Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;51:854-870
DOI: 10.1159/000495382
Published online: 22 November 2018
Accepted: 14 November 2018

Review

Arginine-Depleting Enzymes – An Increasingly Recognized Treatment Strategy for Therapy-Refractory Malignancies

Christin Riess[a,b] Fatemeh Shokraie[b,c] Carl Friedrich Classen[b]
Bernd Kreikemeyer[a] Tomas Fiedler[a] Christian Junghanss[d]
Claudia Maletzki[d]

[a]Virology, and Hygiene, Rostock University Medical Center, [b]University Children's and Adolescents' Hospital, Rostock University Medical Center, [c]Department of General Surgery, Molecular Oncology and Immunotherapy, Rostock University Medical Center, [d]Department of Medicine, Clinic III-Hematology/Oncology/Palliative Care, Rostock University Medical Center, University of Rostock, Germany

Key Words
Arginine-auxotrophy • Arginine deiminase • Therapy resistance • Combination therapy • Tumor microenvironment

Abstract
Arginine auxotrophy occurs in certain tumor types and is usually caused by the silencing of *argininosuccinate synthetase* 1 or *arginine lyase* genes. Such tumors are often associated with an intrinsic chemoresistance and thus a poor prognosis. Arginine auxotrophy however renders these tumors vulnerable to treatment with arginine-degrading enzymes. Among the most frequently applied arginine-degrading agents are bacterial arginine deiminases (ADI). The anti-cancerous effects of ADI derived from different bacteria were extensively studied in numerous preclinical cell culture and xenograft models. *Mycoplasma*-derived ADI-PEG20 is most commonly used and is currently under clinical investigation as a single agent therapeutic as well as in combination with different antineoplastic compounds. Mechanistically, ADI is capable of reducing metabolic activity in tumor cells, contributing to autophagy, senescence and apoptosis in arginine auxotrophic cells. Although clinical trials are promising, the resistance development upon initial treatment response is an increasing challenge. Furthermore, interference of ADI with the tumor microenvironment is poorly understood. In the present review, we outline recent experimental ADI-based treatment approaches and their translation into the clinic. Furthermore, we summarize new insights into the molecular mechanisms

Claudia Maletzki, PhD

Department of Medicine, Clinic III-Hematology/Oncology/Palliative Care
Rostock University Medical Center, University of Rostock, 18057 Rostock (Germany)
Tel. +49 381 494 5764, Fax +49 381 494 5898, E-Mail claudia.maletzki@med.uni-rostock.de

underlying the anti-cancer effects of ADI that might facilitate the refinement of ADI-based combination therapy approaches.

Introduction

Cancer precision medicine is based on the identification of specific biomarkers to predict the individual treatment response of patients prior to intervention. These approaches increasingly rely on metabolic defects that are unique to a particular cancer subgroup. The nutritional requirement for arginine is such a specific feature of some tumor types (=Arginine auxotrophy).

This idea dates back until the early 1930s, when Gilroy showed in his pioneering work, that mice receiving an arginine-enriched diet developed faster and larger tumors than mice on a standard diet. In contrast, an arginine deficient diet reduced tumor incidence and growth [1]. In the 1960s, independent research teams observed the killing of human tumor cells in Mycoplasma contaminated/infected cultures. It is worthy of note that lysis was confined to leukemia and cervical carcinoma cells, while normal embryonic cells remained unaffected [2, 3].

A few years later the Arginine Deiminase (ADI) system was identified as an arginine-degrading pathway not present in mammalian cells [4], and seminal work confirmed that ADI from *Mycoplasma sp. was* responsible for arginine withdrawal from the culture medium and the resulting anti-cancer effect [5]. Thereafter, it was J. B. Jones who first applied purified ADI from *Pseudomonas pudita* as an anticancer agent in her PhD thesis [6]. Since then, numerous studies were initiated to treat arginine auxotrophic cancers and today, *Mycoplasma sp.*-derived PEG-formulated ADI is the bacterial enzyme most frequently used in (pre-) clinical trials with proven efficacy in a variety of arginine-requiring cancers [7–10].

In this review, we give an overview of recent experimental and clinical studies and ways to improve ADI-based treatment by combination with classical and novel drugs. Finally, we shed light on the interference of ADI with the immune system and possible consequences of this treatment strategy on tumor immune surveillance.

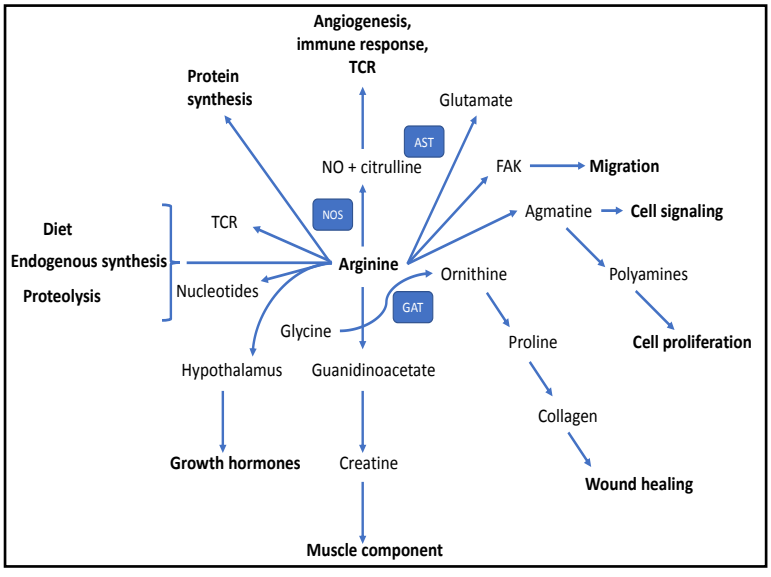

Fig. 1. Schematic overview presenting arginine-mediated involvement in metabolic pathways and functional effects. GAT = glycine amidinotransferase, NOS = NO-synthase, AST = arginine succinyltransferase, FAK = focal adhesion kinase, TCR = T cell receptor, NO = nitric oxide. Enzymes are displayed in blue boxes. Functions/effects are presented in bold.

Sources and metabolism of Arginine

Arginine belongs to the semi-essential amino acids for humans. It is indispensable for synthesis of proteins, urea, polyamines, agmatine and amino acids like glutamate and proline [11–14]. It improves wound healing, stimulates the release of growth hormones and is involved in immunomodulatory effects, such as T cell receptor expression, activation of killer T cells, and development of immunological memory (Fig. 1) [15–17]. Additionally, arginine is crucial for nitric oxide (NO) synthesis by inducible nitric oxide synthase that leads to vasodilatation (Fig. 1) [18]. Extracellular arginine sources for humans originate from plant and animal proteins. In most cells, extracellular arginine crosses the plasma membrane via solute carrier proteins (system y + family) [19].

Arginine is synthesized from citrulline in two steps (Fig. 2): First, argininosuccinate synthetase 1 (ASS1) catalyzes the conversion of L-citrulline and aspartic acid to argininosuccinate, which is then further converted to arginine and fumaric acid by argininosuccinate lyase (ASL). In a second step, arginase degrades arginine to L-ornithine and urea. L-ornithine is converted back to L-citrulline by ornithine transcarbamylase (OTC) and finally recycled back to arginine by ASS1/ASL (Fig. 2). OTC is primarily found in the liver, whereby it is epigenetically silenced via hypermethylation in other tissues [20]. Several other enzymes are also involved in arginine metabolism. These include nitric oxide synthase (NOS) to produce NO from arginine. The glycine amidinotransferase catalyzes the synthesis of ornithine from glycine and arginine and the arginine decarboxylase (ADC) decarboxylates arginine to agmantine and CO_2 (Fig. 2).

A characteristic feature of malignant diseases is a disordered arginine metabolism, playing a critical role in tumor development and progression [21]. Due to increased demand on polyamines and involvement in a number of biosynthetic processes, arginine influences cell growth, proliferation, and carcinogenesis [22] (Fig. 1). Some cancers may even downregulate energy-requiring pathways needed for the biosynthesis of arginine in favor of import from exogenous sources to support rapid growth with minimal energy effort [23].

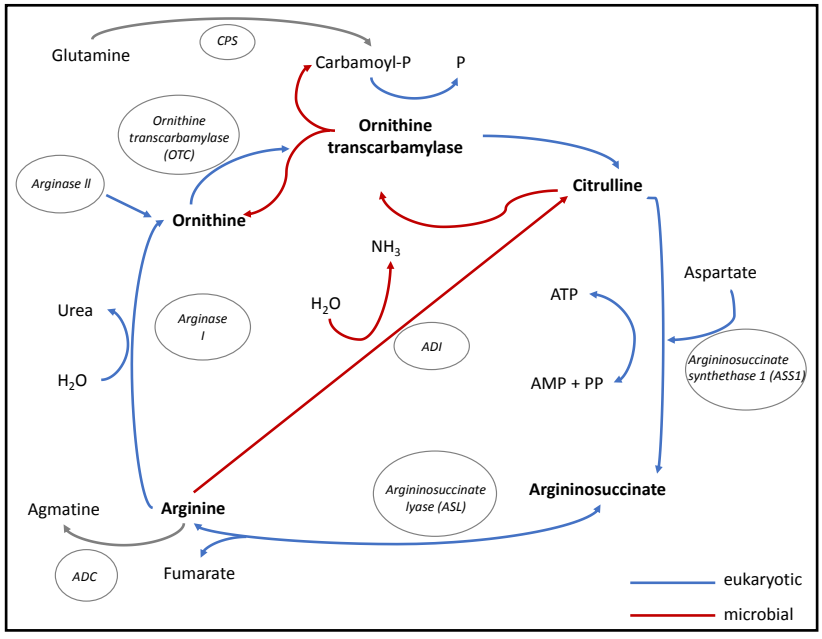

Fig. 2. Eukaryotic and microbial arginine metabolism. Arginine can be synthesized from citrulline in a two-step process via the urea cycle enzymes argininosuccinate synthetase 1 (ASS1) and argininosuccinate lyase (ASL). ASS1 catalyzes the conversion of citrulline and aspartic acid to argininosuccinate, which is cleaved by ASL into arginine and fumaric acid. Arginine is then degraded by arginase to ornithine and urea. OTC metabolizes ornithine into carbamoyl phosphate and citrulline. Arginine can be degraded by several enzymes, among them ADI, which catalyzes the reaction from arginine to citrulline and ammonia. The eukaryotic pathway is indicated by blue lines and microbial pathway by red lines. Enzymes are shown within circles. CPS = Carbamyl phosphate synthetase, ADI = arginine deiminase, ADC = arginine decarboxylase.

Arginine auxotrophy in human malignancies

Arginine auxotrophy is increasingly recognized as a frequent feature of human malignancies. Originally described in malignant melanoma (60-100 %) and hepatocellular carcinoma (HCC, 100 %), arginine auxotrophy has been found in many other advanced, solid tumors. These include: prostate (100 %), pancreatic (>80 %), breast (up to 60 %), and small cell lung cancer (44 %), malignant pleural mesothelioma (~60 %), head and neck squamous cell carcinoma (50 %), as well as Glioblastoma multiforme (GBM, ~20 %) [8,11,24–26]. Among hematological malignancies, 60 – 100 % of all acute myeloid leukemia (AML) cases, as well as primary and relapsed lymphomas, show this metabolic defect [7,11]. Clinically, the majority of arginine auxotrophic tumors are present at an advanced stage when diagnosed, with most of them showing intrinsic chemoresistance. Indeed, this fact has become a prognostic biomarker for reduced metastasis-free survival (=aggressive phenotype) and an accordingly poor outcome. It is, in this regard, still unknown whether arginine auxotrophy is a driving mutational event that initiates tumor escape mechanisms or just constitutes a bystander effect emerging from multiple chromosomal and structural alterations. The observation of arginine auxotrophy in a variety of solid and hematological malignancies, with each of them showing an individual and heterogeneous mutational profile plus different numbers of mutational events, i.e. high [melanoma] vs. low [pancreatic cancer]) makes addressing this question even more complicated.

Silencing of key enzymes for *de novo* arginine biosynthesis constitutes the underlying molecular mechanism. These enzymes include ASS1, ASL, OTC, carbamoyl phosphate synthase I, and arginase I. Genetic as well as epigenetic suppression of *ASS1* is most common and usually presented by CpG island promoter methylation, as proven by (I) methylation-specific PCR and (II) in demethylation experiments using 5-aza-2`-deoxycytidine [11]. Methylation-specific PCR is the method of choice for detecting arginine auxotrophy in tumors [11,26,27], as it is described as being more sensitive than ASS1 immunohistochemistry. Formerly considered a ubiquitously expressed protein in normal cells, a distinct lack of ASS1 protein abundance was observed in non-malignant tonsil and lymphoid tissues [11]. *ASS1* gene regulation is thus complex, being differential according to cell type, differentiation status, and function. Another mechanism is O-glycosylation of the transcription factor Sp1 by glutamine to facilitate nuclear translocation and transactivation of the *ASS1* promoter. In addition, iNOS mediated exploitation of arginine activates the *ASS1* promoter, accompanied by enhanced NF-kB activity under pro-inflammatory stimuli. In some tumors, such as malignant melanomas, *ASS1* promoter repression is a result of enhanced hypoxia-inducible factor-1 alpha (HIF1a) activity [28]. *ASS1* expression is controlled by c-Myc and HIF-1α interacting with an E-box element located at the *ASS1* gene promoter [29] (Fig. 3). From a diagnostic point of view, a combination of several methods is highly desirable to accurately determine susceptibility of the tumors to arginine depleting agents.

The prognostic and predictive value of *ASL* and *OTC* as additional key determinants of the auxotrophic status of tumor cells is largely unknown. As no antibodies recognizing the ASL protein exist, examining its presence or absence by immunohistochemistry is inapplicable. However, the tightly coupled and dynamic control of *ASS1* and *ASL* according to cellular stresses suggests a direct link to both genes' expression. In a previous publication by Syed et al., 22 % of primary GBM cultures were found to harbor a methylated CpG island in the *ASL* core promoter, leading to significant downregulation of *ASL* mRNA [27]. In our own studies on patient-derived GBM cell lines, comparable results were obtained, with reduced *ASL* mRNA levels in half of the examined cases (12 in total) [30]. Additional genes *OTC* and *arginase I* were almost entirely downregulated in this cohort, whereas *carbamoyl phosphate synthase I* was expressed by all cell lines [31]. A reduced transcription level of one of those genes was seen in particular in cells with normal *ASS1/ASL* expression [31]. Supporting these findings, a recent report described significant downregulation of *OTC* in pediatric sarcomas and brain tumors with normal *ASS1* expression [32].

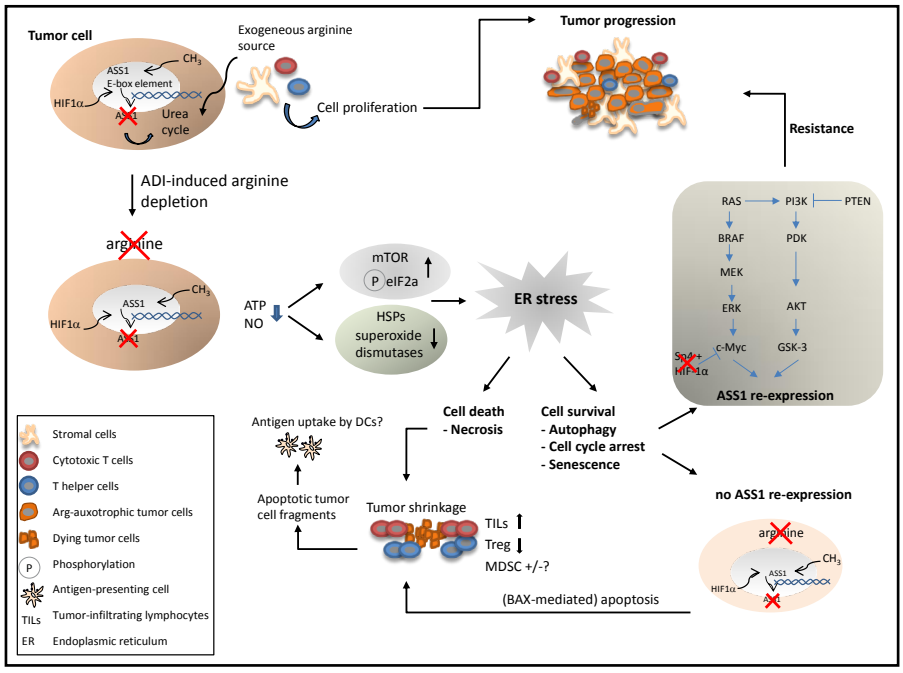

Fig. 3. Scheme illustrating ADI responsiveness and resistance development. Tumor cells become arginine-auxotrophic by either HIF1α-mediated or epigenetic (methylation) silencing of the arginine-synthesizing enzyme ASS1 (for clarity, other key enzymes for *de novo* Arg biosynthesis are not shown). To ensure cell growth, proliferation and survival, arginine-auxotrophic tumor cells obtain arginine from exogenous sources, such as tumor-infiltrating and surrounding normal cells (stromal and immune cells). However, upon ADI-mediated arginine depletion, cells become stressed. Normal cells utilize L-citrulline for growth. Arginine-auxotrophic tumor cells respond with lowered ATP and NO levels, resulting in endoplasmic reticulum (ER) stress. Prolonged stress may lead to necrotic cell death. In most cases, however, stress responses result in autophagy, cell cycle arrest and/or senescence. This has two consequences: (I) apoptotic cell death upon prolonged arginine withdrawal due to the tumor cells' inability to re-express ASS1 or (II) molecular reprogramming (i.e. ASS1 re-expression) to enable regrowth. While the former is associated with enhanced infiltration of (T) lymphocytes, yielding boosted immune response and clinical response, the latter provokes ADI resistance and thus tumor progression.

Conversely, in some *ASS1*-negative tumors an abnormally increased *ASL* expression can be found [33]. This is accompanied by higher clinical aggressiveness mediated by NO and cyclin A2 [34]. Here, blocking *ASL* with shRNA attenuates HCC and breast cancer growth and progression *in vivo* via inhibition of cyclin A2 and NO.

The failure to express at least one of these recycling enzymes renders cells dependent on extracellular arginine and provides another rationale for arginine deprivation based therapies to selectively starve tumors. By culturing tumor cells in media without arginine, up to 80 % killing can be seen in arginine-auxotrophic cells [35, 36].

Strategies for arginine-deprivation therapy - experimental approaches to target cancer

Standard oncologic treatment strategies cover the four main columns including immunotherapy, surgery, radiation, and chemotherapy. In addition, an increasing number of novel and targeted approaches exist, each of them having assets and drawbacks. Though great progress was made for some tumors, e.g. colorectal cancer (CRC), melanoma, and breast cancer, no or little improvement in outcome has been attained for others (e.g. GBM and head and neck cancer) [37, 38]. Innate as well as acquired resistance mechanisms along with spatial (i.e. different response in different lesions) and/or temporal (e.g. stable disease followed by progression) heterogeneity constitute a major challenge. For arginine-auxotrophic tumors,

three (main) different experimental approaches to arginine-deprivation therapy exist. These include (1) dietary restriction of arginine, (2) inhibition of arginine sensing and transport, and (3) enzymatic degradation of arginine. Arginine-restricted diets have only been successfully applied in CRC and skin cancer mouse models so far but did not prove to be effective in humans [1, 39–42]. In CRC, breast cancer and myeloid leukemia cell lines, arginine uptake was decreased, cell growth was inhibited and apoptosis was increased upon knockdown of the energy-independent cationic amino acid transporter CAT-1. CAT transporters, together with L-type amino acid transporters, are responsible for arginine transport in non-malignant tissue [43–45]. To our knowledge there is no competitive inhibitor available which blocks all relevant arginine transporters. Hence, arginine-degrading enzymes are currently the most promising approach to arginine deprivation therapy. Therapeutically relevant arginine-degrading enzymes include (1) arginase 1 catabolizing arginine to ornithine, (2) arginine decarboxylase (ADC) decarboxylating arginine to agmatine and CO_2, and (3) arginine deiminase (ADI) degrading arginine to citrulline and ammonia [19, 30, 46]. The potential of each of these enzymes for arginine degradation not only depends on their efficiency, but also on their immunogenicity, catabolic products as well as stability in patients.

Arginine-depleting enzymes

Arginase

The native mammalian urea cycle enzyme arginase 1 has been used to target arginine-auxotrophic cancer cells since the 1950s [47]. For application in patients, the use of human arginase 1 has an advantage, as immunogenicity is not an issue. However, arginase 1 currently still fails to be clinically applicable due to the following reasons: (I) no significant anti-cancer effects in early small animal experimental models; (II) low affinity for arginine; (III) toxicity because of ornithine accumulation; (IV) a short half-life ($t_{1/2}$ <30 min) as well as (V) an unfavorable pH value in the patient blood stream (7.4), as the enzyme works best at basic pH levels and loses up to 90 % of its activity at pH 7.4 [19, 47]. Still, efforts to safely apply this enzyme are on the way, e.g., PEGylation to increase serum half-life or adapting the pH optimum by using cobalt instead of manganese ions as cofactors [10, 19, 48]. Recombinant human arginase (rhArg) was under clinical investigation as an anticancer drug; the best responses were transient stable disease and a manageable safety profile [49]. In *OTC*-deficient prostate cancer cells, rhArg exhibited significant anti-proliferative activity during pre-clinical evaluation [48]. Comparable effects were seen in HCC and melanoma *in vitro* and *in vivo* [10, 50]. In these tumors, sensitivity towards rhArg is even independent of *ASS1* expression. The above mentioned issues - especially the cytotoxicity towards noncancerous cells - pose major restrictions to the applicability of arginase 1 in cancer therapy.

Arginine decarboxylase

ADC catalyzes the decarboxylation of arginine to agmatine and carbon dioxide. Treatment of tumor cells with ADC can deplete arginine and thus inhibit tumor cell growth. Such effects were seen in the human cervical epithelial carcinoma cell line HeLa. Mechanistically, ADC treatment causes cell cycle arrest and apoptosis [51–53]. Although these data suggest that ADC may provide another interesting agent for Arg-depleting strategies, there are major drawbacks of this enzyme. As for arginase 1, severe side effects on noncancerous cells may occur upon ADC treatment because the cytotoxic amine agmatine is produced by the decarboxylation of arginine. Furthermore, PEGylation – usually used to increase the serum half-life of therapeutic enzymes – completely abolishes the activity of ADC [54]. Hence, ADC is currently far from being clinically applicable.

Table 1. Properties of Arginine deiminase of different bacterial species

	M. hominis	M. arginini	S. pyogenes
MW (kDa)	47 (35)	45	47.2
K_M (L-Arginine)	0.03 mM, 0.1-1 mM	0.2 mM	1.33 ± 0.12 mM (0.67 mmol·L(-1) and 42 s(-1))
Optimal temperature	>37 °C	50 °C	37 °C
Optimal pH	6.0	6.0-7.5	6.5 (6.8)
Circulatory half-life (w/o PEG)	30 mins/4 h	-	-

ADI

Bacterial ADI is the most frequently used and most promising of the arginine-degrading enzymes. The first ADI enzyme was isolated from *Bacillus pyocyaneus* and described as reviewed in [24]. Subsequently, ADI was identified in the species of numerous genera, e.g. *Pseudomonas*, *Bacillus*, *Enterococcus*, *Lactobacillus*, *Streptococcus*, and *Mycoplasma* [55–61]. Numerous bacterial ADI have relatively low affinities to arginine or unfavorable pH or temperature optima for application in humans (Table 1). However, the reaction optima of ADIs from some species fit well with the conditions in humans. Among those are ADI from *Streptococcus pyogenes*, *Pseudomonas plecoglossicida* or *Mycoplasma* species, having their temperature optimum at around 37 °C and their pH optimum in the neutral to slightly acidic range, which corresponds to conditions in the microenvironment of solid tumors [46, 47]. Due to the high affinity to arginine, ADI derived from *Mycoplasma* species are by far best investigated and are the most frequently applied enzymes in experimental and clinical studies. E.g., *Mycoplasma hominis* ADI has an ~1,000-fold higher affinity for arginine (Km ~30 μM) than human arginase 1 (Km ~45 mM) [47, 62] (Table 1). As ADIs are bacterial enzymes, they are highly immunogenic in humans and exhibit a short half-life (rapidly cleared from circulation within 30 min via glomerular filtration). For clinical applications, ADI is being conjugated with up to 16 polyethylene glycol molecules of 20 kDa (ADI-PEG20, Polaris, Inc (Phoenix Pharmacologics)). This modification reduces antigenicity while greatly enhancing its pharmacokinetic circulatory half-life of approximately 4 hours in serum; it also displays optimal activity at a physiological pH [62, 63].

In March 1999, the US Food and Drug Administration approved ADI-PEG20 as an orphan drug for treatment of HCC and malignant melanomas. Only six years later, in July 2005, the European Agency for the Evaluation of Medicinal Products granted orphan drug status to ADI-PEG20 for the treatment of HCC [64].

Preclinical ADI-based approaches

The plethora of experimental data gathered from *in vitro, in vivo,* and *ex vivo* studies are summarized in Table S1 (For all supplemental material see www.karger.com/10.1159/000495382). In most studies, *ASS1* and/or *ASL* were confirmed as frequent targets for (epi-) genetic inactivation in clinical tumor cases. Genetic silencing was virtually always associated with upregulation of *HIF-1α* and downregulation of *c-Myc*, whereas methylation was responsible for epigenetic suppression (Table S1).

In these preclinical analyses, ADI was given as a single agent and in combination with other cytostatic and/or targeted drugs commonly applied in the clinic. Some of the combinational effects are reported later in this review. For determining ADI-based treatment efficacy, several methods have been applied ranging from simple biomass/viability quantification and live/dead analysis, to establishment of stable *ASS1*-overexpressing and/or *ASS1* knockdown cell lines. These cumulative *in vitro* and *in vivo* studies impressively confirm the antitumoral activity of ADI (Table S1). ADI has the ability to interfere with signaling pathways and, in most cases; additive and even synergistic effects were seen after combination therapies. Also, efforts to understand the cellular, biological and molecular effects of arginine-depletion were made. These will be outlined below.

Stress responses upon ADI-based therapy - autophagy, senescence & inhibition of the *Warburg* effect

Autophagy was the first stress response described in arginine-starved tumor cells [27,29,65,66] (Fig. 3). Autophagy defines a non-apoptotic route of programmed cell death to degrade and recycle long-lived organelles and proteins. Although playing a crucial role in cellular survival during starvation, autophagy becomes a cellular suicide pathway when stress conditions are prolonged. Characteristic features of early and late stages of autophagy include: (I) formation of acidic vesicular organelles including autolysosomes and (II) molecular changes resulting in autophagosome formation as evidenced by LC3-I processing to LC3-II, decreased S6K phosphorylation, and activation of MAPkinase [67]. In melanomas, arginine deprivation results in low ATP levels and inactivates the mTOR signaling pathway to reinforce autophagocytic activity [68]. Breast and prostate cancer cell lines respond to ADI treatment by mitochondrial dysfunction, an autophagy-dependent process. As such, autophagy constitutes a stress response, especially in apoptosis-resistant cells – a quite common characteristic of tumor cells, among them GBM. Conversely, autophagy inhibition might lead to apoptosis via the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). TRAIL plays an important role in the cleavage of Beclin-1 (Atg6) and Atg5 in ADI-treated melanoma cells [69]. TRAIL-induced cleavage of Beclin-1 and Atg5 leads to decreased autophagy, thereby increasing apoptosis [67]. Comparable effects were detectable in small-cell lung cancer, leukemia, retinoblastoma, and pancreatic cancer cells [24, 70]. In addition to reducing metabolic activity, arginine starvation induces caspase-dependent and independent apoptosis. The latter type of cell death was inducible by BAX conformation changes and mitochondrial inner membrane depolarization and was found exclusively in *ASS1* negative mesothelioma cells. By contrast, sarcoma cells use sustained autophagy as a period of growth arrest until they reprogram and re-express *ASS1* in a c-Myc-dependent manner, allowing cells to escape the effects of arginine deprivation [68].

Experimentally proven for GBM by our group, autophagy induction is accompanied by the upregulation of stress-related genes belonging to the heat-shock protein and the iron/manganese superoxide dismutase families (*HSPA4/A1A/A8/A9/E1; DNAJA4/JB12; SOD2*). Additionally, arginine-starved tumor cells display endoplasmic reticulum stress and oxidative damage (upregulation of *Calreticulin* and *Glutathione Peroxidase 1*) (Fig. 3). This is accompanied by cell cycle alterations (G_1 or S phase arrest), as proven for pancreatic and breast cancer as well as GBM cells [30, 31, 71].

Another interesting finding related to ADI-based therapy is the induction of cellular senescence (Fig. 3), a specific phenomenon wherein proliferation-competent cells undergo durable states of arrest upon distorted homeostasis [72]. Morphological signs of senescence include flattening, vacuolization and accumulation of stress granules in formerly adherent growing cells [72]. Cells routinely express senescence-associated β-galactosidase, detectable at pH 6.0 with the artificial substrate *X-gal,* as well as several inflammatory cytokines (interleukin-1 (IL-1), IL-6, and IL-8) and signaling molecules ($p16^{INK4a}$, in the case of wild-type *CDKN2A* gene). So far, senescence induction was only confirmed in GBM cells [31]. These tumor cells show intrinsic apoptosis resistance. Hence, it is conceivable that especially apoptosis-resistant tumor cells respond with a high abundance of senescence-associated β-galactosidase under arginine starvation. However, this has to be tested in different tumor entities prospectively.

Finally, Kremer et al. identified a treatment-related switch from dependence on aerobic glycolysis (the *Warburg effect*) to glutamine anaplerosis and mitochondrial oxidative phosphorylation [73]. The *Warburg effect* is imperative in providing rapidly proliferating cells with pyruvate as an electron acceptor, necessary in the oxidative biosynthesis of aspartate. Arginine deprivation diverts glucose into the serine biosynthetic pathway, rendering tumor cells more vulnerable to the inhibition of serine biosynthesis and downstream folate-dependent enzymes [73].

Prolonged ADI treatment induces apoptosis, depending on exposure time and ability to undergo apoptotic cell death. In the study of Kremer et al., *ASS1*-deficient leiomyosarcoma (SKLMS1 and SKUT1) and melanoma cells (SKMEL2) were cultured for three months with ADI-PEG20 to identify potential synthetic lethal interactions. In our own studies, chronic arginine starvation of apoptosis-resistant GBM either *in vitro* (= eight repetitive cycles) or *in vivo* (= six peritumoral injections) with *S. pyogenes* ADI boosted cell death. *In vitro* cultured cells treated for a long term had elevated numbers of senescent cells, most likely a result of prolonged and durable cellular stress [31].

Another so far incompletely addressed side effect of *in vivo* arginine starvation is anti-angiogenesis caused by impeding tube formation of endothelial cells and neovascularization [74]. This can be attributed to suppressed nitric oxide (NO) production [73]. NO is synthesized by NO synthases. Under conditions of low arginine, NO synthase generates peroxynitrite due to reduced levels of NO reacting with NOS-generated superoxide. Both superoxide and peroxynitrite trigger cell damage that is largely reversible by adding arginine exogenously. Arginine deprivation additionally impairs synthesis of arginine-rich nuclear histones as well as *de novo* protein synthesis. Collectively, arginine withdrawal induces several therapeutically targetable metabolic alterations to persuade synthetic lethality in susceptible cancers.

Clinical ADI-based trials

In early clinical trials with ADI-PEG20, patients that suffered from HCC, metastatic melanoma, and mesothelioma were included; the majority of these had been heavily pre-treated and had failed prior cytostatic or targeted therapy. Up to now, virtually all arginine-auxotrophic subtypes were included in phase I and ll trials (Tables S2 and S3). In all studies, ADI-PEG20 was administered repetitively. The application route was mainly intramuscular on a weekly schedule and at different doses to determine the maximum tolerated doses.

Clinical application of a ADI-PEG20 monotherapy is well tolerated, with only mild to moderate side effects. Most adverse events are local injection reactions, rash, hyperuricemia and fatigue, while adverse events such as myelosuppression, gastrointestinal toxicity or other major organ toxicities – commonly seen under standard cytostatic drug therapy – were never observed (Table S2). Table S2 provides a comprehensive overview on clinical trials along with information of intervention, treatment-related toxicities, and outcomes. The latest clinical trials record stable disease as the best response, with evidence for a rebound in mean plasma arginine levels due to the development of drug-neutralizing antibodies that limit the duration of arginine depletion (median of 50 days) and the number of treatment cycles. Still, in most cases, even as single agent, ADI-PEG20 therapy yields extended progression-free and overall survival with response rates ranging from 25 (melanoma) to 47 % (HCC) (Table S2). Recent trials focus on a combination strategy, backboned by cytostatic drugs (Table S3).

Intrinsic and acquired resistance mechanisms towards ADI

As outlined above, clinical application of ADI is hampered by cancer drug resistance. Resistance mechanisms to arginine deprivation include: (I) re-expression of the once-silenced *ASS1* gene; (II) development of neutralizing antibodies, (III) *ASL* upregulation in ASS1-negative cells, and (IV) enhanced glycolysis [24]. ASS1, catalyzing the rate-limiting step in arginine production, may be mildly upregulated in a sufficient minority of tumor cells to permit resistance. In addition, the local tumor environment may have enough arginine from surrounding normal cells to maintain the viability of malignant cells with a subsequent disease progression [75]. Hence, metabolic alterations are required for the adaptation of and subsequent escape from arginine starvation-induced growth arrest.

In most patients, however, the ASS1-negative status of tumor cells turns into a positive one upon relapse. This suggests that *ASS1* re-expression is likely to be a mechanism of ADI

resistance [28]. On a molecular level, this is attributable to the close interaction of *c-Myc* and *HIF-1a* with an E-box element located at the promoter of the *ASS1* gene [28] (Fig. 3). Both genes are involved in regulation of cancer energy metabolism [76].

Tumor cells re-acquire *de novo* arginine biosynthesis capabilities by *ASS1* re-expression [73, 77]. It is worth noting that metabolic changes are mostly negligible between ADI-PEG20 treated and untreated cells, providing further evidence for resistance development. As mentioned above, chronic arginine starvation by *S. pyogenes* ADI boosted the cell death of apoptosis-resistant GBM cells or xenografts without developing resistance. Gene expression patterns of urea cycle genes –including *ASS1* and *ASL*– remained nearly unchanged and might provide an explanation for sustained ADI-susceptibility [30,31]. These *in vitro* and *in vivo* data are promising. Subsequent studies will show whether ADI from *S. pyogenes* might be superior to the "classical" ADI-PEG20, especially for long-term *in vivo* application.

An open question remains of how to deal with the innately resistant tumors or tumor cell subpopulations, such as "cancer-initiating or stem-like cells". These rare subpopulations are involved in self-renewal and maintenance of cancer stem cell properties, i.e. they divide slowly. In theory, arginine depletion preferentially affects growth and thus division of cells. In one study, susceptibility of $CD133^+$ cancer stem-like GBM cells with partial sensitivity towards ADI-PEG20 (*ASL* gene promoter methylation) was examined and compared to their non-stem-like counterpart. Here, both cell populations were equally inhibited by ADI-PEG20 [27]. However, CD133 is a weak and only putative GBM stem cell marker with limited informative value. In ADI-PEG20-sensitive AML cells with a stem cell phenotype ($CD34^+CD38^-$), presence of ASS1 was examined and compared with their bulk population counterpart (10 $CD34^+$ AMLs analyzed) [7]. ASS1 abundance was similar in both subpopulations (stem cells + bulk culture), suggesting responsiveness towards arginine depletion and thus ADI-PEG20. Experimental confirmation is, to the best of our knowledge, still missing.

Strategies to improve ADI-based therapy

Arginine degradation has multiple effects on biological processes, in some cases interfering with or even counteracting effects of anti-neoplastic or targeted therapies. Principally, and as discussed above, the anti-tumor activity of arginine deprivation includes the induction of cell cycle arrest, apoptosis, autophagy, senescence, and inhibition of angiogenesis. Its anti-angiogenic activity suggests that ADI could become a novel anti-cancer drug targeting neovascularization-related tumors. In other studies, ADI-PEG20 treatment downregulated thymidylate synthase and interfered with pyrimidine synthesis [65, 78]. This is of particular relevance for combination approaches using the standard chemotherapeutic drug 5-Fluorouracil (5-FU). The most common (acquired) resistance mechanism towards 5-FU is elevated expression of thymidylate synthase. The impact of a combined ADI/5-FU application to affect tumor growth in (pre-clinical) studies is therefore of particular relevance. Even more interesting in this context is the question of whether the observed interference with thymidylate synthase provides a potential novel use of ADI in intrinsically resistant tumors to act as "chemosensitizer". However, this hypothesis has to be proven. Several other studies tested the combination of ADI-PEG20 and Gemcitabine to treat pancreatic cancer [71, 79]. The prognosis of this disease is as bad as for GBM, making improvement of treatment options necessary. Of note, ADI-PEG20 and Gemcitabine boosted pancreatic cancer growth inhibition by suppressing survivin and blocking NF-κB p65 phosphorylation (serine 536). Similar effects were seen when ADI-PEG20 was combined with the platinum drug Cisplatin to treat malignant melanomas, HCC and ovarian cancer [80, 81]. In ASS1-positive HCC cell lines, Cisplatin treatment down-regulated ASS1, rendering those cells sensitive. Among the more targeted substances, histone-deacetylase inhibitor SAHA (Vorinostat) has shown favorable activity in conjunction with ADI-PEG20 as well as *S. pyogenes* ADI in GBM therapy. In the latter, GBM xenograft growth was completely inhibited (4/5 cases) and one tumor even totally disappeared [30].

Finally, ADI-treatment modulates phosphoinositide 3-kinase (PI3K) via suppression of phosphatase and tensin homolog (Fig. 3). Several (pan and isoform-specific) PI3K inhibitors [37] are close to entering or have already entered the clinic and may constitute ideal combination partners to improve efficacy. This pathway, described to be the master regulator of aerobic glycolysis and cellular biosynthesis [23], represents an acquired resistance mechanism to anticancer therapy. Implementation of these agents into multimodality drug regimens along with the application of imaging and tissue/fluid-based biomarkers as predictors of response will prospectively pave the way for successful ADI-based therapy.

Arginine and the complex interplay with the tumor microenvironment

In the 1970s, it was found that arginine prevented thymic involution after surgery and appeared to increase the number of lymphocytes [82]. Arginine availability is thus essential for normal T-cell proliferation and function. Upon T-cell activation, its uptake increases in amounts exceeding the requirements for protein synthesis, mainly due to elevated CAT activity. These transporters are highly regulated and co-induced with arginine-metabolizing enzymes to generate downstream metabolites [82]. Once activated, T cells showed a drop in their intracellular arginine concentration [83].

Under low arginine conditions, T cells have decreased numbers of T-cell receptors on the cell membrane and show an endoplasmic reticulum stress response (through *ERN1* signaling) as well as cell cycle arrest at G_0/G_1 [84]. Resulting from translational downregulation of the z-chain peptide, the principal signal-transduction element [85], this contributes to impaired proliferation and decreased cytokine production accompanied by autophagy to prevent the onset of apoptosis [84]. Indeed, CD3 z is more profoundly decreased in tumor-infiltrating than in circulating T lymphocytes [17, 85], partially explaining their compromised antitumor efficacy as a result of nutrient starvation. A mechanism to counteract functional impairment is increased citrulline uptake and upregulated *ASS1* expression to generate endogenous arginine – very similar to that seen in tumor cells. Hence, under certain conditions, T cells have evolved strategies to conquer acute arginine depletion. Replenishment of arginine drives T lymphocytes to proliferate again and they recover their normal cell cycle profile, whereby autophagy is inhibited and no longer required [84, 86].

In contrast, elevated intracellular arginine levels directly induce metabolic changes and longevity of human T cells ($CD4^+$ and $CD8^+$), independent of mTOR signaling or downstream metabolites [83].

The complex interplay between tumor and immune cells in the microenvironment, however, is challenging for the maintenance of normal T cell function. Arginase I and II, produced by tumor and myeloid-derived suppressor cells (MDSC), force nutrient deficiency in the tumor microenvironment. This additionally contributes to immune escape by triggering T-cell dysfunction and apoptosis [87]. Inhibiting arginase I with a small-molecule inhibitor shifts the immune landscape towards a pro-inflammatory environment by dampening MDSC-mediated immune evasion [88]. Another study even proposed specific accumulation of MDSC in arginine-deprived tumor-bearing mice [89]. It is noteworthy that ADI-PEG20 did not impair activation processes or mitochondrial respiration in T cells.

It is still relatively unclear how arginine availability is regulated during an immune response and how ADI-PEG20 therapy affects activity of (tumor-infiltrating) immune cells. Metabolic fitness and T-cell survival are particularly crucial in anti-tumor responses. Only recently, Brin et al. described no adverse side effects on human peripheral blood mononuclear cells upon arginine starvation [90]. This study thus supports recent findings stating that ADI-PEG20 does not significantly impair the viability of normal cells - especially when high amounts of citrulline are present [89]. Nevertheless, the following is worth mentioning in this context: (I) in this study normal, un-primed T cells from healthy donors were used and (II) the time of arginine removal was critical; the complete withdrawal during T cell stimulation leads to anergy (through mTOR inhibition), while at a later phase activation is

relatively arginine-independent. This can be explained by the extraordinarily high metabolic need for arginine during T-cell priming (between 24 and 48 hours after T-cell activation *in vitro*) [83]. Since tumor patients often have high amounts of primed T cells infiltrating their tumors, interference of ADI-PEG20 with those cells is rather unlikely. Though not clinically proven, ADI-PEG20 boosted tumor immunogenicity in a syngeneic B16-F10-melanoma mouse model and thus contradicts previous findings [90]. Effects were further enhanced when ADI-PEG20 was combined with immune-checkpoint inhibiting antibody PD-1 [90]. To the best of our knowledge, neither study examined the influence on antigen-presenting and antibody-producing B-cells in the context of arginine deprivation. Depending on the tumor type and density of lymphocytic infiltration at the start of therapy, one may speculate that, similar to T cells, interference with B-cell functions is rather low. Whether antigen uptake is affected in arginine-deprived tumors is another unanswered question. Accordingly, it is still not known by what means migration to draining lymph nodes and thus long-lasting antitumoral immunity with the potential to control (micro-) metastases is influenced. Still, the promising data from a combined ADI/PD-1 application encourage further efforts.

Summing up, there is a link between the availability of certain (non-) essential amino acids and the immune response, with most of them apparently inhibiting normal T-cell proliferation. By contrast, ADI-PEG20-based therapy seems to favor T-cell infiltration by metabolic adaptations as yet to be discovered. Subsequent (pre-) clinical studies will show whether these findings apply to other tumor models as well or are rather specific for melanoma– an outstandingly immunogenic tumor entity.

Perspectives

Arginine-depleting strategies represent promising approaches for the treatment of certain cancers. However, the clinically recognized resistance towards ADI-based therapy constitutes a major challenge and adds to a long list of experiences in which experimentally proven substances are facing limits upon clinical application. Recent observations provide some novel aspects and potential strategies for dealing with resistance development. Surviving arginine-starved tumor cells respond with a shift in molecular pathways that may render tumor cells vulnerable to other (targeted) strategies. Activation of the PI3K/Akt/mTOR pathway constitutes one of the classical and frequently observed "resistance" mechanisms. Bearing that in mind, the huge number of selective (pan-) PI3K-inhibitors forthcoming will hopefully ameliorate treatment options for affected patients. Ways to precision medicine, i.e. "the right drugs (=adequate pharmacologic inhibition), for the right patient (=molecular-based preselection), at the right time (neo-adjuvant, sequential, multimodal intervention)" include genomic sequencing (next generation sequencing) from patients' tumors or even "liquid biopsies" to cover virtually all mutational events. Also, re-sequencing under therapy at multiple time points to assure real-time monitoring of resistance development would be desirable. This improved treatment design may finally provide the patients with an adequate therapy, and ultimately, a cure.

Abbreviations

ADI (Arginine deiminase); ADC (arginine decarboxylase); AML (acute myeloid leukemia); ASS1 (argininosuccinate synthetase 1); ASL (argininosuccinate lyase); CRC (colorectal carcinoma); GBM (Glioblastoma multiforme); HCC (hepatocellular carcinoma); HIF1α hypoxia-inducible (factor-1 alpha); MDSC (myeloid-derived suppressor cells); NO (nitric oxide); NOS (nitric oxide synthase); OTC (Ornithine transcarbamylase); PI3K (Phosphatidylinositol-4, 5-bisphosphate 3-kinase); 5-FU (5-Fluorouracil); rhArg (Recombinant human arginase).

Acknowledgements

This work was supported by a grant from the German research foundation to CM [grant number MA5799/2-1].

Disclosure Statement

The authors declare no potential conflicts of interest.

References

1 Gilroy E: The influence of arginine upon the growth rate of a transplantable tumour in the mouse. Biochem J 1930;24:589–595.
2 Kraemer PM, Defendi V, Hayflick L, Manson LA: Mycoplasma (PPLO) strains with lytic activity for murine lymphoma cells in vitro. Proc Soc Exp Biol Med 1963;112:381–387.
3 Kenny GE, Pollock ME: Mammalian cell cultures contaminated with pleuropneumonia-like organisms. I. Effect of pleuropneumonia-like organisms on growth of established cell strains. J Infect Dis 1963;112:7–16.
4 Schimke RT, Berlin CM, Carroll WR: The Generation of Energy by the Arginine Dihydrolase Pathway in Mycoplasma The Generation of Energy by the Arginine Pathway in Mycoplasma hominis Dihydrolase. J Biol Chem 1966;241:2228–2237.
5 Simberkoff MS, Thorbecke GJ, Thomas L: Studies on PPLO Infection : Inhibition of lymphocyte mitosis and antibody formation by mycoplasmal extract. J Exp Med 1969;129:1163–1181.
6 Jones JB: The effect of arginine deiminase on murine leukemic lymphoblasts (Ph. D. dissertation), University of Oklahoma Oklahoma City, OK 1981
7 Miraki-Moud F, Ghazaly E, Ariza-McNaughton L, Hodby KA, Clear A, Anjos-Afonso F, Liapis K, Grantham M, Sohrabi F, Cavenagh J, Bomalaski JS, Gribben JG, Szlosarek PW, Bonnet D, Taussig DC.: Arginine deprivation using pegylated arginine deiminase has activity against primary acute myeloid leukemia cells in vivo. Blood 2015;125:4060–4068.
8 Ascierto PA, Scala S, Castello G, Daponte A, Simeone E, Ottaiano A, Beneduce G, De Rosa V, Izzo F, Melucci MT, Ensor CM, Prestayko AW, Holtsberg FW, Bomalaski JS, Clark MA, Savaraj N, Feun LG, Logan TF: Pegylated arginine deiminase treatment of patients with metastatic melanoma: Results from phase I and II studies. J Clin Oncol 2005;23:7660–7668.
9 Ensor CM, Holtsberg FW, Bomalaski JS, Clark MA: Pegylated arginine deiminase (ADI-SS PEG20,000mw) inhibits human melanomas and hepatocellular carcinomas in vitro and in vivo. Cancer Res 2002;62:5443–5450.
10 Lam T-L, Wong GK, Chow HY, Chong HC, Chow TL, Kwok SY, Cheng PN, Wheatley DN, Lo WH, Leung YC.: Recombinant human arginase inhibits the in vitro and in vivo proliferation of human melanoma by inducing cell cycle arrest and apoptosis. Pigment Cell Melanoma Res 2011;24:366–376.
11 Delage B, Luong P, Maharaj L, O'Riain C, Syed N, Crook T, Hatzimichael E, Papoudou-Bai A, Mitchell TJ, Whittaker SJ, Cerio R, Gribben J, Lemoine N, Bomalaski J, Li CF, Joel S, Fitzgibbon J, Chen LT, Szlosarek PW: Promoter methylation of argininosuccinate synthetase-1 sensitises lymphomas to arginine deiminase treatment, autophagy and caspase-dependent apoptosis. Cell Death Dis 2012;3:1–9.
12 Wu G, Morris SM: Arginine metabolism: nitric oxide and beyond. Biochem J 1998;336:1–17.
13 Husson A, Brasse-Lagnel C, Fairand A, Renouf S, Lavoinne A: Argininosuccinate synthetase from the urea cycle to the citrulline-NO cycle. Eur J Biochem 2003;270:1887–1899.
14 Morris SM: Recent advances in arginine metabolism: Roles and regulation of the arginases. Br J Pharmacol 2009;157:922–930.
15 Stechmiller JK, Childress B, Cowan L: Arginine supplementation and wound healing. Nutr Clin Pract 2005;20:52–61.
16 Alba-Roth J, Müller OA, Schopohl J, Von Werder K: Arginine stimulates growth hormone secretion by suppressing endogenous somatostatin secretion. J Clin Endocrinol Metab 1988;67:1186–1189.

17 Bronte V, Zanovello P: Regulation of immune responses by L-arginine metabolism. Nat Rev Immunol 2005;5:641–654.
18 Luiking YC, Ten Have GAM, Wolfe RR, Deutz NEP: Arginine de novo and nitric oxide production in disease states. AJP Endocrinol Metab 2012;303:E1177–E1189.
19 Fultang L, Vardon A, De Santo C, Mussai F: Molecular basis and current strategies of therapeutic arginine depletion for cancer. Int J Cancer 2016;139:501–509.
20 Feun L, You M, Wu CJ, Kuo MT, Wangpaichitr M, Spector S, Savaraj N: Arginine Deprivation as a Targeted Therapy for Cancer. Curr Pharm Des 2008;14:1049–1057.
21 Delage B, Fennell DA, Nicholson L, McNeish I, Lemoine NR, Crook T, Szlosarek PW: Arginine deprivation and argininosuccinate synthetase expression in the treatment of cancer. Int J Cancer 2010;126:2762–2772.
22 Guoyao W: Arginine metabolism and nutrition in growth, health and disease. Aminoacids 2009;37:153–168.
23 DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB: The Biology of Cancer: Metabolic Reprogramming Fuels Cell Growth and Proliferation. Cell Metab 2008;7:11–20.
24 Qiu F, Huang J, Sui M: Targeting arginine metabolism pathway to treat arginine-dependent cancers. Cancer Lett 2015;364:1–7.
25 Lowery MA, Yu KH, Kelsen DP, Harding JJ, Bomalaski JS, Glassman DC, Covington CM, Brenner R, Hollywood E, Barba A, Johnston A, Liu KC, Feng X, Capanu M, Abou-Alfa GK, O'Reilly EM.: A phase 1/1B trial of ADI-PEG 20 plus nab-paclitaxel and gemcitabine in patients with advanced pancreatic adenocarcinoma. Cancer 2017;123:4556–4565.
26 Wu L, Li L, Meng S, Qi R, Mao Z, Lin M: Expression of argininosuccinate synthetase in patients with hepatocellular carcinoma. J Gastroenterol Hepatol 2013;28:365–368.
27 Syed N, Langer J, Janczar K, Singh P, Lo Nigro C, Lattanzio L, Coley HM, Hatzimichael E, Bomalaski J, Szlosarek P, Awad M, O'Neil K, Roncaroli F, Crook T: Epigenetic status of argininosuccinate synthetase and argininosuccinate lyase modulates autophagy and cell death in glioblastoma. Cell Death Dis 2013;4:1–11.
28 Kuo MT, Savaraj N, Feun LG: Targeted cellular metabolism for cancer chemotherapy with recombinant arginine-degrading enzymes Abstract : Glycolysis TCA cycle. Oncotarget 2010;1:246–251.
29 Tsai WB, Long Y, Park JR, Chang JT, Liu H, Rodriguez-Canales J, Savaraj N, Feun LG, Davies MA, Wistuba II, Kuo MT: Gas6/Axl is the sensor of arginine-auxotrophic response in targeted chemotherapy with arginine-depleting agents. Oncogene 2016;35:1632–1642.
30 Fiedler T, Strauss M, Hering S, Redanz U, William D, Rosche Y, Classen CF, Kreikemeyer B, Linnebacher M, Maletzki C: Arginine deprivation by arginine deiminase of Streptococcus pyogenes controls primary glioblastoma growth in vitro and in vivo. Cancer Biol Ther 2015;16:1047–1055.
31 Maletzki C, Rosche Y, Riess C, Scholz A, William D, Classen CF, Kreikemeyer B, Linnebacher M, Fiedler T: Deciphering molecular mechanisms of arginine deiminase-based therapy – Comparative response analysis in paired human primary and recurrent glioblastomas. Chem Biol Interact 2017;278:179-188.
32 Vardon A, Dandapani M, Cheng D, Cheng P, De Santo C, Mussai F: Arginine auxotrophic gene signature in paediatric sarcomas and brain tumours provides a viable target for arginine depletion therapies. Oncotarget 2017;8:63506–63517.
33 Huang HL, Chen WC, Hsu HP, Cho CY, Hung YH, Wang CY, Lai MD: Argininosuccinate lyase is a potential therapeutic target in breast cancer. Oncol Rep 2015;34:3131–3139.
34 Hung YH, Huang HL, Chen WC, Yen MC, Cho CY, Weng TY, Wang CY, Chen YL, Chen LT, Lai MD: Argininosuccinate lyase interacts with cyclin A2 in cytoplasm and modulates growth of liver tumor cells. Oncol Rep 2017;37:969–978.
35 Wheatley DN, Campbell E: Arginine deprivation, growth inhibition and tumour cell death: 3. Deficient utilisation of citrulline by malignant cells. Br J Cancer 2003;89:573–576.
36 Dillon BJ, Prieto VG, Curley SA, Ensor CM, Holtsberg FW, Bomalaski JS, Clark MA: Incidence and Distribution of Argininosuccinate Synthetase Deficiency in Human Cancers: A Method for Identifying Cancers Sensitive to Arginine Deprivation. Cancer 2004;100:826–833.
37 Cai Y, Dodhia S, Su GH, Cai Y, Dodhia S, Su GH: Dysregulations in the PI3K pathway and targeted therapies for head and neck squamous cell carcinoma. Oncotarget 2017;5:1–15.

38 Li X, Wu C, Chen N, Gu H, Yen A, Cao L, Wang E, Wang L: PI3K/Akt/mTOR signaling pathway and targeted therapy for glioblastoma. Oncotarget 2016;7:33440–33450.
39 Yeatman TJ, Risley GL, Brunson ME: Depletion of dietary arginine inhibits growth of metastatic tumor. Arch Surg 1991;126:1372–1376.
40 Gonzalez GG, Byus C V: Effect of dietary arginine restriction upon ornithine and polyamine metabolism during two-stage epidermal carcinogenesis in the mouse. Cancer Res 1991;51:2932–2939.
41 Castillo L, Ajami A, Branch S, Chapman TE, Yu YM, Burke JF, Young VR: Plasma arginine kinetics in adult man: response to an arginine-free diet. Metabolism 1994;43:114–122.
42 Lind DS: Arginine and cancer. J Nutr 2004;134:2837S–2841S; discussion 2853S.
43 Closs EI, Simon A, Vekony N, Rotmann A: Plasma membrane transporters for arginine. J Nutr 2004;134:2752S–2759S; discussion 2765S–2767S.
44 Abdelmagid SA, Rickard JA, McDonald WJ, Thomas LN, Too CKL: CAT-1-mediated arginine uptake and regulation of nitric oxide synthases for the survival of human breast cancer cell lines. J Cell Biochem 2011;112:1084–1092.
45 Shima Y, Maeda T, Aizawa S, Tsuboi I, Kobayashi D, Kato R, Tamai I: L-arginine import via cationic amino acid transporter CAT1 is essential for both differentiation and proliferation of erythrocytes. Blood 2006;107:1352–1356.
46 Hering S, Sieg A, Kreikemeyer B, Fiedler T: Kinetic characterization of arginine deiminase and carbamate kinase from Streptococcus pyogenes M49. Protein Expr Purif 2013;91:61–68.
47 Dillon BJ, Holtsberg FW, Ensor CM, Bomalaski JS, Clark MA: Biochemical characterization of the arginine deiminase degrading enzymes arginase and arginine deiminase and their effect on nitric oxide production. Med Sci Monit 2002;8:BR248-253.
48 Hsueh EC, Knebel SM, Lo W-H, Leung Y-C, Cheng PN-M, Hsueh C-T: Deprivation of arginine by recombinant human arginase in prostate cancer cells. J Hematol Oncol 2012;5:17.
49 Yau T, Cheng PN, Chan P, Chan W, Chen L, Yuen J, Pang R, Fan ST, Poon RT: A phase 1 dose-escalating study of pegylated recombinant human arginase 1 (Peg-rhArg1) in patients with advanced hepatocellular carcinoma. Invest New Drugs 2013;31:99–107.
50 Cheng PNM, Lam TL, Lam WM, Tsui SM, Cheng AW, Lo WH, Leung YC: Pegylated recombinant human arginase (rhArg-peg5,000mw) inhibits the in vitro and in vivo proliferation of human hepatocellular carcinoma through arginine depletion. Cancer Res 2007;67:309-317.
51 Philip R, Campbell E, Wheatley DN: Arginine deprivation, growth inhibition and tumour cell death: 2. Enzymatic degradation of arginine in normal and malignant cell cultures. Br J Cancer 2003;88:613–623.
52 Wheatley DN, Scott L, Lamb J, Smith S: Single amino acid (arginine) restriction: growth and death of cultured HeLa and human diploid fibroblasts. Cell Physiol Biochem 2000;10:37–55.
53 Patil MD, Bhaumik J, Babykutty S, Banerjee UC, Fukumura D: Arginine dependence of tumor cells: targeting a chink in cancer's armor. Oncogene 2016;35:4957–4972.
54 Wheatley DN, Campbell E: Arginine catabolism, liver extracts and cancer. Pathol Oncol Res 2002;8:18–25.
55 Lüthi E, B Baur H, Gamper M, Brunner F, Villeval D, Mercenier A, Haas D: The arc operon for anaerobic arginine catabolism in Pseudomonas aeruginosa contains an additional gene, arcD, encoding a membrane protein. Gene 1990;87:37–43.
56 Gamper M, Zimmermann A, Haas D: Anaerobic regulation of transcription initiation in the arcDABC operon of Pseudomonas aeruginosa. J Bacteriol 1991;173:4742–4750.
57 Maghnouj A, Abu-Bakr AAW, Baumberg S, Stalon V, Vander Wauven C: Regulation of anaerobic arginine catabolism in Bacillus licheniformis by a protein of the Crp/Fnr family. FEMS Microbiol Lett 2000;191:227–234.
58 Zúñiga M, Champomier-Verges M, Zagorec M, Pérez-Martínez G: Structural and Functional Analysis of the Gene Cluster Encoding the Enzymes of the Arginine Deiminase Pathway of Lactobacillus sake. J Bacteriol 1998;180:4154–4159.
59 Zuniga M, Perez G, Gonzalez-Candelas F: Evolution of arginine deiminase (ADI) pathway genes. Mol Phylogenet Evol 2002;25:429–444.
60 Griswold A, Chen Y-YM, Snyder JA, Burne RA: Characterization of the arginine deiminase operon of Streptococcus rattus FA-1. Appl Environ Microbiol 2004;70:1321–1327.
61 Dong Y, Chen Y-YM, Burne RA: Control of expression of the arginine deiminase operon of Streptococcus gordonii by CcpA and Flp. J Bacteriol 2004;186:2511–2514.

62 Holtsberg FW, Ensor CM, Steiner MR, Bomalaski JS, Clark MA: Poly(ethylene glycol) (PEG) conjugated arginine deiminase: Effects of PEG formulations on its pharmacological properties. J Control Release 2002;80:259–271.
63 Takaku H, Misawa S, Hayashi H, Miyazaki K: Chemical modification by polyethylene glycol of the anti-tumor enzyme arginine deiminase from Mycoplasma arginini. Jpn J Cancer Res 1993;84:1195–1200.
64 Ni Y, Schwaneberg U, Sun Z-H: Arginine deiminase, a potential anti-tumor drug. Cancer Lett 2008;261:1–11.
65 Allen MD, Luong P, Hudson C, Leyton J, Delage B, Ghazaly E, Cutts R, Yuan M, Syed N, Lo Nigro C, Lattanzio L, Chmielewska-Kassassir M, Tomlinson I, Roylance R, Whitaker HC, Warren AY, Neal D, Frezza C, Beltran L, Jones LJ, Chelala C, Wu BW, Bomalaski JS, Jackson RC, Lu YJ, Crook T, Lemoine NR, Mather S, Foster J, Sosabowski J, Avril N, Li CF, Szlosarek PW: Prognostic and therapeutic impact of argininosuccinate synthetase 1 control in bladder cancer as monitored longitudinally by PET imaging. Cancer Res 2014;74:896–907.
66 Kim RH, Coates JM, Bowles TL, McNerney GP, Sutcliffe J, Jung JU, Gandour-Edwards R, Chuang FY, Bold RJ, Kung HJ: Arginine deiminase as a novel therapy for prostate cancer induces autophagy and caspase-independent apoptosis. Cancer Res 2009;69:700–708.
67 Rybstein MD, Bravo-San Pedro JM, Kroemer G, Galluzzi L: The autophagic network and cancer. Nat Cell Biol 2018;20:243–251.
68 Long Y, Tsai WB, Wangpaichitr M, Tsukamoto T, Savaraj N, Feun LG, Kuo MT: Arginine Deiminase Resistance in Melanoma Cells Is Associated with Metabolic Reprogramming, Glucose Dependence, and Glutamine Addiction. Mol Cancer Ther 2013;12:2581–2590.
69 You M, Savaraj N, Wangpaichitr M, Wu C, Kuo MT, Varona-Santos J, Nguyen DM, Feun L: The combination of ADI-PEG20 and TRAIL effectively increases cell death in melanoma cell lines. Biochem Biophys Res Commun 2010;394:760–766.
70 Bowles TL, Kim R, Galante J, Parsons CM, Virudachalam S, Kung HJ, Bold RJ: Pancreatic cancer cell lines deficient in argininosuccinate synthetase are sensitive to arginine deprivation by arginine deiminase. Int J Cancer 2008;123:1950–1955.
71 Liu J, Ma J, Wu Z, Li W, Zhang D, Han L, Wang F, Reindl KM, Wu E, Ma Q: Arginine deiminase augments the chemosensitivity of argininosuccinate synthetase-deficient pancreatic cancer cells to gemcitabine via inhibition of NF-κB signaling. BMC Cancer 2014;14:1–17.
72 Sharpless NE, Sherr CJ: Forging a signature of in vivo senescence. Nat Rev Cancer 2015;15:397–408.
73 Kremer JC, Prudner BC, Lange SES, Bean GR, Schultze MB, Brashears CB, Radyk MD, Redlich N, Tzeng SC, Kami K, Shelton L, Li A, Morgan Z, Bomalaski JS, Tsukamoto T, McConathy J, Michel LS, Held JM, Van Tine BA: Arginine Deprivation Inhibits the Warburg Effect and Upregulates Glutamine Anaplerosis and Serine Biosynthesis in ASS1-Deficient Cancers. Cell Rep 2017;18:991–1004.
74 Buijs N, Oosterink JE, Jessup M, Schierbeek H, Stolz DB, Houdijk AP, et al.: A new key player in VEGF-dependent angiogenesis in human hepatocellular carcinoma: dimethylarginine dimethylaminohydrolase 1. Angiogenesis 2017;20:557–565.
75 Glazer ES, Piccirillo M, Albino V, Di Giacomo R, Palaia R, Mastro AA, Beneduce G, Castello G, De Rosa V, Petrillo A, Ascierto PA, Curley SA, Izzo F: Phase II Study of Pegylated Arginine Deiminase for Nonresectable and Metastatic Hepatocellular Carcinoma. J Clin Oncol 2010;28:2220–2226.
76 Zwaans BMM, Lombard DB: Interplay between sirtuins, MYC and hypoxia-inducible factor in cancer-associated metabolic reprogramming. Dis Model Mech 2014;7:1023–1032.
77 Bean GR, Kremer JC, Prudner BC, Schenone AD, Yao JC, Schultze MB, Chen DY, Tanas MR, Adkins DR, Bomalaski J, Rubin BP, Michel LS, Van Tine BA: A metabolic synthetic lethal strategy with arginine deprivation and chloroquine leads to cell death in ASS1-deficient sarcomas. Cell Death Dis 2016;7:e2406.
78 Thongkum A, Wu C, Li YY, Wangpaichitr M, Navasumrit P, Parnlob V, Sricharunrat T, Bhudhisawasdi V, Ruchirawat M, Savaraj N: The combination of arginine deprivation and 5-fluorouracil improves therapeutic efficacy in argininosuccinate synthetase negative hepatocellular carcinoma. Int J Mol Sci 2017;18.
79 Daylami R, Muilenburg DJ, Virudachalam S, Bold RJ: Pegylated arginine deiminase synergistically increases the cytotoxicity of gemcitabine in human pancreatic cancer. J Exp Clin Cancer Res 2014;33:1–12.

80 Savaraj N, Wu C, Li Y-Y, Wangpaichitr M, You M, Bomalaski J, He W, Kuo MT, Feun LG: Targeting argininosuccinate synthetase negative melanomas using combination of arginine degrading enzyme and cisplatin. Oncotarget 2015;6:6295–309.
81 McAlpine JA, Lu HT, Wu KC, Knowles SK, Thomson JA: Down-regulation of argininosuccinate synthetase is associated with cisplatin resistance in hepatocellular carcinoma cell lines: Implications for PEGylated arginine deiminase combination therapy. BMC Cancer 2014;14:1–12.
82 Popovic PJ, Zeh HJ, Ochoa JB: Arginine and immunity. J Nutr 2007;137:1681S–1686S.
83 Geiger R, Rieckmann JC, Wolf T, Basso C, Feng Y, Fuhrer T, Kogadeeva M, Picotti P, Meissner F, Mann M, Zamboni N, Sallusto F, Lanzavecchia A: L-Arginine Modulates T Cell Metabolism and Enhances Survival and Anti-tumor Activity. Cell 2016;167:829–842.e13.
84 García-Navas R, Munder M, Mollinedo F: Depletion of L-arginine induces autophagy as a cytoprotective response to endoplasmic reticulum stress in human T lymphocytes. Autophagy 2012;8:1557–1576.
85 Rodriguez PC, Zea AH, DeSalvo J, Culotta KS, Zabaleta J, Quiceno DG, Ochoa JB, Ochoa AC: L-Arginine Consumption by Macrophages Modulates the Expression of CD3 Chain in T Lymphocytes. J Immunol 2003;171:1232–1239.
86 Rodriguez PC, Quiceno DG, Ochoa AC: L -arginine availability regulates T-lymphocyte cell-cycle progression. Blood 2016;109:1568–1574.
87 Chang CH, Qiu J, O'Sullivan D, Buck MD, Noguchi T, Curtis JD, Chen Q, Gindin M, Gubin MM, van der Windt GJ, Tonc E, Schreiber RD, Pearce EJ, Pearce EL: Metabolic Competition in the Tumor Microenvironment Is a Driver of Cancer Progression. Cell 2015;162:1229–1241.
88 Steggerda SM, Bennett MK, Chen J, Emberley E, Huang T, Janes JR, Li W, MacKinnon AL, Makkouk A, Marguier G, Murray PJ, Neou S, Pan A, Parlati F, Rodriguez MLM, Van de Velde LA, Wang T, Works M, Zhang J, Zhang W, Gross MI: Inhibition of arginase by CB-1158 blocks myeloid cell-mediated immune suppression in the tumor microenvironment. J Immunother Cancer 2017;5:1–18.
89 Fletcher M, Ramirez ME, Sierra RA, Raber P, Thevenot P, Al-Khami AA, Sanchez-Pino D, Hernandez C, Wyczechowska DD, Ochoa AC, Rodriguez PC: L-Arginine depletion blunts antitumor T-cell responses by inducing myeloid-derived suppressor cells. Cancer Res 2015;75:275–283.
90 Brin E, Wu K, Lu H, He Y, Dai Z, He W: PEGylated arginine deiminase can modulate tumor immune microenvironment by affecting immune checkpoint expression, decreasing regulatory T cell accumulation and inducing tumor T cell infiltration. Oncotarget 2017;26:58948-58963.