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REVIEW ARTICLE

Arginine depriving enzymes: applications as emerging therapeutics in cancer treatment

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Abstract

Cancer is the second leading cause of death globally. Chemotherapy and radiation therapy and other medications are employed to treat various types of cancer. However, each treatment has its own set of side effects, owing to its low specificity. As a result, there is an urgent need for newer therapeutics that do not disrupt healthy cells' normal functioning. Depriving nutrient or non/semi-essential amino acids to which cancerous cells are auxotrophic remains one such promising anticancer strategy. L-Arginine (Arg) is a semi-essential vital amino acid involved in versatile metabolic processes, signaling pathways, and cancer cell proliferation. Hence, the administration of Arg depriving enzymes (ADE) such as arginase, arginine decarboxylase (ADC), and arginine deiminase (ADI) could be effective in cancer therapy. The Arg auxotrophic cancerous cells like hepatocellular carcinoma, human colon cancer, leukemia, and breast cancer cells are sensitive to ADE treatment due to low expression of crucial enzymes argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL), and ornithine transcarbamylase (OCT). These therapeutic enzyme treatments induce cell death through inducing autophagy, apoptosis, generation of oxidative species, i.e., oxidative stress, and arresting the progression and expansion of cancerous cells at certain cell cycle checkpoints. The enzymes are undergoing clinical trials and could be successfully exploited as potential anticancer agents in the future.

Keywords Auxotrophic cancer · Argininosuccinate synthetase · Therapeutic enzyme · Deprivation therapy · Arginase · Arginine deiminase · Arginine decarboxylase

Abbreviations

Arg	L-Arginine
ADE	Arginine depriving enzymes
ADC	Arginine decarboxylase
ADI	Arginine deiminase
ASS	Argininosuccinate synthetase
OCT	Ornithine transcarbamylase
OAT	Ornithine aminotransferase
ODC	Ornithine decarboxylase
PEG	Polyethylene glycol
hArg	Human arginase
rhArg	Recombinant human arginase
NO	Nitric oxide

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Introduction

Cancer is a deadly disease and is considered the major cause of high mortality worldwide [1]. Cancer is caused due to defective cellular processes, which result in uncontrolled cell growth and thus affect normal cellular function. Amino acids are the primary building blocks of life required by both normal and diseased cells for their growth, help in the proliferation of cellular events, and help them to meet the physiological demands of cells [2, 3]. Cancerous cells require more energy and nutrients (amino acids) for their rapid growth as compared to normal healthy cells, so to halt their growth, two strategies can be explicitly opted for the removal of diseased cells, i.e., targeting the cellular metabolism or altering the microenvironment around them [4, 5]. Targeting cancer cells' metabolic activities by amino acid deprivation is already a well-established therapeutic approach and previously well-studied for asparaginase, methionase, glutaminase, and arginase enzymes. These enzymatic approaches rely on the ameliorated cancer cells' requirement for semi-essential amino acids such as asparagine,

methionine, glutamine, and arginine, respectively. Targeting semi-essential amino acids is fascinating in anticancer therapy without affecting normal cellular function. The normal cellular function can also be restored by adding an alternative source of amino acids [5–7].

Arg is a semi-essential standard amino acid because cellular cells have metabolic machinery that can synthesize amino acids to meet half of their demand. The remainder is obtained from the diet [8, 9]. Arg scarcity is targeted as it involves multiple functions in cellular metabolism. L-Arg is included in the various cellular processes, mainly in the urea cycle and biosynthetic pathways of polyamines, nitric oxides, improvement in reproduction and wound healing in the normal physiological state, but in the case of undergrowth and diseased conditions (cancer), the demand for an amino acid increases [2, 3, 8].

Gilroy did the pioneering work in this direction in the 1930s, which observes that Arg-rich diet resulted in progressive tumor growth in mice and, contrary to Arg deficient diet, results in regressive tumor growth [10]. As the auxotrophic cancer cells have a defective cellular mechanism, they are dependent on the extracellular nutritional pool of healthy cells to get essential amino acids and all other nutrition. Many cancer cells are auxotrophic to enzymes involved in Arg synthesis, i.e., argininosuccinate synthetase (EC 6.3.4.5; ASS), argininosuccinate lyase (EC 4.3.2.1; ASL), and ornithine transcarbamylase (EC 2.1.3.3; OCT), rendering them incapable of synthesizing Arg [9, 11, 12]. As a result, such auxotrophic cancer cells then fulfill their demand from the nutritional pool. Considering the importance of Arg as a critical metabolite, these auxotrophic cancers can be treated by ADE which opens a potential window for cancer treatment without disturbing the growth of normal healthy cells [5, 7].

Arginine decarboxylase (ADC; EC 4.1.1.19), Arginine deiminase (ADI; EC 3.5.3.6), and arginase (EC 3.5.3.1) are the three major enzymes that deplete Arg. Microorganisms utilize the ADI pathway to synthesize ornithine which serves as an energy source for their growth. *Mycoplasma arginini* is the first reported ADI source, which was being used for preclinical and clinical evaluation against various cancerous cell lines [13–16]. Another Arg depriving enzyme, ADC, expressed majorly in mammals, is not encouraged to be used as an anticancer enzyme owing to its cytotoxic effect on healthy normal cells [17]. In contrast to these two enzymes, arginase has come out as a potential candidate for efficient treatment of hepatocellular carcinoma [18], human prostate cancer cells [19], pancreatic cancer [20, 21], leukemia [22], glioblastoma [23], breast cancer [24], and non-Hodgkin's lymphoma [25]. This article will endeavor to provide information and discussion regarding the different roles and demerits of various ADE along with their budding therapeutic applications. The review also discusses the current status of ADE's preclinical and clinical trials with its scenarios as an effective approach for cancer therapy.

L-Arginine (Arg) metabolism

Arg is a semi-essential amino acid that plays very imperative roles in various metabolic physiochemical functioning (protein, urea, agmatine, proline, glutamine synthesis) of normal healthy cells. Arg is endogenously synthesized by the intestinal-renal axis pathway. Arg is synthesized in the liver via the urea cycle as well as in the kidney [8, 26]. Arg is a vital metabolite that acts as a precursor of many bioactive molecules like polyamines and proline via ornithine decarboxylase (EC 4.1.1.17; ODC) and ornithine aminotransferase (EC 2.6.1.13; OAT) enzymes, respectively. Nitric oxide, nucleotide and immunity precursors are also formed by this multifarious acting precursor molecule Arg. Argininosuccinate synthetase (EC 6.3.4.5; ASS) and argininosuccinate lyase (EC 4.3.2.1: ASL) are two limiting enzymes (absent in cancer cells) working properly in normal healthy cells, replenishing the normal pool of Arg. These are the key enzymes making healthy normal cells unaffected by Arg deprivation.

In contrast, both these enzymes are not expressed in cancer cells rendering them to undergo starvation and eventually cell death (Fig. 1). These enzymes determine the efficacy of Arg deprivation therapy as re-expression could lead to resistance to therapeutic enzyme treatment [7, 21, 27].

Arginine scarcity: a potential window for cancer therapy

Chemotherapy and radiotherapy are allied with several significant shortcomings, like lack of precision, non-specificity, and, moreover, radiotherapy is applied only on solid tumors. These shortcomings can be overcome by supplementary precise therapeutic enzyme treatment [29]. The enzymatic treatment seemed to be a promising therapy as these biomolecules are involved in various metabolic and proliferative cell pathways and specific towards cancerous cells [30]. Enzymes asparaginase, arginase, methionase and Arginine deiminase have already been explored for their anticancer properties and many of them are in clinical trials [5, 31–34]. Normal healthy cells have various checkpoints that keep them under controlled cell division conditions, lost in cancerous cells. Enzymatic therapies will act particularly on these checkpoints, ultimately involved in cellular metabolism and proliferation activities [35–37].

A single amino acid starvation strategy is well studied and characterized by previous studies [6, 38]. Auxotrophic cancerous cells are targeted by starvation therapy and

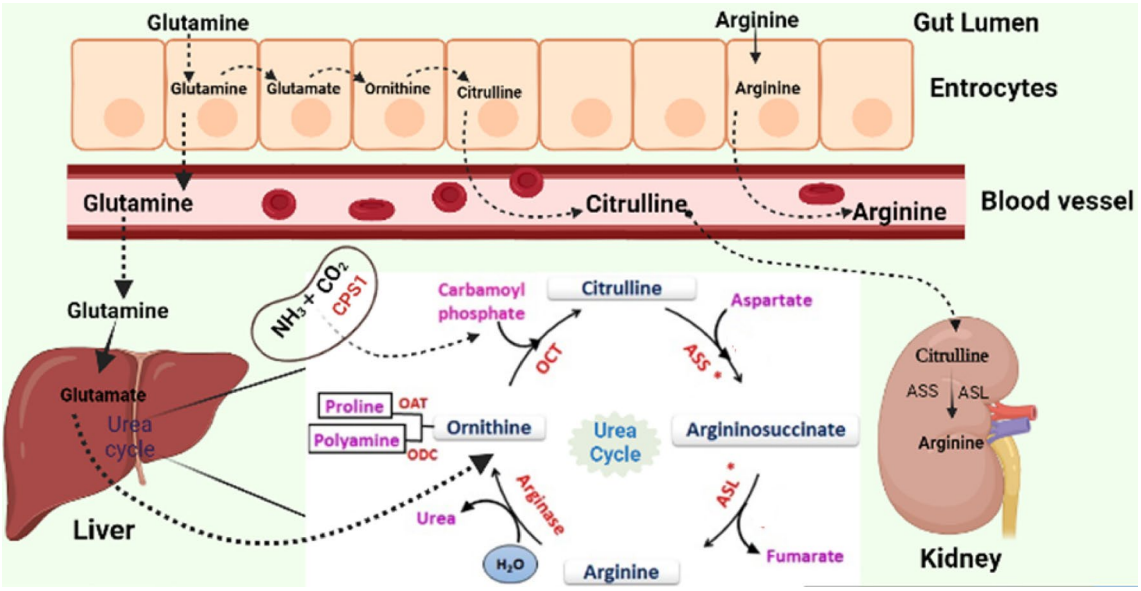

Fig. 1 Schematic representation of the Arg metabolism. Arg from dietary sources absorbed directly from the lumen and performed its normal metabolic functioning in healthy cells. Liver catabolizes Arg by urea cycle where L-Ornithine along with carbamoyl phosphate (pooled by the action of carbamoyl phosphate synthetase 1 (CPS1) of mitochondria) is transformed into L-citrulline by ornithine transcarbamylase (EC 2.1.3.3; OCT), highly expressed in the liver and small intestine. Ornithine is a precursor for polyamine and proline biosynthesis and is formed by the enzymes ornithine decarboxylase (EC 4.1.1.17; ODC) and ornithine aminotransferase (EC 2.6.1.13; OAT), respectively. L-Ornithine is recycled back by argininosuccinate synthetase (EC 6.3.4.5; ASS) and argininosuccinate lyase (EC 4.3.2.1: ASL). Both ASS and ASL are the key rate-limiting enzymes in Arg metabolism, working well in normal healthy cells whereas absent in Arg auxotrophic cancer (enzymes missing in cancer cells shown with an asterisk over them). Arg is a versatile amino acid involved in various biomolecules' metabolism (Proline, NO, Hormone, creatine, and polyamine) [7, 27, 28]. The figure was prepared using www.biorender.com

these findings are already under clinical trials. Researchers have been interested in Arg deprivation for the last few years because of its semi-essential nature and role in tumor proliferation. A very earlier study also showed the association between Arg and tumor in a mouse [10]. Since Arg is a versatile biomolecule and studies have shown that ASS is the crucial enzyme in its metabolism and most cancer cells are auxotrophic to this amino acid. The ASS auxotrophic nature of cancerous cells opens the gateway for novel therapy by altering the microenvironment (restricted Arg) around uncontrolled cell proliferation. The primary cause of this auxotrophic nature is either due to metabolic defects or a molecular mechanism based on the silencing of key Arg metabolizing enzymes (ASS 1, ASL, OTC, Carbamoyl phosphate synthetase I, and Arginase 1).

The ASS 1 expression is suppressed by methylation of the CpG island promoter region. The ASS gene is differentially expressed in different cell types. Arg auxotrophic due to ASS 1 observed in solid tumors like HCC (100%), prostate (100%), pancreas (less than 80%), breast cancer (60%), small lung cancer (44%), bladder cancer (45%), Hodgkin lymphoma (97%), non-Hodgkin lymphoma (95%), malignant pleural mesothelioma (63%), head and neck squamous cell (56%), glioblastoma multiforme (36%), osteosarcoma (63%), and malignant melanoma (100%) [8, 39–41]. Another major factor responsible for transactivation of ASS promoter is O-glycosylation of transcription factor SP1 by glutamine. ASS expression is also controlled by cMyc and hypoxia-induced factor-1 alpha (HIF1 alpha) interaction with E-box element located at ASS 1 gene promoter. The efficacy of deprivation therapy imparts on the susceptibility of all the factors mentioned earlier [27, 40].

Studies have also shown the high expression of another key enzyme, ASL, in ASS negative tumor cells. Inhibition of NO and cyclin A2 blocks the ASL expression in HCC and breast cancer. Low ASL mRNA expression observed in GBM (Glioblastoma) cell lines due to methylation of CpG island in the ASL promoter region [42, 43]. OTC expression is also downregulated in pediatric sarcoma and brain tumors [44]. The mRNA expression of ASS 1 in normal and cancer cells is elucidated by the GENT2 bioinformatics tool shown in Fig. 2.

Three strategies could be opted for altering the concentration of Arg in microenvironments of Arg auxotrophic cancers [11, 45]. The first approach is to restrict the dietary intake of Arg, but the success is achieved only to treat colorectal cancer (CRC) and skin cancer progression in a mouse model. This approach has been dropped from the list

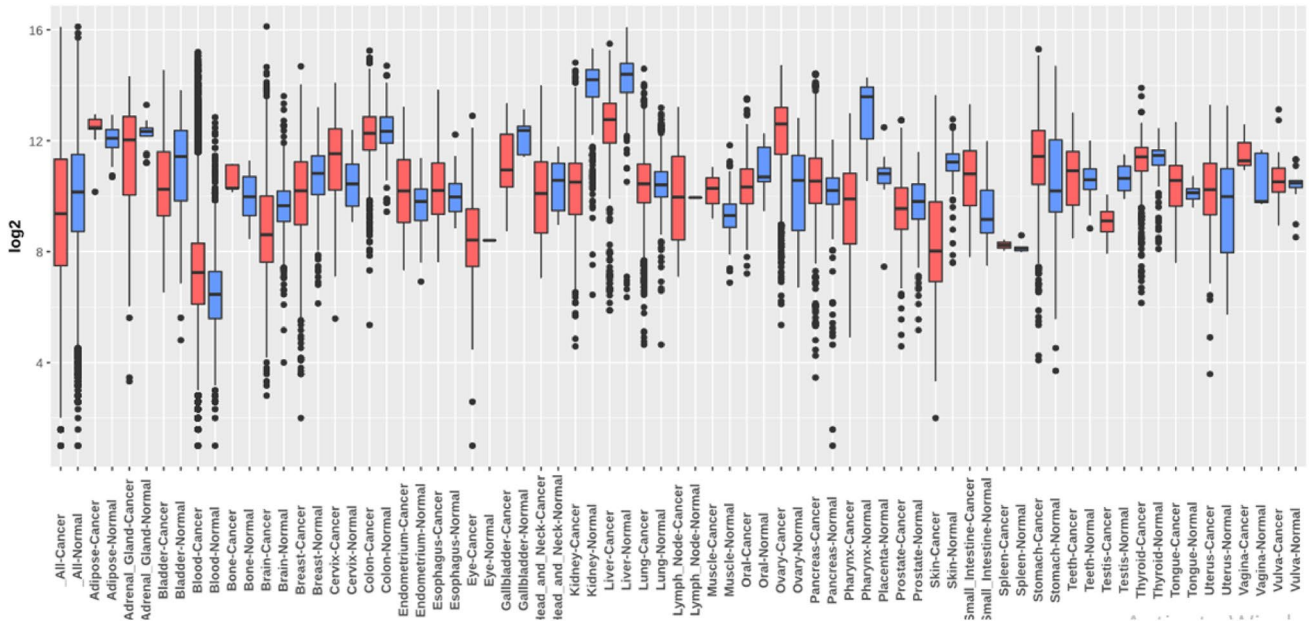

Fig. 2 Estimated mRNA expression in normal (represented in blue) and cancer cells (represented in red) (http://gent2.appex.kr/gent2)

as it proves to be ineffective against human cell lines [46, 47]. The second approach, inhibiting the Arg sensors and transporters, was also ineffective since no agent has yet been discovered to block all Arg transportation [48]. The third and most fascinating approach is applying Arg depriving enzymes such as Arg decarboxylase (ADC), Arg deiminase (ADI), and arginase. These enzymes have different catalytic properties, products formed, immunogenicity, stability, and pharmacokinetic properties [38].

Out of three ADEs, ADI and arginase are in clinical trials for their anticancer properties. This Arg starvation results in auxotrophic cancer's death due to impairment in mitochondrial functions, disturbances in the nucleus (inhibition of DNA and RNA biosynthesis), and chromatin autophagy. As ASS auxotrophic cancer cells procure Arg from their microenvironments, Arg depletion results in the induction of autophagy pathways or cell growth arrest at several checkpoints. The mTOR (Mechanistic Target of Rapamycin) pathway gets inhibited, whereas the AMPK (AMP-activated protein kinase) pathway is activated to unregulate cancer cells apoptotic destruction. Normal cells divert their metabolic machinery to citrulline as growth promoters, whereas cancer cells become stressed due to Arg deprivation. Short term Arg deprivation therapy resulted in a low level of ATP and NO leads to Endoplasmic reticulum (ER) stress by activating the mTOR pathway and consequently leading to autophagy induction. Prolonged Arg deficient cancer cells either provoke a strong immune response by activating the Bax-mediated apoptotic pathway or cells may become resistant to enzymatic therapy due to re-expression of the ASS gene. The autophagic inhibitors (chemotherapeutic drugs) exerts their effects by activating tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which leads to cleavage of Beclin 1 and finally, the instigate apoptotic pathway [49–51] (Fig. 3).

Cancer cells behave very smartly to protect themselves from Arg deficiency. One of the leading causes is the re-expression of key limiting enzymes of Arg metabolism (ASS and ASL), making arginine available for cancer proliferation [52]. Moreover, cell proliferation is sometimes sustained by an auxiliary mechanism like an aspartate molecule that acts as a precursor not only for pyrimidine nucleotide but also substrate for argininosuccinate, due to which Arg is abundantly available to cancer cells (Fig. 1). In such a scenario, aspartate deprivation should be implemented along with ADEs [53]. Autophagy induction also enhances cell survival by activating AMPK and ERK1/2 (extracellular signal-regulated protein kinase) protein cascade pathways which can be overcome using autophagy inhibitors like chloroquine [27, 54].

Arg decarboxylase (ADC)

ADC (EC 4.1.1.19) catalyzes the transformation of Arg to agmatine and carbon dioxide. Agmatine is supposed to induce anti-tumor activity by non-specifically obliterating OCT expression to elicit apoptotic pathways [24]. Agmatine has a versatile function in polyamine and NO metabolism and as an ADC inhibitor (Feedback inhibition) [11]. Previous studies have concluded the role of ADC against solid

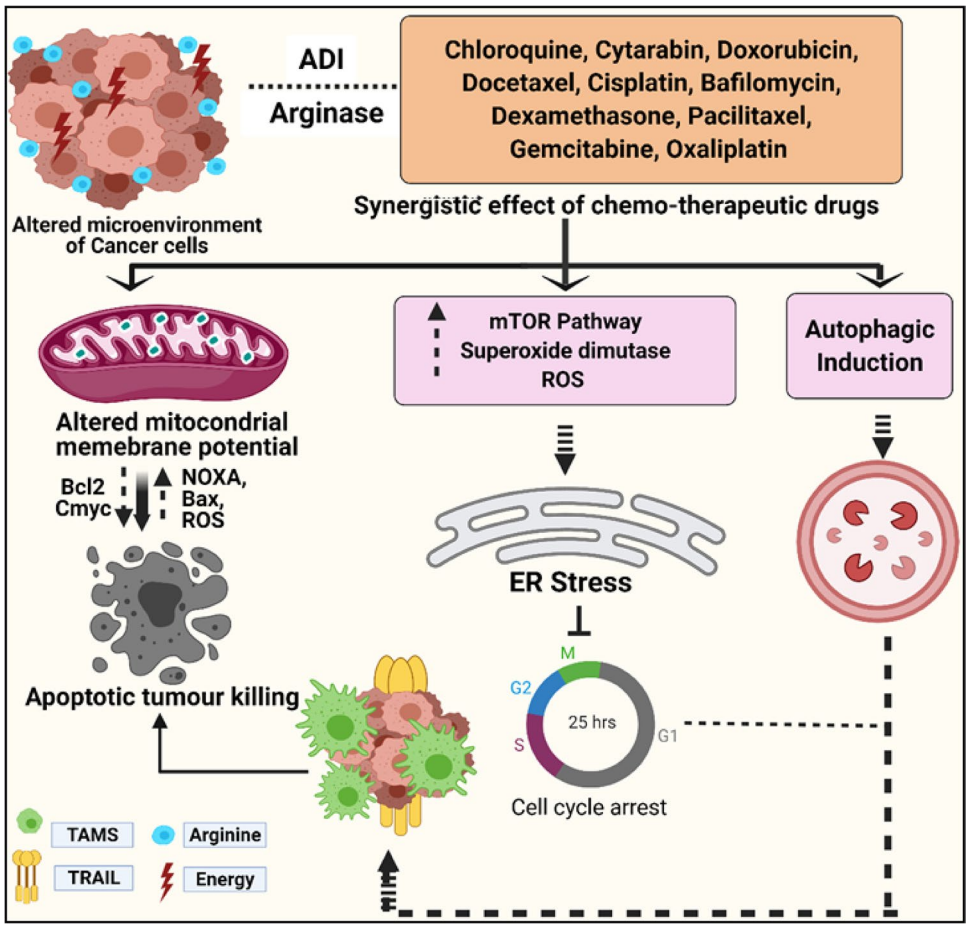

Fig. 3 Arg starvation on tumor cells: altered metabolic microenvironment of cancerous cells leads to increased demand for nutrients (Arg, asparagine, methionine, and energy fuel), which counter them as prospective targets for treatment [37, 55]. Alteration in the microenvironment by ADEs leads to enhanced activity of activated TAMS (Tumor Activated Macrophages), TRAIL (Tumor Necrosis Factor Related Apoptosis-inducing Ligand), and ROS (Reactive Oxygen Species) production, which is a marker for apoptosis [49]. Low levels of energy fuel ATP (Adenosine Tri-Phosphate) and NO (Nitric Oxide) activate Manganese Superoxide Dismutase, Calreticulin, Glutathione Peroxidase enzymes, activates the mTOR pathway, that leads to ER (Endoplasmic reticulum) stress and, consequently, results in autophagic induction. Long-term therapy results in cell growth arrest at various checkpoints. Senescence eventually leads to apoptosis by Bax-mediated pathway. Chemotherapeutic drugs, along with ADEs, also induce autophagy and mediate apoptotic pathways for cancer cell death [8, 27]. The figure is prepared with the help of www.biorender.com

tumors' proliferation by interfering with polyamine biosynthesis and the cell cycle. Impairment in agmatine homeostasis shows antineoplastic activity in human colon cell lines (Caco2, Cx1, Colo320, HT29, Colo205E, and SW480) [56]. The anti-proliferative activity of agmatine shows a cytostatic effect on the human colon adenocarcinoma HT29 $Glc^{-/+}$ cell line [57]. Inhibition in the progression of human hepatoma cells (HepG2) was also observed with agmatine dosage due to the abolition of the ODC expression [58]. Exogenous agmatine administration inhibited the human leukemia cells HMC-1 and HL-60 proliferation by causing cell-specific effects (low agmatinase expression) and interfering with G1 and G2 phases of the cell cycle without any evident sign of apoptosis. Only cytostatic events were observed at the G2 phase of the cell cycle [59]. Besides these functions, agmatine is retained in healthy cells and begins to have toxic effects on their growth. The non-metabolic agmatine, when non-selectively stored in cells, inhibits polyamine biosynthesis, eliminates ODC actions, and arrests cells at different checkpoints without being selective. ADC is no longer considered a potential cancer treatment candidate as it has non-selective toxicity against cancer cells and healthy normal cells [17] (Fig. 4).

Arginase deiminase (ADI)

ADI (Arg iminohydrolase, EC 3.5.3.6) catalyzes the semi-essential amino Arg to L-citrulline and ammonia. ADI is present mainly in a prokaryotic system (mycoplasma) and absent in the mammalian system [60]. The ADI pathway

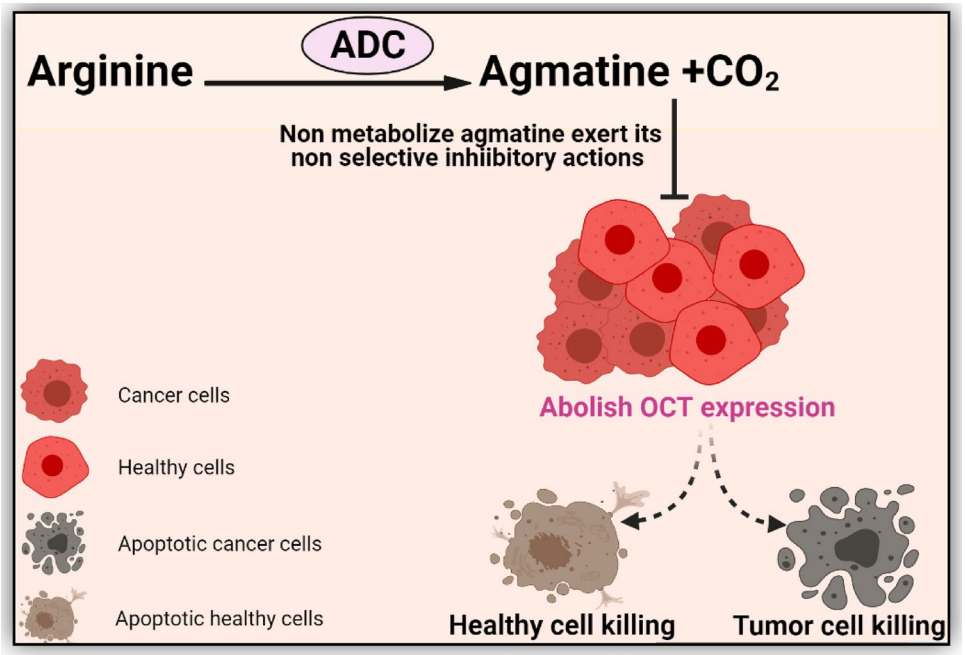

Fig. 4 Schematic representation of the action of arginine decarboxylase (ADC): non-selective destruction of normal healthy cells and cancerous cells due to the action of non-metabolic agmatine produced by ADC. The figure is prepared with the help of biorender.com

in microbes is the major non-glycolytic pathway for the energy source. ADI plays an imperative role in the bacterial system as the critical factor for survival under stress conditions and determines the bacterial system's virulence potency. The ADI pathway in the bacterial system is most studied in prokaryotes and plays a detrimental role in cell survival under acidic environmental conditions [28]. The ADI pathway is regulated at the transcriptional level by Arc operon and ArcR regulators. Arc operon encodes multiple enzymes by ArcA (Arg deiminase; ADI), ArcB (Ornithine carbamoyltransferase EC 2.1.3.3; OCT), and ArcC (Carbamate kinase EC 2.7.2.2; CK) [61]. Arg enters the bacterial system with the efflux of ornithine by Arg ornithine antiporter and gets metabolized to citrulline and ammonia by ADI, which is then phosphorylated to Carbamoyl phosphate (EC 6.34.16) by the action of OCT. Energy generated during the ADI pathway provides energy for bacterial survival. In the end, the ADI pathway also generates ammonia and carbon dioxide by the action of carbamate kinase. The ammonia generated further undergoes an oxidoreduction to form ammonium ions, which elevates the pH of the cytosol and allows it to withstand hostile acidic conditions through its neutralizing effect [28, 62] (Fig. 5). Thus, the enzyme ADI plays a significant role in the pathogenesis of *Streptococcus pyogenes* [63], *Laribacter hongkongensis* [64], *Listeria monocytogenes* [62], and *Salmonella enterica* serovar *Typhimurium* [65].

Various studies in the early '90s established ADI's antitumor properties for ASS-negative cancerous cells [13, 66]. The most common ADI sources are *Bacillus*, *Pseudomonas*, *Enterococcus*, *Lactobacillus*, *Streptococcus*, and *Mycoplasma* [66–70]. In vivo and in vitro anti-tumor activity of mycoplasma ADI was studied by Takaku et al. 1992, 1993. The group reported higher antigenicity and shorter half-life as the significant shortcomings of the ADI during in vivo experiments [66]. To address the issue of a shorter half-life, the group also recommended multiple doses of ADI. To deal with the other shortcoming of ADI, i.e., higher immunogenicity, ADI was chemically modified using polyethylene glycol (PEG) [71, 72]. Earlier studies showed that ADI potency against tested melanoma cell lines depends upon the expression of key enzymes of the urea cycle. The group interpreted that the auxotrophic nature of these cancerous cells was attributed due to a lack of ASS enzyme [73].

One of the significant findings of ADI was its anti-proliferative effect against human leukemia cells and is 100 times more effective than asparaginase. Before that, asparaginase was the only enzyme used for leukemia therapy [74]. ADI-PEG with reduced immunogenicity has been studied against human melanoma, hepatocellular carcinoma (HCC), small lung cancer, pancreatic cancer, prostate cancer, as shown in Table 1 [14–16, 54, 75, 76]. Pegylation significantly improves the pharmacokinetics properties of ADI. ADI-PEG 20 has been clinically approved by the US Food and Drug Administration (US-FDA) for HCC treatment [27, 77]. ADI-PEG agents were found ineffective as targeted therapy when screened against HCC and metastatic melanomas. The studies were done as a monotherapy injected intramuscularly in multiple doses in phase I/II clinical trials. Although cytostatic and anti-proliferative effects were successfully achieved, there were also mild to severe side

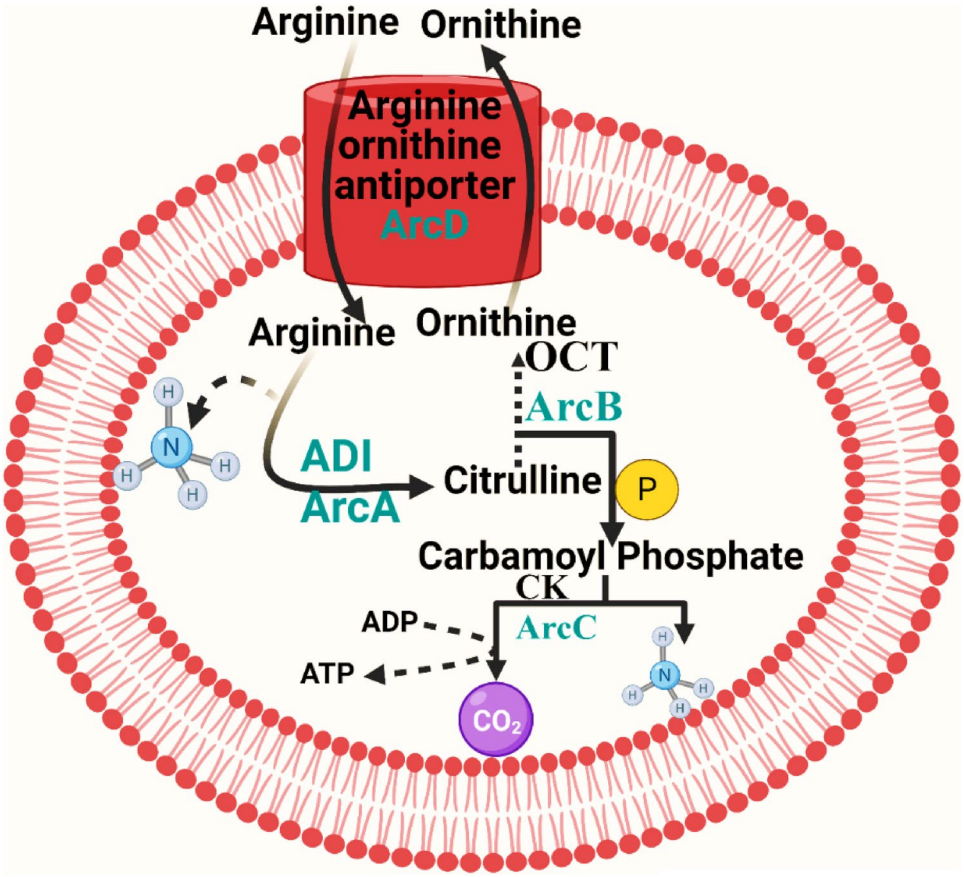

Fig. 5 ADI pathway showing the catabolism of Arg to ornithine with the generation of ammonia and urea (Biorender.com)

effects. Fatigue, local rash at the injection site, and hyperuricemia were all mild side effects, whereas gastrointestinal toxicity was observed as one of the adverse side effects after therapy. Trials also concluded that the major drawback of ADI-PEG treatment was that its effects could be reversed by neutralizing antibodies in the circulating serum [14, 16, 39]. As monotherapy was not effective in overcoming cancer progression, combinatorial approaches are being studied in phase I of clinical trials (Clinical studies are listed in Table 2). One of the most commonly studied chemotherapeutic drugs, 5-Fluorouracil (a potent anticancer drug), is being investigated along with ADI for its interfering potential against cancer [78]. Another study has shown that the combined administration of ADI-PEG-Gemcitabine inhibits the development of pancreatic cancer growth by inhibiting the phosphorylation of NF-κB p65 [79].

Other studies have shown that the combination of ADI-PEG and cisplatin increases the sensitivity of ASS-positive HCC cell lines and melanomas [80]. The combination of ADI-PEG and Doxorubicin inhibits breast cancer progression. ADI-PEG combinatorial therapy with chemotherapeutic agents (Cisplatin, pemetrexed, doxorubicin) is also studied against prostate and small lung cancers [11]. More preclinical extended studies are needed to confirm the success stories of these ADI-PEG combinatorial cancer-fighting strategies. The mechanism for ADI that results in cancer progression inhibition relies on the fact that it reduces the Arg from the microenvironment of cancer cells. This physiological change in Arg homeostasis triggers a range of changes within cells, such as AMPK sensing the cellular AMP/ATP ratio, reduced nutritional content activating mTOR/ Akt, ERK1/2 pro-apoptotic proteins, which in turn undergo a series of sequential activation and deactivation, eventually leading to cancerous cell death (Fig. 3) [11, 54].

Arginase

Arginase (Arg amidohydrolase EC 3.5.3.1) is a metalloenzyme, i.e., a manganese-dependent hydrolase that catalyzes the conversion of the semi-essential amino acid Arg to ornithine (a non-standard amino acid) and urea (waste of ureotelic) [99]. Arginase is a ubiquitous enzyme expressed from single-celled bacterial communities to complex higher vertebral organisms. It is crucial for nitrogen metabolism [100]. Simpler organisms like bacteria and yeast typically have one form expressed in mitochondria whereas, in the mammalian system, arginase 1 and arginase 2 are well characterized and expressed in the liver (cytosolic location) and kidney (mitochondrial location), respectively. In humans, two isoforms of arginase, arginase 1 and arginase 2, are mainly expressed

Table 1 Prior preclinical studies of ADI as therapeutic enzymes for cancer remedy

Preclinical study	Cell line/animal model	Results	Short-comings	References
ADI application on murine leukemia lymphoblasts	Murine leukemia lymphoblasts cell lines	The first study of ADI as a therapeutic enzyme	NA	[81]
Purified ADI from a mycoplasma-infected cell line showed growth inhibition of human tumor cells	HLE, HSC-3, HSC-4, C4I, A549, CaSki, SCC, KB, T98G, RPMI-8226, VMRC 5	ADI was 1000 times more efficient with lower K_M than that of bovine liver arginase	From RSV-BRL infected cells, the mycoplasma strain was not identified	[13]
Purified ADI from *mycoplasma arginini* showed in vivo anti-tumor activity	Mouse tumor cell lines Colon 26, MH134 Meth A (fibrosarcoma), B16 (melanoma), and mice	First report of ADI as the anti-tumor agent from *mycoplasma arginini* No toxic effect was observed on mice with an in vivo half-life of 4 h A strong inhibitory effect on Meth A, Colon 26, S-180, and MH134 at a minimum concentration of 5 ng/ml, whereas B16 and L1210 were resistant to treatment	Only the mouse cell line study, no human cancer cell line was studied Not effective towards a solid tumor	[66]
In vitro growth inhibition by mycoplasmal ADI for susceptible human melanoma cell lines	A375, G361, Mewo, VMRC-MELG, C32TG, HeLa	Cell lines were susceptible, excluding HeLa cells The sensitivity of cell lines varies with ASS expression	The effect was reversed with the addition of L-ornithine	[73]
High-level mycoplasma ADI expression in *Escherichia coli* for its effectual renaturation as an anti-tumor enzyme	MH134 and Meth A cells and mice	ADI gene, highly expressed in *E. Coli*, purified under denaturation conditions using Gdn-HCl extraction while maintaining all catalytic and biological efficiency	The study was limited to mice cell lines only	[82]
Assessing anti-tumor activity of *Mycoplasma arginini* ADI as well as its growth-inhibitory mechanism	Mouse hepatoma cell line MH134, MethA fibrosarcoma	*Mycoplasma arginini* ADI treatment resulted in 50% growth inhibition in cancerous cell lines at a concentration (20 ng/ml) of the enzyme	A control cell line was not there The supplementation of ornithine reversed the inhibitory effect of ADI	[72]
ADI hinders cell propagation by arresting checkpoints of cell cycles and induce apoptosis	Human umbilical vein endothelial (HUVE) cells, SH-EP, WAC2, SaOS, and Y-79 Cells	A study proved that ADI showed not only the anti-proliferative effect by depriving Arg, but also it arrests them at G1 and S phase and renders them to apoptosis	No in vivo study was carried out	[83]
ADI constrains the growth of human leukemia cells more effectually than asparaginase by inducing programmed cell death and arresting checkpoints of cell cycles	Human cell lines acute pediatric T-(Jurkat) or B-(Tanoue) leukemia and HL-60	The very first report of ADI against leukemic cells Provide positive checkpoints at G1 and S phases and lead to faulty cell death The 5–10 ng/ml concentration was an effective biological dose, which is 100 times lower than asparaginase	In vivo toxicity experiment was not done to validate the results thoroughly	[74]

Table 1 (continued)

Preclinical study	Cell line/animal model	Results	Short-comings	References
PEG conjugated ADI: PEG formulations and assessment of its pharmacological properties	Mice and rabbits	ADI-PEG succinimidyl succinate conjugate was found to be among the various linkers Decreased the immunological reaction after PEG linking Enhancement in pharmacokinetic (PK) and pharmacodynamic (PD) properties were observed	Overall 50% enzymatic activity was lost	[77]
The anti-angiogenic potential of rADI	Human umbilical vein endothelial (HUVE) cells	ADI gene from *Mycoplasma arginini* was successfully expressed in *E. coli* This was the first report suggesting rADI is anti-angiogenic	Not clear from the study whether ADI induces an anti-proliferative effect or it acts as anti-angiogenic	[84]
In vitro and in vivo inhibition of human melanomas and HCC by ADI-SS PEG-20,000 MW treatment	The nude and SCID mice Melanoma and HCC human cell lines	The specificity of ADI towards Arg was established The study was extended to a human cancer cell line Arg auxotrophic nature of these cell lines was due to the lack of ASS, so all these cell lines were sensitive in vitro ADI-SS PEG-20,000 showed enhanced in vivo efficacy and prolonged half-life up to 7 days compared to naïve one, i.e., 5 h	The exact mechanism of in vitro and in vivo inhibition was not explained	[76]
rADI treatment resistance to anti-proliferative activity in cell culture correlates with the endogenous enzyme ASS	Mouse fibroblast (L929), Human umbilical vein endothelial (HUVE), human cervix adenocarcinoma (HeLa), human lung carcinoma (A549), human prostate carcinoma (LNCaP), Madin–Darby canine kidney (MDCK), human mammary adenocarcinoma (MCF 7), and Chinese hamster ovarian (CHO) cells	Cell lines require Arg for their growth; however, in MCF 7, HeLa, and LNCaP cell lines, rADI treatment failed to inhibit their growth due to the high expression of ASS, which converts citrulline to Arg	The exact mechanism and role of ASS induction for tumor cells by rADI therapy were not precise	[85]
Dexamethasone doses along with ADI treatment leads to Cytotoxic effects in human T-lymphoblastic leukemia CCRF-CEM cells	Human T-cell acute lymphoblastic leukemia CCRF-CEM	ADI showed a synergistic effect with dexamethasone (DEX) by suppressing c-myc expression and upregulating p27Kip1	The in vivo study was not carried out	[86]

Table 1 (continued)

Preclinical study	Cell line/animal model	Results	Short-comings	References
Renal cell carcinoma (RCC) lack ASS expression and is extremely sensitive to Arg deprivation via ADI	RCC cell lines (Caki-1, Caki-2, ACHN, SN12C, SN12PM6, RENCA) and Mice	RCC cells do not show any ASS expression, thus incapable of synthesizing Arg from citrulline, making them more prone to ADI treatment in a dose-dependent manner Allografted renal cell carcinoma (RENCA) tumor cells are also inhibited in vivo in mice models Reduced mechanism of neovascularization due to reducing NO and polyamine synthesis	ASS expression profiling from different populations must be done to validate the results thoroughly	[87]
ASS deficient pancreatic cancer cell lines sensitive to Arg deficiency by ADI treatment	47 human pancreatic adenocarcinoma specimens, 20 non-neoplastic pancreatic tissues Nude Mice	ADI-PEG treatment resulted in a cytotoxic effect in vitro due to autophagy (caspase activation) Pancreatic mice xenograft showed 50% reduced tumor growths	ASS^+ tumor cells, almost 87%, were resistant to ADI	[88]
ADI treatment as a novel therapy for prostate cancer that results in programmed cell death of diseased cells	PC3 cells, CWR22Rv1 Nude BALB/c mice	ADI-PEG-20 induces cytotoxic effect alone as well as with docetaxel (chemotherapeutic drug) by mechanism mTOR/Akt autophagic pathway	Either ASS^- or mild ASS^+ cancer cell lines were sensitive A limited number of cell lines were studied	[54]
Arg deprivation a new approach to head and neck cancer	Head and neck cancer cell lines FaDu, HONE-1, KB, OECM1, UMSCC-1, SCC-4, SCC-15, SCC-25	Showed that Arg is the pre-requirement for the progression of head and neck cancer Very first report where Arg deprivation with ADI is employed for head and neck cancer cell lines that are ASS negative	It cannot halt the growth of an ASS^+ tumor (as its capability to restore Arg from ornithine)	[9]
A combinatorial approach for Arg deprivation with ADI treatment along with cisplatin against melanoma cell lines	A375, A2058, and SK-Mel-2 from ATCC; Mel-1220, Mel-F, and Mel-114	The study showed that the anti-proliferative effect of ADI-PEG-20 was not only because of the non-availability of Arg but also due to apoptosis induction This study showed the enhancement in the expression of pro-apoptotic protein Noxa and diminished expression of anti-apoptotic protein Survivin in a cell line study Synergistic effects of treatment were observed with cisplatin	Effective against ASS-negative cells only The in vivo study was not carried out	[89]

Table 1 (continued)

Preclinical study	Cell line/animal model	Results	Short-comings	References
ADI-PEG-20 obstructs the progression of small cell lung cancers deficient in the expression of ASS	Human small cell lung cancer (SCLC) xenografts	The very first report showed the anti-proliferative effect of ADI-PEG-20 against SCLC Overall, 45% of SCLC tumors and 50% of cell lines under study showed ASS expression and thus resistant towards ADI treatment SCLC xenografts also showed a positive effect	Less efficient	[75]

NA not assigned

in cytosol and mitochondria, respectively. Both isoforms are encoded by two different genes located on different human chromosomes [101–103]. Human arginase 1 and 2 consists of 322 and 354 amino acid residues, respectively. Both isoforms are closely homologous and share common identical crucial areas that are responsible for metal ion binding. Both isoforms require manganese ions as the cofactor for their activity [104]. The last enzyme in the urea cycle, human arginase 1, involved in eliminating excessive ammonia generated primarily by the amino acid catabolism [105], is expressed mainly in hepatic tissues (Fig. 6). Human arginase 2 is mainly expressed in mitochondria of extra-hepatic tissues, especially the kidney (Fig. 7). It is primarily involved in the biosynthesis of nitric oxide and polyamines. [7, 8].

Arginase sources

Arginase is found in various organisms in nature and well-characterized in bacteria, fungi, lichens, plants, and higher mammals [106] (Table 3). The functional roles of the arginase enzyme are as diverse as their occurrence. In lichens, arginase acts as lectin binding firmly with algae's cell wall, assisting cell-to-cell communication and maintaining a symbiotic balance between algal and fungal counterparts to sustain the organism's vitality [107]. In plants, it plays a diverse role, from seed germination, nitrogen mobilization to defensive functions. Arginase expression has been seen both in biotic as well as abiotic conditions. Arg is the key storage form of amino acid in seedlings and during germination, arginase is expressed in mitochondria and helps in the amalgamation of nitrogen-containing biomolecules [108–110].

Arginase as a therapeutic agent

The earlier study began when arginase was purified from the ox liver by precipitation. Afterwards, an anti-proliferative study was first established when purified arginase resulted in a 77% decrease in tumor growth [142, 143]. However, much success was not achieved due to the high K_M value, which required a very high dose. Pegylation was done to improve its catalytic efficacy [144, 145]. Several studies have shown that using human arginase, covalent binding the human arginase to PEG, and replacing the metal ion with cobalt resulted in immunogenicity, optimum pH, and a low circulatory half-life in serum [20, 146, 147]. Both ASS^- and OCT^- cancer cells can be treated successfully with arginase administration. Yoon et al. also pointed out that melanoma cells lack ASS, so they require additional Arg from outside sources to meet their needs [148]. The only clinically tested prescribed chemotherapeutic drug for melanoma is dacarbazine, but it is ineffective in most cancer cases [149]. These prior studies have established arginase as a viable treatment option for melanoma. HCC is a fatal neoplastic condition prominently

Table 2 Clinical studies carried out using ADI as therapeutic enzymes for cancer remedy

Clinical trial	Cancer patients	Results	Side effects	References
Phase I/II trial ADI-PEG-20 in unresectable HCC	Patients with advanced HCC	160 IU/m^2 was used as an optimal biologic dose for phase I study and the higher dose of 240 IU/m^2 for phase II showed positive results	Allergic reaction	[14]
Phase I study of PEG-ADI for patients suffering from metastatic melanoma	Stage IV inoperable metastatic melanoma patients In the US phase I study, 14 patients out of 15 completed the treatment: injected at doses of 20, 40, 80 and 160 U/m^2 12 out of 24 patients from the Italian phase I to II study completed treatment	160 U/m^2 biologic dose once in the week was effective for removing plasma Arg in US phase I This was the first study that showed that NO synthesis inhibition (due to ADI-SS PEG 20,000 MW) has no effects on heart rate and blood pressure US patients were unresponsive, whereas in Italian population showed a 25% response rate. No neutralizing antibodies were observed	Side effect like hyperuricemia was observed Enzymatic profiling of patients involved in Arg metabolism was not done	[39]
Phase II study of ADI-PEG-20, a new approach for melanoma	Patients with melanoma	A biological dose of 160 IU/m^2 was injected intramuscularly and was active against melanoma cells The efficacy of the treatment depends on ASS expression	Side effects at the site of injection like discomfort and slightly elevated serum uric acid were observed	[15]
Phase II study of pegylated ADI for nonresectable and metastatic HCC	Patients with unresectable HCC	Prolonged the survival rate in patients Arg level remains at baseline	Immunogenic effects	[20]
Pegylated ADI Treatment of Patients with unresectable HCC: results from phase I/II studies	HCC patients (19 patients)	This is the first report regarding Phase I/II Studies using ADI-SSPEG 20,000 MW, which successfully showed the 160 U/m^2 as an optimum biological dose with no side effects No neutralizing body, so no allergic reaction was observed	The sample size was small	[90]
Phase II study of ADI-PEG-20 in Asian population suffering from advanced HCC	Patients with advanced HCC	Intramuscular injections of ADI-PEG-20 at doses of IU 160 were given weekly Arg level was significantly depleted in plasma after the first dose of ADI-PEG-20, but Arg level was noticed before the second dose, making this approach less significant	Neutralizing antibodies formed Multinational studies need to be done	[91]
ADI-PEG-20 for patients with advanced melanoma Clinical trial identifier: NCT00520299	31 previously treated patients	Patients treated in three cohorts with 40, 80 or 160 IU/m^2 ADI-PEG-20 doses weekly Circulatory arginine reduced in 30/31 patients by day 8. Mean plasma levels of ADI-PEG-20 correlated inversely with ADI-PEG-20 antibody levels	AEs grade 1 and 2 including injection site pain, rash, and fatigue, were observed	[16]

Table 2 (continued)

Clinical trial	Cancer patients	Results	Side effects	References
1/1B non-randomized open-label study of ADI-PEG-20 along with gemcitabine and nab-paclitaxel against advanced pancreatic adenocarcinoma Clinical trial Identifier: NCT02101580	Total 18 patients out of which 9 Patients had metastatic pancreatic cancer (1 line of prior treatment; the dose-escalation cohort) and other 9 have no prior treatment (the expansion cohort)	Patients received both gemcitabine (1000 mg/m^2) and Nab-paclitaxel (125 mg/ m^2) for 3–4 weeks cohort 1 treated intramuscular with ADI-PEG-20 at 18 mg/m^2 weekly; cohort 2 (expansion cohort) treated with 36 mg/m^2 weekly Recommended Phase 2 dose (RP2D) was 36 mg/m^2 Overall 45% response rate achieved with median overall survival (OS) of 11.3 months	All patients experienced grade 3/4 AEs (Adverse events) (regardless of any drugs: 67% neutropenia, 56% leukopenia, 44% anemia, and 33% lymphopenia)	[92]
Phase 1 dose-escalation study of ADI-PEG-20 combined with pemetrexed and cisplatin (ADIPemCis) in the first-line treatment of s malignant pleural mesothelioma (MPM) or non-small-cell lung cancer (NSCLC) Clinical trial Identifier: NCT02029690	Total nine patients; four with NSCLC and five with MPM	RP2D was weekly, intramuscularly injected at ADI-PEG-20 36 mg/m^2 plus three-weekly intravenous cisplatin 75 mg/m^2 and pemetrexed 500 mg/m^2 Circulating Arg concentrations dropped rapidly, and high citrulline levels; both changes persisted at 18 weeks	AE's grade 1/2 (Vomiting, nausea, dizziness and rash) were (83% of total) were observed	[93]
Phase 2 randomized clinical trial of ADI-PEG-20 for malignant pleura mesothelioma Clinical trial Identifier: NCT01279967	68 patients with advanced malignant pleural mesothelioma	Weekly intramuscularly vaccinated with 36.8 mg/m^2 ADI-PEG-20 PFS hazard ratio was 0.56 with a median of 3.2 months with an OS of 15.7 months	AE's grade 3/4 were observed	[94]
Phase 1, single-centre, open-label trial of ADI-PEG-20 and modified FOLFOX6 (mFOLFOX6) against HCC and other advanced gastrointestinal tumors Clinical trial identifier: NCT02102022	Twenty-seven patients enrolled 23 with advanced HCC and 4 with other gastrointestinal tumors	Patients were treated with mFOLFOX6 (oxaliplatin 85 mg/m^2 IV on day 1; leucovorin 400 mg/m^2 over 2 h on day 1; 5-fluoro-uracil 400 mg/m^2 IV bolus on day 1 then 1200 mg/m^2 over 24 h for 2 days) intravenously biweekly and ADI-PEG-20 intramuscularly weekly at 18 (Cohort 1) or 36 mg/m^2 (Cohort 2 and RP2D expansion) Median progression-free survival (PFS) and OS were 7.3 and 14.5 months, respectively	Grade ≥ 3 AE's were neutropenia (47.8%), thrombocytopenia (34.7%), leukopenia (21.7%), anemia (21.7%), and lymphopenia (17.4%) were observed	[95]

Table 2 (continued)

Clinical trial	Cancer patients	Results	Side effects	References
Phase I of ADI-PEG-20 with pemetrexed and cisplatin (ADIPEMCIS) to patients with HGGs Clinical trial identifier: NCT02029690	Ten treated patients with high-grade gliomas (HGG), six had glioblastoma, one of which had transformed from a grade II astrocytoma and three had anaplastic astrocytoma	ADI-PEG-20 was intramuscularly injected at 36 mg/m^2 weekly plus pemetrexed 500 mg/m^2 and cisplatin 75 mg/m^2 intravenously once every 3 weeks (six cycles) The best overall response was stable disease in eight patients (80%). The anti-ADI-PEG-20 antibody titer escalated during the first 4 weeks of therapy. Median progression-free survival (PFS) was 5.2 months and OS was 6.3 months ADI-PEG-20 interrupts pyrimidine pools in ASS1-negative HGGs	Grade 1/2 AE,s observed	[96]
Phase I study of ADI-PEG20 plus low-dose cytarabine (LDC) for the treatment of acute myeloid leukemia (AML) Clinical trial identifier: NCT02875093	18 AML patients	Patients were treated with 20 mg LDC subcutaneously twice daily for 10 days every 28 days and ADI-PEG-20 intramuscularly weekly injected at 18 or 36 mg/m^2 (dose levels 1 and 2) No dose-limiting toxicities were observed An overall response rate (ORR) of 44.4% and a median OS of 8.0 months among the 18 evaluable patients and an ORR of 71.4% and CR of 57.1% in the 7 treatment naïve patients were observed	AE's grade 3/4 were observed	[97]
Phase 1 trial of ADI-PEG-20 plus cisplatin in patients with metastatic melanoma Clinical trial Identifier: NCT01665183	99 patients with metastatic ASS-negative malignancies	No dose-limiting toxic effects or mortality 3% of patients withdrawn due to toxic effects. After treatment, 5% (5/99) of patients had partial responses, and 41% had stable disease. The median progression-free and OS were 3.62 and 8.06 months, respectively	AE's grade 3/4 were observed	[98]

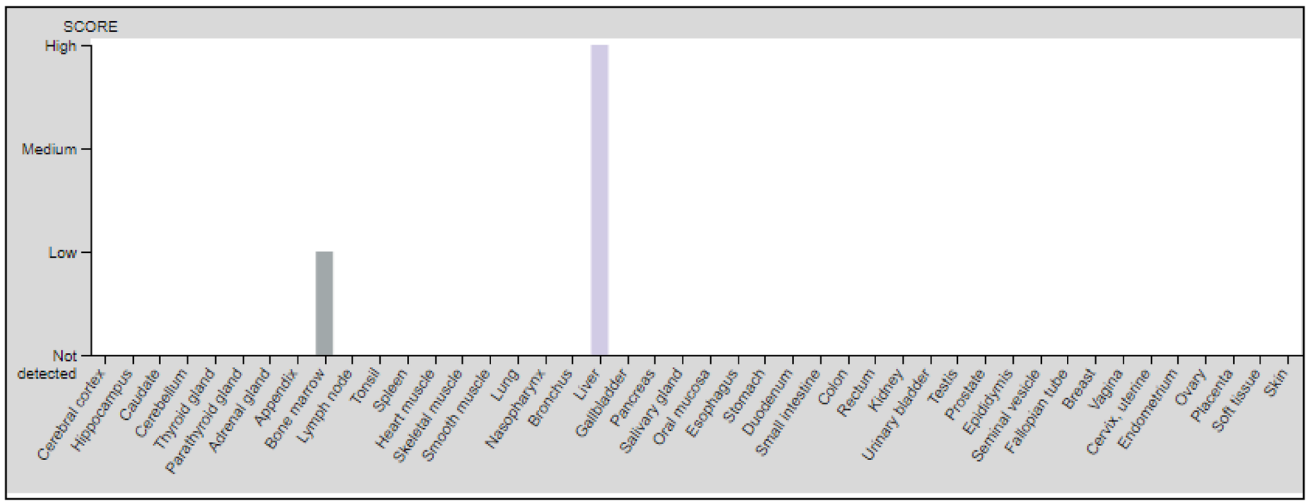

Fig. 6 Estimated arginase1 expression in normal tissues (https://www.proteinatlas.org)

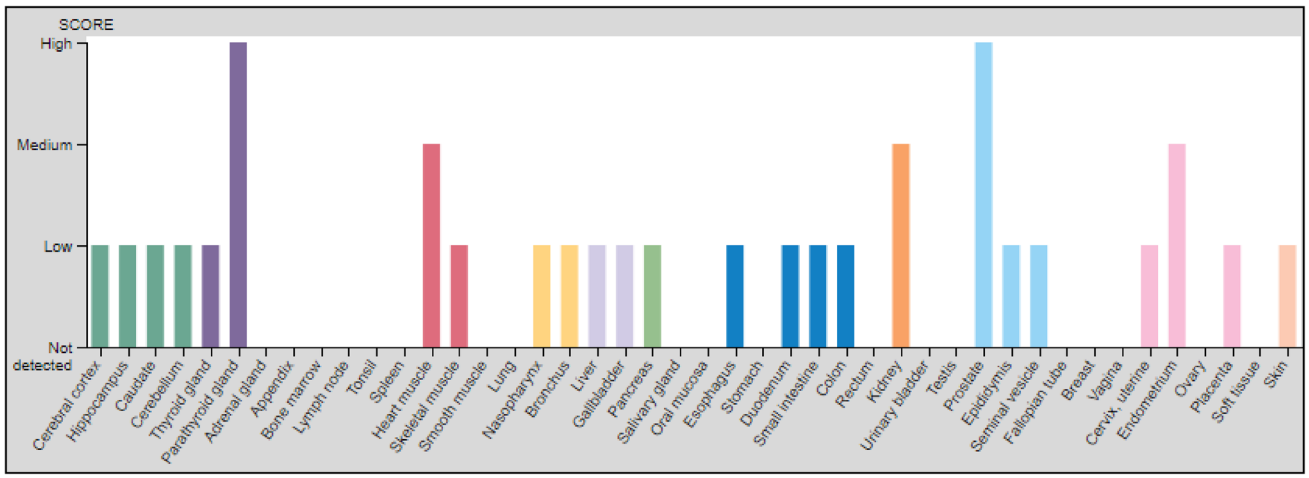

Fig. 7 Estimated arginase 2 expression in normal tissues (https://www.proteinatlas.org)

caused due to cirrhosis, leading to death [150]. There are a variety of treatments for HCC, including surgical procedures such as tumor resection and liver transplantation; percutaneous interventions such as ethanol injection and thermal ablation with radiofrequency, transarterial interventions, but all of these treatments are only effective for early-stage detection with a small diseased lesion (diameter less than 5 cm). Radiation therapy is less effective because of its non-specificity and toxicity. As a result, several potential drug molecules have been identified and are currently being tested in clinical trials [151].

Arginase has emerged as one of the potential drug candidates for the HCC treatment as previous studies revealed the L-Arg auxotrophic nature of the HCC [152]. Various studies conducted so far in cell lines and animal models have suggested that arginase is an effective agent with high specificity and no side effects [153, 154]. Earlier studies showed how arginase depletes arginine in HCC ASS positive and Hela cell lines [18]. Recent studies have revealed how arginase inhibits cancer cells' growth without affecting normal healthy cells. Arginase induces autophagy (programmed cell death) by increasing the membrane potential, ROS, Akt/mTOR signaling pathway, Erk1/2 activation, activation of tumor-associated macrophages, and various pro-apoptotic factors. All these events eventually lead to the death of cancer cells [8, 123, 155]. The preclinical studies carried out using arginase as Arg depriving enzyme are tabulated in Table 4. Previous studies have suggested that arginase is preferred over ADI for treatment, as the former can also be implemented for ASS-positive tumors. Clinical studies have indicated that arginase works well at physiological pH without eliciting any adverse effects for treating solid

Table 3 Prokaryotic and eukaryotic sources of the arginase enzyme

Sources	References
Bacteria	
Lactobacillus Plantarum	[111]
Lactobacillus hilgardii	[112]
Leuconostoc oeni	[113]
Lactobacillus lactis	[114]
Helicobacter pylori	[115]
Weissella koreensis	[116]
Bacillus caldovelox	[116]
Bacillus subtilis	[117, 118]
Bacillus brevis TT02-8	[119]
Bacillus brevis	[120]
Bacillus subtilis 168	[121]
Rummeliibacillus pycnus SK31.001	[122]
Pseudomonas aeruginosa IH2	[123]
Fungi	
Neurospora crassa	[124]
Penicillium chrysogenum KJ185377.1	[125]
Aspergillus nidulans	[126]
Yeast	
Saccharomyces cerevisiae	[127, 128]
Kluyveromyces lactis	[129]
Lichens	
Evernia prunastri	[107]
Xanthoria parietina	[130]
Peltigera canina	[131]
Plants	
Iris hollandica	[132]
Panax ginseng	[133]
Pinus taeda	[110]
Cucurbita pepo	[134]
Vicia faba	[135]
Arabidopsis thaliana	[136]
Solanum lycopersicum	[137]
Glycine max	[138, 139]
Arachis hypogaea	[140]
Coriandrum sativum L	[141]

tumors [146, 156, 157]. Completed clinical studies are listed in Table 5. As prior studies indicated, arginase could be a potential candidate for treating head and neck cancer cells more efficiently than ADI. More studies on cell lines and the animal model need to be done to validate these findings and be approved by the FDA.

Therapeutic potential of human arginase (hArg) and recombinant human arginase (rhArg)

Arginase (EC. 3.5.3.1), a metalloenzyme, and Arg deiminase could be used as depriving agents for Arg auxotrophic melanomas and HCC [38, 155]. The potential role of human arginase (hArg) for the treatment of hyperarginemia (autosomal recessive disorder resulted due to defective arginase 1 enzyme) is well established and administration of exogenous arginase would reduce the level of Arg in serum [176]. Arg auxotrophic cancers treated with hArg result in the activation of apoptotic pathways, leading to their death [11, 177]. hArg has a tremendous therapeutic value, but its lower physiological pH and short half-life activity are significant shortcomings. Moreover, therapy becomes ineffective because small animal models and toxicity arise due to ornithine accumulation, which is also a concern [27, 178]. These shortcomings can be overcome by the PEGylation of rhArg and replacing manganese ions with cobalt and covalently attaching them to polymers [179]. PEG-rhArg has been widely studied to treat HCC, prostate cancer, breast cancer, and non-Hodgkin lymphoma and melanoma [19, 20, 25, 154]. A phase I clinical trial of PEG-rhArg has been successfully carried out against HCC in humans [172]. hArg has metal-binding efficiency for both Co^{2+} and Mn^{2+} ions and thus stabilizes the tertiary and quaternary structure. However, with Co^{2+} ions, the enzyme showed a ten-fold higher activity due to increasing substrate binding capacity (D'Antonio, Hai, and Christianson 2012). Recently, attempts have been made to prolong the life and efficacy of rhArg by fusing the enzyme with the F_c region of immunoglobulin (IgG) (rhArg-F_c). Both in vivo and in vitro studies have shown that cancer cells' proliferation and migration potential has been inhibited. rhArg-F_c acts synergistically with cisplatin to retard HCC [180].

Various chemotherapeutic drugs (chloroquine bafilomycin, oxaliplatin, and capecitabine) have been tested in combination with bioengineered pegylated human arginase showing synergistic effects on tumor progression [164]. The chemotherapy medications synergistically (rhArg-chloroquine) resulted in the induction of autophagic genes like Beclin 1 and retarded the progression of non-Hodgkin lymphoma [25] (Fig. 3). The enhanced cytotoxic potential of rhArg against laryngeal squamous cell carcinoma was observed with combination therapy of chloroquine and bafilomycin [167]. More clinical studies need to be executed to decipher the mechanism of these synergistic approaches.

Arginase assay

Both quantitative and qualitative assays are available for determining arginase activity. It is essential to monitor the Arg level while administrating arginase as a therapeutic drug. In addition to spectrophotometric methods, advanced bioanalytical techniques such as fluorometric and biosensing methods are also used [181]. Table 6 summarizes some of these assay methods.

One of the most common conventional methods is thin layer chromatography (TLC) which relies on the use of

Table 4 Preclinical studies of arginase as potential therapeutic enzymes

Preclinical studies	Cell line/model study	Results	Reference
A small approach for the purification of arginase from ox liver: acetone dried ox liver arginase purified with ammonium sulfate precipitation	NA	It requires less time and can be easily reproducible	[142]
The effect of arginase on the obstruction of tumor growth	Walker 256 carcinoma injected with purified arginase formulation	The growth of a tumor after 2–4 days of injection reduced up to 77%	[143]
The effects of Arg deficit on lymphoma cells	L5178Y and L1210 mouse lymphosarcoma cell line treatment with rat and beef liver arginase	The growth of all the tumor cells gets halted, whereas normal thymus cells remain unaffected	[158]
Growth Inhibitory properties and arginase activities observed in liver and hepatoma extracts	Hep 2 cells	An inhibitory effect on cells was shown by rat liver arginase	[159]
Preparation of non-immunogenic arginase by the covalent attachment of polyethylene glycol (PEG)	Mice	Bovine liver arginase was attached covalently to Methoxypolyethylene glycol of 5000 D (PEG). It was injected intravenously into mice which reduced the problem of immune reaction But the K_M value was high for covalently attached arginase. Moreover, it reduced catalytic activity than a native one	[144]
Arginase enzyme therapy against cancer cell lines and enhancing the effectiveness of treatment by pegylation of arginase	L5178Y mouse leukemia treated with both arginase and PEG-arginase	PEG-arginase more effective for destruction than unmodified arginase during the in-vitro study, whereas both remain unsuccessful during in vivo studies	[145]
rhArg-PEG 5000 MW has in vitro and in vivo anti-proliferative potential	BALB/c nude mice bearing human HCC xenografts Hep3B, HepG2 & PLC	The activity was moderate against diseased cells	[153]
Pharmaceutical formulation and method of treatment of human malignancies with Arg deprivation. US Patent no. 20050244398. 2005 November 3	NA	The human His-tagged arginase I (liver arginase) enzyme (rhArg) gene was introduced in the *Bacillus subtilis* and improved expression was achieved	[160]
rhArg-PEG 5000 MW inhibits in vitro and in vivo proliferation of HCC	HepG2, Hep3B, PLC/PRF/5, Huh7, and SK-HEP-1 HeLa cells (cervix adenocarcinoma Mice transfected with Hep3B tumor cells (ASS-positive and ADI resistant	Both native and rhArg-PEG 5000 MW showed in-vitro efficacy against HCC cell lines (OCT^-) as well as in HeLa cells HCC xenograft mice showed altered growth patterns after treatment makes them prone to death WiDr (colorectal cancer) and A549 (lung cancer) were found to be resistant to rhArg-PEG 5000 MW due to the presence of OCT Cycle-dependent drugs such as 5-Fluorouracil (5-FU) along with rhArg-PEG 5000 MW could remove the resistant tumor cells No side effects like free ammonia production were observed. Combined therapy may be more effective than a single one	[18]
rhArg inhibits the progression of HCC by inducing cell cycle arrest	Hep3B, HepG2 cells, biopsy specimen from HCC patients	Tumor gene expression for ASS, ASL, and OCT was studied for all specimens and found that rhArg could inhibit more sensitive HCC cells than ADI treatment Growth halted at G2/M and S phases	[161]

Table 4 (continued)

Preclinical studies	Cell line/model study	Results	Reference
Alternatively, activated macrophages possess anti-tumor cytotoxicity activity that is induced by interleukin IL-4 and mediated by Arginase 1	B16-F1 melanoma cells	IL-4 inhibits the growth of B16-F1 melanoma cell proliferation along with arginase produced by activated macrophages itself that will reduce Arg from its pool and render them to show the anti-tumor property	[162]
rhArg inhibits in vitro and in vivo propagation of human melanoma by promoting cell cycle arrest and apoptosis	Human malignant melanomas A375, SK-MEL-2, SK-MEL-24, and SK-MEL-28, mouse melanoma B16-F0, Mouse melanoma B16, BALB/c nude mice	Due to ASS^+ expression in tested cell lines, ADI proved ineffective, whereas with rhArg remarkable cell cycle arrest in S and G2/M phases was observed mRNA expression showed that cyclin D1, cyclin E1, cyclin A1, CDK6, CDK2, and caspase-3 were up-regulated, cyclin B1 downregulated	[163]
rhArg 1 for sustained in vivo activity in cancer therapy: formulation, characterization, and study of their pharmacodynamics properties in vivo and in vitro against HCC	Human HCC cell lines (HepG2 and Hep3B)	The resultant rhArg1-PEG 5000 MW showed 94% relative arginase activity as compared to the native one Arg depletion was achieved in vivo K_M value comparable to the native one showed a sufficient affinity for substrate binding Circulatory half-life increased for pegylated one for 6 days compared to the native one, which was just 2 h Pegylated one has higher anti-tumor activity	[154]
Bioengineered human arginase I with augmented activity and stability against controls HCC and pancreatic carcinoma xenografts	HepG2, Panc-1, and nude BALB/c mice	In both cell line study and mice study cases, successfully arginase was used without eliciting the formation of neutralizing antibodies (was formed due to non-human protein when introducing in a body) The only limiting factor was the limited number of cell lines were studied	[20]
Deprivation of L-Arg by rhArg1against prostate cancer cells	LNCaP, DU-145, PC-3	The cytotoxic effect in all three cell lines was due to the autophagy mechanism	[19]
[HuArgI (Co)-PEG 5000]-lead to Arg depletion, which is cytotoxic to human acute myeloid leukemia cells	Human AML cell lines HL-60, U937, ML1, ML2, Mono-Mac-1, Mono-Mac-6, TF1-vRaf, TF1-vSrc and TF1-HaRas Human CD34+progenitor bone marrow blasts (PBMBs), Human peripheral blood mononuclear cells (PBMCs)	This study revealed the auxotrophic nature of AML cell lines and treatment with HuArgI (Co)-PEG 5000 resulted in 75% cytotoxicity in all tested AML cells Further, no cytotoxic effect was observed with PBMB and $CD34^+$ cell lines revealed that normal cells remain unaffected Synergistic effects with chloroquine, cytarabine, and doxorubicin (chemotherapeutic drugs) were observed	[147]
Role of autophagy in rhArgI: induced cell apoptosis and progression inhibition of malignant melanoma cells	A375 cells lines (malignant melanoma cell line)	The very first study showed that rhArg induces autophagy in melanoma cells via the mitochondrial pathway The autophagic potential of rhArg increased along with the involvement of chloroquine	[164]
Substituting Mn^{2+} with Co^{2+} in hArg I resulted in boosted cytotoxicity toward Arg auxotrophic cancer cell lines	HCC cell line Hep3b and the melanoma cell line A375	*E. coli* BL21 cells were used to express human arginase and the replacement of Mn^{2+} ion with Co^{2+} resulted in a lower K_M value without affecting the catalytic activity Overall in vivo activity increased up to 10 folds against Hep3b and A375 at physiological pH 7	[146]

Table 4 (continued)

Preclinical studies	Cell line/model study	Results	Reference
Pegylated arginase 1: a potential therapeutic approach in T-ALL	Human T-acute lymphoblastic leukemia (T-ALL)	PEG-Arg 1 was found to be effective both in vivo and in vitro without affecting normal cells Block T-cell growth by arresting cells at the G0-G1 phase by Arg starvation and deleting cyclin D3 leading to cell death by apoptosis	[22]
Arginase therapy inhibits the retrieval of canine lymphoma and osteosarcoma cells resistant to the lethal effects of protracted L-Arg treatment	The canine lymphoid cell lines: GL-1 (B-cell leukemia), CLBL-1 (B-cell lymphoma), 17–71 (B-cell lymphoma), CL-1 and OSW (T-cell leukemia), the murine lymphocytic leukemia cell line L1210, canine osteosarcoma cell lines D-17, OSCA-2, OSCA-8, OSCA-29, OSCA-40, OSCA71, and OSCA-78, Multipotent Adult Progenitor Cells (MAPC)	Arginase (from the bovine liver) was explored for animal (dogs) treatment Arg scarcity in cell culture renders all the cell lines more susceptible to arginase treatment in a time-dependent manner. None of them was able to recover when supplemented with again Arg-rich culture conditions Arginase was more operative than asparaginase for arresting the growth of lymphoid cell lines	[165]
rhArg (human arginase gene in BL21 (DE3) *E. coli* cells) induced caspase-dependent apoptosis and autophagy in non-Hodgkin's lymphoma cells	Human NHL cell lines (Raji and Daudi)	mTOR/S6K pathway of autophagy, autophagosomes (observed by TEM) confirmed the inhibitory effect of rhArg Combined therapy with 3-methyladenine, chloroquine inhibitor, and genetic tactics (small interfering RNA) resulted in the induction of autophagy-related gene 5 and Beclin 1, ultimately turning on the autophagic potential for cancerous cells	[25]
rhArg a potential target against Triple-negative breast cancers	MDA-MB-231, BT-549, HCC-1806, HCC-1937 and HS-578 T	rhArg along with autophagic inhibitors showed enhanced cytotoxicity against cancerous cells	[166]
HuArgI(Co)-PEG 5000-induced Arg deprivation, which selectively shows cytotoxic to human glioblastoma cells	Human glioma cell lines U251, U87-MG, T98, U118-MG, Hs683, SF, A172, H4, SW1088 (astrocytoma) and Human fetal glial cells SVG p12	Very first report where GBM (human glioblastoma) selectively showed cytotoxic effects on its progression when treated with HuArgI(Co)-PEG 5000 The sensitivity of cell lines depends upon the concentration of supplemented citrulline (for rescue) and expression of ASS and thus, in a knockdown ASS experiment, all cell lines showed cytotoxicity when the medium is supplemented with citrulline	[23]
rhArg treatment in laryngeal squamous cell carcinoma induces cytotoxicity and autophagy	Tu212 cells	The very first study showed the cytotoxic effects of rhArg along with pharmacological inhibitors of autophagy, i.e., chloroquine (CQ) and bafilomycin A1 (Baf A1), which showed enhanced cytotoxicity against laryngeal squamous cell carcinoma	[167]
Purified arginase from endophytic *Pseudomonas aeruginosa* IH2induce cell death of human cancer cell lines	Human cancer cell line LS-180, HCT-116, MCF-7, BT-549, MOLT-4, HL-60, K-562, PC-3 and non-cancer cell lines HEK-293 AND FR-2	Arginase induces anti-proliferative effects by arresting cell cycles at G0/G1 phase and undergoes mitochondrial autophagy, which was confirmed by a high level of MMP loss, ROS formation, Bax protein, and low protein expression of Bcl-2, SOD, CAT, GPx, and GSH	[123]
Novel PEGylated human arginase (PT01) against cancer lines of breast and pancreas and its cytotoxic assessment	MIA PaCa-2 pancreatic and PC-3 prostate tumor xenograft models	PT01 administration at nM level results in 44–67% tumor growth inhibition	[168]

Table 4 (continued)

Preclinical studies	Cell line/model study	Results	Reference
rhArg, BCT-100) a novel agent for bladder cancer treatment	Bladder cancer cell lines (J82, SCaBER, T24, and 5637 and T24 nude mice xenograft models	Circulatory arginine level was observed to be sharply declined with the onset of apoptotic events BCT-100 was found to induce H_2O_2 production, mitochondrial membrane depolarization The expression of LC3B and Becllin-1 were up-regulated, while p62 downregulated AKT/mTOR pathway was suppressed in a time-dependent manner. While N-acetyl-L-cysteine mediate apoptosis and autophagy	[169]
HuArgI (Co)-PEG 5000 induce death by inducing autophagy in ovarian cancer cells	SKOV3 and Caov-3 ovarian cells	Elucidated mechanism revealed that RhoA involves in autophagy and invasiveness potential	[170]

NA Not assigned

reagent ninhydrin, which produces an orange color when combined with the amino acid ornithine and has a specific retention factor (Rf). Although this is a qualitative method, it can also be used for quantitative analysis [113]. Another most reliable quantitative and qualitative analysis is the spectrophotometric method, which relies on forming a colored complex based on recording absorbance at a specific wavelength [119, 182]. High throughput techniques such as High-Performance Liquid Chromatography (HPLC) and biosensors are used for quantitative analysis as they are more sensitive and reliable [183, 184].

Arginase has been used in food, clinical and environmental analysis to detect metal ions and Arg concentration. First, for metal detection, recombinant arginase is used as an apoenzyme for Mn^{2+} and Co^{2+} metal ions. The enzymes get converted to active holoenzyme and subsequently act on Arg to give urea and ornithine. Further, urea reacts with a 2, 3-butanedione monoxime (DMO) reagent quantified by fluorometric and spectrophotometric techniques. The amount of urea detected in the sample is equivalent to the number of ions present [184]. The Arginase enzymatic system is used as a biosensor to detect Arg levels with high sensitivity and specificity. Both potentiometric and amperometric biosensors have been developed using recombinant arginase. Besides these biosensors, arginase immobilized over gold and silver nanoparticles gives higher sensitivity through fluorometric detection [183].

Why arginase is better than ADI?

ADI is expressed mainly in the bacterial system to meet its energy requirements. ADI is an FDA-approved enzymatic therapy with low K_M value and available in a pegylated form for human use. Despite so many clinical studies conducted so far, there are so many demerits associated with them. First, ADI administration leads to eliciting an immune response due to its recognition as a foreign molecule. Consequently, immunogenic neutralizing antibodies will be formed, which will reduce ADI's efficacy by neutralizing the enzyme. Second, the action of the ADI enzyme releases citrulline and ammonia as a by-product. In the long-term treatment, ammonia can be toxic and leads to hyper ammonia (psychological problems). The third major shortcoming of this therapy is the ineffectiveness in ASS expressing tumors due to ASS expression, which converts citrulline back to Arg [27, 185, 186]. Only ASS-negative tumor cells are susceptible to ADI therapy [187] (Fig. 8).

Currently, efforts are focused on reducing ADI's immunogenicity using a computational approach that targets hot spots in the B-cell epitome [186]. Because of the interaction between cMyc and HIF-1α with the ASSI gene's promoter region, tumor cells developed ADI resistance. Moreover,

Table 5 Clinical studies carried out using arginase as a therapeutic agent

Clinical study	Patients	Results	References
Arginase is a potent drug candidate for HCC	7 Patients with unresectable HCC	Arginase found to be a better candidate than ADI-PEG because it removes plasma Arg effectively at physiological pH with low immunogenic and no ammonia toxicity The study also shows that Insulin also reduces the Arg from the nutritional pool	[171]
A phase 1 clinical trial of dose-escalating PEG-rhArg1 against patients suffering from HCC Clinical trial identifier: NCT01092091	15 patients of advanced HCC	Very first human clinical study of peg-rhArg1 in the treatment of advanced human HCC The prime therapeutic dose of pEG-rhArgI was found to be 1600 U/kg in humans Concluded the effectiveness of arginase in HCC without any side effects	[172]
Synergistic effects of PEG-BCT-100 with 5-FU and Oxaliplatin against advance HCC Clinical trial identifier: NCT02089633	15 HCC patients	Patients were given 3-weekly cycles: PEG-BCT-100 2.7 mg/kg on Day 1, 8 and 15 of each cycle; oral capecitabine 1000 mg/m^2 twice daily for Day 1 to Day 14 of each cycle and IV oxaliplatin on Day 1. The most common AEs were levels 1 and 2). Liver function derangement was encountered in 6% of enrolled patients. The clinical benefit rate was 33%, with one patient achieved a partial response and four patients had stable disease. The median time to progression (TTP) was 6.8 weeks and the median OS was 7.2 months	[173]
rhArg for arginine auxotrophic solid tumors Trial registration number: NCT02285101	A 65-year-old patient with metastatic melanoma who progressed through two failed immunotherapy strategies, one with an immune checkpoint and the second with inhibitor antibodies 23 patients enrolled	Patient in phase 1 treated with 2 mg/kg rhArg1 intravenously Altered transcriptomic pathway in diseased condition ASS and OTC expression lacking, indicating a state of arginine auxotrophy	[174]
A phase II clinical study of pegylated rArg on HCC Trial registration number: NCT01092091	27 sorafenib-failure HCC patients	Weekly intravenously PEG-BCT-100 at 2.7 mg/kg was given The median TTP and progression-free survival (PFS) were both 6 weeks [95% CI (Confidence interval), 5.9–6.0 weeks] Ten patients with ASS-negative tumors had OS of 35 weeks and 15.14 weeks for three patients with ASS-positive tumor	[175]

Table 6 Assay for analyzing arginase activity

Assay type	Substrate utilized	Product analyzed	Principle	Major outcomes	Major shortcomings	References
Spectrophotometric	Arg	Urea	The Thio-Acetyl Monoxime urea method is established to develop a colored complex with a diacetyl reagent, which was being recorded at 520 nm	Specific towards urea	Volatile nature of the reagent Low sensitivity	[119, 123]
Spectrophotometric	Arg	Urea	Under acidic conditions, urea reacts with α-nitrosopropiophenone reagent to form red color, which showed maximum absorbance at 540 nm	Very much specific towards urea	Dark conditions required to maintain red color complex	[182]
Spectrophotometric	Arg	Ornithine	Based on the reaction between ornithine and ninhydrin at a very low pH which results in an orange color complex (read at 515 nm). The Amount of orange complex formed is correlated with arginase activity	Specific towards ornithine	Hinder by reaction conditions like pH as the reaction is pH specific	[182]
TLC	Arg	Ornithine	With the mobile phase flow, ornithine has a specific Rf value visualized as an orange spot when sprayed with ninhydrin	Qualitative analysis is done	Quantitative determination is not possible	[113]
HPLC	Arg	Ornithine	Reverse-phase chromatography was employed to elute bounded ornithine	Both qualitative and quantitative analysis is done	Expensive	[113]
Potentiometric Biosensors	Arg	Ammonia	Biosensors based on bi-enzyme system arginase and urease having an ammonia detecting electrode giving out the signal	Gives signal over a wide range	High price	[183]

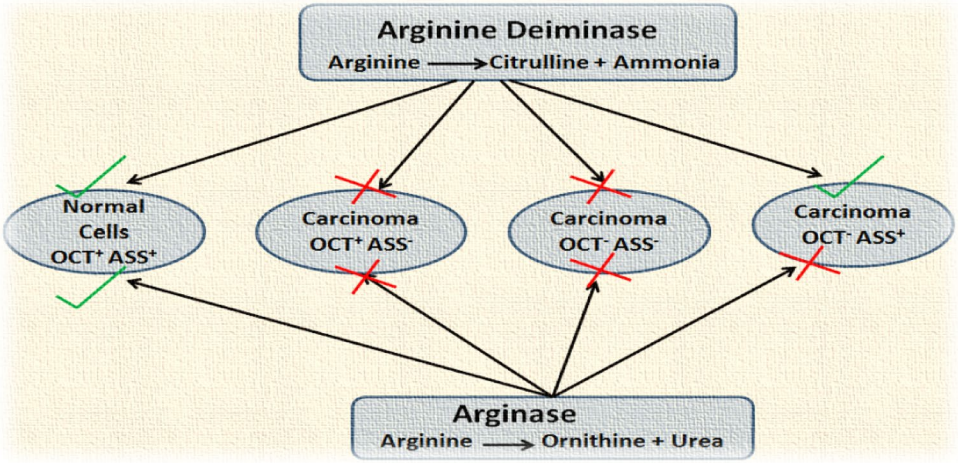

Fig. 8 Effect of treatment of arginase and ADI on the normal healthy cell as well as on L-Arg auxotrophic carcinoma

up-regulation of ASL has also been observed in ASS^- cells [188]. All these drawbacks can be overcome with the arginase enzyme as it is of human origin, so there is no such problem of immunogenicity. As the end products of arginase action are non-toxic, it has a wide range of efficacy against tumor-like ASS-positive cells. Besides, the K_M value is significantly lowered and reaction pH shifts near to physiological pH 7.5 by substituting manganese ions with cobalt. At the same time, its pegylated form has shown an improvement in catalytic properties [22, 146]. All these valuable characters have made arginase more gripping as a therapeutic application against cancerous cells over ADI.

Table 7 shows how the enzyme expression involved in the metabolism of Arg affects enzymatic therapy. Based on these enzyme expressions, appropriate immunotherapy alone or in combination can opt-out.

ADI and arginase application for the biosynthesis of other bioactive molecules

Arg is the mutual substrate for both arginase and nitric oxide (NO) biosynthesis with the enzyme nitric oxide synthase (NOS). NO is a biological mediator that plays a crucial role in several biological and pathological processes such as stroke, multiple sclerosis, diabetics, and inflammation. NO also exerts its lethal effects by activating effector molecules such as tumor necrosis factor-alpha and endotoxin [189]. Neuronal NOS (nNOS) and endothelial NOS (eNOS) are two of the three isozymes of NOS that are constitutively expressed, while iNOS is an inducible isozyme. Arginase and ADI have proven to be much more specific for eliminating NO-associated toxicities. Previous data also demonstrate the effectiveness of these enzymes in lowering NO production [190, 191]. The homeostasis of Arg and NO (Arg: NO) is recently monitored and utilized for the early diagnosis of breast cancer [192].

L-ornithine is a non-protein amino acid formed by Arg's enzymatic hydrolysis and has excellent value in the food and pharmaceutical industries. L-Ornithine acts as a precursor to the biosynthesis of polyamines. Its commercial production has primarily relied on chemical, fermented, and enzymatic hydrolysis. Enzymatic hydrolysis by arginase is much more preferable for ornithine production over the other two methods, i.e., chemical and fermentative methods, due to the ease of getting purified products. Previous studies have concluded arginase from *Bacillus thuringiensis*, *Bacillus amyloliquefaciens*, *Bacillus caldovelox*, and *Sulfobacillus acidophilus* are ideal sources for L-ornithine production [164, 193]. The arginase gene from *Bacillus caldovelox* was expressed in *Escherichia coli* for L-ornithine production so that the resulting product could be easily extracted [194]. The prior study revealed that L-ornithine production was enhanced by the over-expressing hArg 1 in *Pichia pastoris* [195]. Proline and

Table 7 Sensitivity of cells according to their ASS and OCT expression

S. No	Cell type	ASS expression	OCT expression	ADI treatment	Arginase treatment
1	Healthy cell	+	+	Resistant	Resistant
2	Cancer cell	+	+	Resistant	Sensitive
3	Cancer cell	−	+	Sensitive	Sensitive
4	Cancer cell	+	−	Sensitive	Sensitive

polyamine are the resultant products formed during Arg metabolism, which induces cells' proliferation [155].

Conclusion

In summary, arginase treatment seems to be a promising approach for designing newer and better cancer therapeutics. Before therapeutic enzyme treatment, mRNA expression profiling of ASL, ASS, and OCT of cancer tumors should be essential. Arginase-based enzymatic therapy of cancer cells is a more specific treatment strategy with better efficacy and minimal side effects. Moreover, using enzymes combined with other novel chemotherapeutic drugs, pharmacological inhibitors could efficiently induce autophagy in tumor cells. Furthermore, neutralizing antibodies can be reduced by expressing the gene from the normal human flora to the expression system with all the desired properties. At this stage, more extensive expression profiling of ADEs, cell-based studies along animal models based on in vivo testing is needed. These combination treatment strategies could increase the expected pharmacokinetic properties of the novel therapeutic agents. Overall, systematic, global concurrent studies and clinical trials will be needed to effectively implement arginase-based mono-immunotherapy or combination drug therapy against cancers.

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Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest to disclose.

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