	BCNU	[33]
	Temozolomide	[33]
	Cisplatin	[85]
	TRAIL-R2 agonist	[86]
Leucine	Epidermal growth factor receptor (EGFR) inhibitors	[87]
	Tamoxifen	[88]
Cysteine	BSO	[40]
	Curcumin	[40]

acid. For example, tumors may induce ASS1 expression upon ADI treatment [55] while ARGase treatment may promote ornithine recycling into Arg [11]. ASNase sensitive NCSLC cell lines become resistant by inducing ASNS expression in a KRAS dependent manner. Combining ASNase treatment with KRAS pathway inhibition *in vitro* and *in vivo*, re-sensitizes cells to ASNase-induced cell death [56].

Autophagy, the stress activated catabolism of macromolecules and even complete organelles in order to preserve and recycle energy and nutrients is a potent rescue mechanism for cells to overcome periods of limited availability of resources [57]. ASNase is known to induce cytoprotective autophagy in ovarian cancer, chronic myeloid leukemia (CML), and ALL [57] and also ADI therapy

promotes autophagosome formation *in vitro* [58]. Autophagy inhibitors such as chloroquine (CG) can re-sensitize cells to those amino acid depletion therapies [57], although this may deprive normal cells from this cytoprotective process as well.

Many mechanisms by which cells can acquire resistance are related to a switch in metabolic dependencies, frequently leading to the formation of another Achilles heel. For example, breast cancer cell lines resistant to the GLS inhibitor CD-839 show downregulated Gln consumption, but an increased dependence on exogenous Asn [59]. Conversely, increased activity of Gln transporters through post-translational modifications induces a Gln dependent resistance against ASNase [60] and similar mechanisms were found in ADI resistant cell lines [61]. Both the ASNase [60,62] and ADI [61,63] resistant tumor cells could be re-sensitized by targeting Gln metabolism.

Our growing understanding of tumor cell metabolism also allows for rational design of combination therapies. Targeting two or more nutrients simultaneously could prevent cells from compensating one addiction with another. Furthermore, targeting modulators of the integrated stress response [64], mTORC1 [55,65], redox homeostasis [40], or oxidative phosphorylation [66,67] can enhance the antitumor response of amino acid depletion therapies.

Other resistance mechanisms developing in response to nutrient depletion therapies have been observed, such as upregulation of eEF2 kinase by blocking translation elongation [68] or drug-resistant mutations, such as GLS-K325A, which leads to resistance towards GLS inhibitors (BPTES, CD-839) [69]. Current efforts include drug screens and CRISPR/Cas9 based screens to identify actionable pathways that may enhance tumor cell killing in combination with amino acid depletion strategies [70,71]. These approaches recently led to the identification of ZBTB1 [72] and Wnt/STOP signaling [73] and BTK [74] as possible targets to enhance the efficiency of ASNase.

Challenges Remaining

As with all drugs, resistance, either intrinsic or acquired, remains a formidable challenge. Resistance can not only occur as a result of cell autonomous factors, as explained previously, but can also be induced by extrinsic factors such as the tumor environment. Suboptimal amino acid depletion may be sufficient to maintain tumor cells in a state of cellular quiescence rather than to induce apoptosis, increasing the chance of relapse once the treatment is discontinued. This can occur when amino acid depletion enzymes cannot reach the tumor cells because of poor penetrance of the drug at so-called sanctuary sites, such as the central nervous system or the bone marrow [89]. Furthermore, amino acids can be provided by cells present in the tumor microenvironment (Figure 4). For leukemia it was shown that mesenchymal stem cells (MSCs) in the bone marrow niche protect leukemic blasts from ASNase induced cytotoxicity [90], whereas bone marrow stromal cells provide Cys for CLL cells [91]. Moreover, obesity is known to impair the efficacy of therapy in ALL as adipocytes can release Gln causing leukemia cell resistance to ASNase [92]. Similarly, cancer associated fibroblasts (CAFs) support solid tumor growth by providing Asp to carcinoma cells [93]. Interestingly, in this model, the tumor cells return the favor by providing glutamate that allows the CAFs to balance their redox state. Also, Ser-starved pancreatic ductal adenocarcinoma were shown to attract peripheral axons secreting Ser into the tumor [94] while in neuroblastoma, tumor associated macrophages release interleukin 1β and tumor necrosis factor α in response to Arg depletion, leading to upregulation of Arg2 in the neuroblastoma cells, creating an immune suppressive environment, which correlates with a poor outcome [95] (Figure 4). Hence, there is a close interaction between tumor cells and cells within their tumor environment, altering the amino acid availability upon treatment.

Another major challenge of therapies using therapeutic enzymes of non-human origin, is the immune response that is mounted following recognition of these therapeutic proteins as non-self. The development of inhibitory antibodies results in enhanced clearance of the enzyme, allowing amino acid concentrations to rapidly return to baseline levels [12,96]. In addition, allergic reactions, ranging from mild hypersensitivity reactions up to anaphylactic shock, can prevent the continuation of treatment [39,96]. To increase the half-life and reduce immunogenicity, therapeutic enzymes have been modified by conjugation to polyethylene glycol (PEG) [96], nanoparticle encapsulation [78], or erythrocyte encapsulation [97], and the treatment can be combined with immune-suppressive agents such as prednisone and dexamethasone. Furthermore, *in silico* and *in vitro* immunogenicity prediction tools can be used to assess the immunogenicity potential of a protein drug beforehand [98].

Concluding Remarks

Amino acid depletion strategies show great promise in the treatment of cancer. A major advantage over other therapies is their limited toxicity and the absence of late effects as a result of DNA damage. However, before amino acid depletion can be applied more broadly in the clinic, the metabolic dependencies of particular cancer types and its tumor environment need to be investigated in detail, allowing selection of the right amino acid target. Also, therapeutic agents including metabolic inhibitors are unlikely to be effective as a single agent, as metabolic changes in tumor cells exposed to amino acid starvation may render cells resistant to the therapy (see Outstanding Questions). Therefore, future applications of amino acid depletion therapies will likely involve a combination with (targeted) agents in order to prevent resistance mechanisms to occur. In order to prevent tumor regrowth by the persistence of residual cancer cells, a deep initial response will have to be achieved. This implies a multidrug strategy that will simultaneously inhibit different (metabolic) pathways within cancer cells and takes into account reciprocal interactions with the tumor microenvironment. Therefore, amino acid depletion sensitizers will need to be developed that increase both the efficiency and durability of the response.

Declaration of Interests

No interests are declared.

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Published online February 17, 2021. https://doi.org/10.1016/j.cmet.2021.02.001

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