



Arginine dependence of tumor cells: targeting a chink in cancer's armor

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Citation	Patil, M D, J Bhaumik, S Babykutty, U C Banerjee, and D Fukumura. 2016. "Arginine Dependence of Tumor Cells: Targeting a Chink in Cancer's Armor." <i>Oncogene</i> 35 (38) (April 25): 4957–4972. doi:10.1038/onc.2016.37.
Published Version	doi:10.1038/onc.2016.37
Citable link	http://nrs.harvard.edu/urn-3:HUL.InstRepos:37133883
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1 **Arginine Dependence of Tumor Cells: Targeting a Chink in**
2 **Cancer's Armor**

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1 **Abstract**

2 Arginine, one among the twenty most common natural amino acids, plays a pivotal
3 role in cellular physiology as it is being involved in numerous cellular metabolic and
4 signaling pathways. Dependence on arginine is diverse for both tumor and normal cells. Due
5 to decreased expression of argininosuccinate synthetase (ASS) and/or ornithine
6 transcarbamoylase (OTC), several types of tumor are auxotrophic for arginine. Deprivation of
7 arginine exploits a significant vulnerability of these tumor cells and leads to their rapid
8 demise. Hence, enzyme-mediated arginine depletion is a potential strategy for the selective
9 destruction of tumor cells. Arginase, arginine deiminase (ADI) and arginine decarboxylase
10 (ADC) are potential enzymes that may be used for arginine deprivation therapy. These
11 arginine catabolizing enzymes not only reduce tumor growth but also make them susceptible
12 to concomitantly administered anti-cancer therapeutics. Most of these enzymes are currently
13 under clinical investigations and if successful will potentially be advanced as anti-cancer
14 modalities.

15

16 **Keywords: cancer, arginine deprivation, arginase, arginine deiminase, arginine**
17 **decarboxylase**

18

1 **Introduction**

2 Amino acids play a major role in regulating important cellular events in both normal
3 and malignant cells. Besides their role in the synthesis of hormones and peptides, amino acids
4 also function as cell signaling molecules, playing a modulatory role in gene expression.¹
5 Amino acids regulate RNA synthesis by diverse mechanisms ranging from regulating
6 transcription factors assembly,² to total mRNA turnover.^{3,4} Amino acids are major
7 determinants of a normal cellular physiology, therefore potential signaling pathways such as
8 amino acid response (AAR) pathway sense their altered metabolism [Figure 1]. Hence, amino
9 acid levels in the body are critical for important cellular functions.⁵⁻⁹

10 There is a significant difference between the metabolism of normal and malignant
11 cells.¹⁰ For instance, bio-energetic requirements for homeostasis in normal cells are fulfilled
12 by catabolic metabolism. On the other hand, the majority of the tumor cells alter their
13 metabolic program (“*metabolic remodeling*”) and consume additional nutrients in order to
14 maintain a balance between elevated macromolecular biosynthesis¹¹ and adequate levels of
15 ATP for survival.^{12,13} However, the endogenous supply of nutrients becomes inadequate
16 during intense growth. Thus tumor cells depend on exogenous nutrients in their
17 microenvironment to fulfill the elevated energy requirements *i.e.* they become auxotrophic
18 for nutrient and energy sources.¹⁴⁻¹⁶ Deprivation of amino acids results in growth inhibition
19 or death of tumor cells by the modulation of various signaling cascades.^{6-9,17,18}

20 Exogenously incorporated enzymes that deprive amino acids could be a novel
21 strategy for the treatment of auxotrophic tumors. The first FDA approved heterologous
22 enzyme for the treatment of cancer was *E. coli* L-asparaginase.¹⁹ L-asparaginase exploits the
23 differences on their dependence of normal and leukemic cells towards L-asparagine.²⁰ L-
24 asparaginase has been proven to be a promising agent for the treatment of L-asparagine
25 auxotrophic T-cell acute lymphoblastic lymphoma (T-ALL). Use of L-asparaginase in T-

1 ALL opened up new windows of ‘amino acid-depriving therapy’. Currently, there is a
2 resurgence of interest in enzyme-mediated amino acid deprivation as a new therapeutic
3 approach for cancer treatment.^{6,7,21,22} For example, arginine depletion can inhibit tumor cell
4 proliferation and induce cell death pathways. Here we endeavor to provide a basic
5 understanding of the roles of arginine in normal and tumor cell with emphasis on current
6 knowledge and developments in the application of enzyme-mediated arginine depriving
7 therapy as a potential anticancer approach.

8 **Enzyme-mediated arginine deprivation: a potential anti-cancer approach**

9 Arginine is involved in the regulation of various molecular pathways and thus
10 availability of arginine can modulate key metabolic, immunological, neurological and
11 signaling pathways of the cells [Figure 2 and 3].^{23,24} Auxotrophy towards arginine by certain
12 tumor cells (particularly that of hepatocellular carcinoma and melanoma) has been well
13 characterized.^{25,26} Normal cells, when deprived of arginine, undergo cell cycle arrest at G₀/G₁
14 phase and become quiescent. If reinstated with arginine, the majority of the normal cells
15 recover to their normal proliferation status. However, arginine deprivation in tumor cells does
16 not arrest cell cycle at G₁ phase and continue to be in a cell cycle, leading tumor cells to
17 undergo unbalanced growth and eventually lead to the activation of apoptotic pathways.^{27,28}

18 Owing to the involvement of arginine in a plethora of cellular pathways, arginine
19 dependence of tumor cells has rapidly emerged as a potential target for cancer.²⁹ However,
20 dietary restriction results in the reduction of only 30% of plasma free arginine.³⁰ Thus,
21 arginine degrading enzyme-mediated arginine deprivation has been proposed as a potential
22 anti-cancer therapy by various research groups.²⁷⁻³⁵ Enzymes that can be used for arginine
23 deprivation therapy (ADT) include arginine deiminase (ADI), arginase and arginine
24 decarboxylase (ADC) as discussed below [Figure 3].

25

1 **1. Arginine deiminase**

2 Arginine deiminase (ADI) (E.C.3.5.3.6) is a prokaryotic enzyme originally isolated
3 from *Mycoplasma*, which catalyzes an irreversible deimination of the guanidine group of L-
4 arginine to citrulline and ammonium ion.³⁶ Normal cells are able to convert citrulline into
5 arginine through argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL),
6 expression of which are tightly regulated. However, the expression of ASS/ASL is down-
7 regulated in certain tumor cells by unknown mechanisms and these cells are unable to
8 convert citrulline to arginine.^{30-33,37} This makes the tumor cells auxotrophic for arginine for
9 their growth and cellular functioning. ADI-mediated arginine deprivation leads to apoptotic
10 cell death, selectively of arginine auxotrophic ASS (-) tumor cells sparing the ASS (+) ADI
11 resistant normal cells³⁸ [Table 1]. Incidence of ASS deficiency varies depending on the
12 tumor type and expression level of ASS has been proposed as a biomarker for identification
13 of ADI sensitive tumors.^{24,25,39-42}

14 In 1990, Miyazaki and co-workers⁴³ were the first to report the growth inhibition of
15 *Mycoplasma* infected human tumor cells. The cause of growth inhibition of human tumor cell
16 lines was identified as a ADI produced by *Mycoplasma*. *In vitro* growth-inhibitory dose of
17 *Mycoplasma* ADI appeared to be 1000 times lower than that of bovine liver arginase.
18 Subsequently in 1992, growth inhibitory activity of ADI was demonstrated in ASS-
19 downregulated human melanoma cells.⁴⁴ These pioneering studies established ADI as a
20 potential anti-cancer enzyme [Figure 4].

21 **1.1 PEGylated ADI**

22 Being microbial in origin, ADI has serious disadvantages of eliciting strong
23 antigenicity and rapid plasma clearance (half-life of 4 h). To circumvent these limitations,
24 several studies have aimed to extend the plasma half-life of ADI and to minimize its
25 antigenicity. In 1993, Takaku *et al* addressed these problems for the first time by

1 polyethylene glycol (PEG) modification.⁴⁵ Remarkably, PEGylation of *Mycoplasma arginini*
2 ADI enhanced its cytotoxic potential *in vivo* and once a week intravenous injection of PEG-
3 ADI at a dose of 5 U/mouse (10 mg protein/Kg) depleted plasma arginine to an undetectable
4 level at least for a week, whereas native enzyme required 10 daily injections to achieve
5 similar effects. Nevertheless, PEGylation of *Mycoplasma hominis* ADI also resulted in
6 significant enhancement of arginine lowering potential of native *Mycoplasma hominis*
7 ADI.^{46,47} Recently, PEGylation and pharmacological properties of an engineered ADI
8 originated from *Pseudomonas plecoglossicida* have been studied. PEGylated *Pseudomonas*
9 *plecoglossicida* ADI remarkably improves the systemic half-life (by 11-folds) and found to
10 exhibit superior efficacy than native ADI in depleting plasma arginine.⁴⁸

11 PEG-ADI has also shown promising outcomes for the treatment of human
12 malignancies. In March 1999, ADI-PEG20, PEGylated recombinant *Mycoplasmal* ADI was
13 approved as an orphan drug by US-FDA for the treatment of HCC and malignant melanomas.
14 Subsequently in July 2005, European Agency for the Evaluation of Medicinal Products
15 (EMA) granted orphan drug status to ADI-PEG20 for the treatment of HCCs.⁴⁹

16 ADI-PEG20 is currently undergoing clinical investigation as a randomized double-
17 blind Phase III trial in patients with advanced HCC (NCT 01287585), Phase II studies in
18 patients with ASS-negative metastatic melanoma (NCT 01279967) and Phase II studies in
19 patients with relapsed small-cell lung cancer (SCLC) (NCT 01266018)⁵⁰ [Table 2]. Outcomes
20 of the previous clinical studies were also encouraging, achieving response rates of 25% and
21 47% in melanoma and HCC, respectively [Table 2]. Moreover, grade III and IV toxicities
22 have not been observed in clinical investigations involving ADI-PEG20 in metastatic
23 melanoma and HCC patients.^{51,52} Therefore, clinicians are looking forward to the
24 establishment of ADI-PEG20 as a potent anti-cancer modality.

25

1 **1.2 Tumor sensitivity towards ADI**

2 The auxotrophicity of tumors towards arginine and their sensitivity towards **it can be**
3 **attributed** to the lack or reduced expression of ASS in tumors.^{25,37-39,53} **Notably**, numerous
4 tumor cells which are deficient in ASS expression, are sensitive towards ADI treatment
5 **[Table 1]**. Transfection of an expression plasmid containing human ASS cDNA in HCC and
6 melanoma cells confers severe resistance to ADI treatment compared to ASS-negative cells.⁴⁷
7 **Till** date, most promising targets for ASS expression dependent ADT identified are human
8 melanoma and HCCs. Other promising targets include malignant pleural mesothelioma
9 (MPM), renal cell carcinoma, prostate cancer, T-ALL and osteosarcoma.⁵⁰ However,
10 molecular mechanisms underlying tumor sensitivity towards ADI treatment, by down-
11 regulation of ASS expression in tumor cells, are still elusive. Promoter hypermethylation-
12 dependent silencing of ASS gene is an endorsed mechanism of ASS gene repression.^{37,54-56}
13 Methylation frequency of the ASS promoter upto 50-80% level at the CpG loci is
14 documented across a broad range of lymphomas. **In contrast**, normal lymphoid samples were
15 found unmethylated.²⁶ Treatment of ADI-PEG20 to ASS-methylated lymphoma cell lines
16 **revealed** dramatic decrease in **the** proliferation rate and viability count, by inducing caspase-
17 dependent apoptosis, without **affecting** normal lymphoblastoid cell lines. Demethylation-
18 induced resistance to ADI-PEG20 treatment has also been confirmed in Cutaneous T-cell
19 Lymphoma (CTCL) cell lines, as their incubation with 5-Aza-dC (demethylating agent) for 8
20 days **which** resulted in partial demethylation, followed by transcriptional activation and
21 synthesis of ASS protein.²⁶

22 **Recently Rabinovich *et al* have confirmed that proliferation of the osteosarcoma cells**
23 **is supported by down-regulation of ASS, by facilitating pyrimidine synthesis via activation of**
24 **CAD (carbamoyl-phosphate synthase 2, aspartate transcarbamylase and dihydroorotase)**
25 **complex.⁵⁷ As cytosolic aspartate serves as a substrate for both ASS and for CAD complex,**

1 ASS down-regulation can enhance aspartate availability for CAD for the synthesis of
2 pyrimidine nucleotides to promote proliferation. Thus, aspartate transport can be exploited as
3 an additional therapeutic target in tumors with ASS down-regulation, especially in those ones
4 which develop resistance to arginine-depriving enzymes.

5 **1.3 Tumor resistance towards ADI**

6 ASS-deficient tumors are sensitive to ADI treatment; however, arginine deprivation
7 eventually up-regulates ASS expression in tumor cells and thereby confers resistance towards
8 ADI.^{25,58} Transcriptional induction of ASS expression and increase in ASS mRNA level is
9 reported in human embryonic kidney cells and melanoma cells during arginine starvation.^{59,60}
10 Transcription factors such as c-Myc and HIF-1 α are involved in the up-regulation of ASS
11 expression under arginine depleted conditions.⁶⁰ E-box and GC-box are the important
12 sequences located between -85 and -35 nucleotides in the ASS promoter region that modulate
13 ASS expression through their interactions with c-Myc and HIF-1 α . Under the normal
14 concentrations of arginine, HIF-1 α (but not c-Myc) binds to E-box and thus acts as a negative
15 regulator of ASS expression. Under the conditions of arginine depletion, HIF-1 α is degraded
16 and replaced by up-regulated c-Myc, which directly binds to E-box; thus, c-Myc acts as a
17 positive regulator of ASS expression [Fig. 6 of Ref. 60]. Recently reported in melanoma
18 cells, inhibition of ubiquitin-mediated protein degradation is a molecular mechanism
19 responsible for the stabilization and accumulation of c-Myc.⁶¹ Furthermore, various cellular
20 pathways, such as Ras and its downstream ERK/PI3K/AKT kinase cascade are associated
21 with the post-translational modifications of c-Myc, leading to its phosphorylation and
22 stabilization during ADI-PEG20-mediated arginine deprivation conditions. Involvement of
23 Ras/PI3K/ERK signaling pathway in the development of resistance towards ADI treatment
24 suggests that combination of ADI with Ras/ERK, PI3K/AKT inhibitors is a potential
25 therapeutic strategy to improve the anti-cancer response.^{62,63}

1 Development of anti-drug neutralizing antibodies is another possible mechanism of
2 resistance towards ADI-PEG20 treatment.⁶⁴ Arginine concentrations were recovered up-to
3 pre-treatment levels in a patient with malignant pleural mesothelioma and in Asian patients
4 with advanced hepatocellular carcinoma following the ADI-PEG20 treatment. This recovery
5 in arginine concentration was found concomitant with an increase in anti-ADI-PEG20
6 antibody titer.⁶⁵ These studies suggest the involvement of drug-associated resistance i.e. anti-
7 drug neutralizing antibodies, rather than tumor-related factors as another possible mechanism
8 of resistance of some tumor cell types towards ADI-PEG20 treatment.^{62,63}

9

10 **1.4 Anti-tumor mechanisms of ADI treatment**

11 *1.4.1 Role of autophagy and apoptosis in ADI-mediated arginine deprivation therapy*

12 Due to the involvement of arginine in numerous cellular pathways [Figure 2], the
13 exact anti-proliferative mechanisms of ADI treatment, besides that of arginine depletion, are
14 still elusive. One of the potential pathways involved in the cytostatic and cytotoxic potential
15 of ADI is TRAIL (tumor necrosis factor-related apoptosis-inducing ligand).⁶⁶⁻⁶⁸ TRAIL plays
16 an important role in the cleavage of Beclin-1 (Atg6) and Atg5 in arginine deprived melanoma
17 cells.⁶⁹ Beclin-1 and Atg5 are essential for the formation of autophagosomes and thus crucial
18 for autophagy. Since autophagy serves as a mean to evade apoptosis in arginine depleted
19 cells, TRAIL induced cleavage of Beclin-1 and Atg5 leads to decreased autophagy, thereby
20 increasing apoptosis.⁶⁹ Additionally, these two drugs (ADI and TRAIL) complement each
21 other by activating the intrinsic apoptosis pathways. ADI-PEG20 increases cell surface
22 receptors DR4/5 for TRAIL thereby binding TRAIL to these death receptors. As a result,
23 caspase-8 or 10 are activated.⁶⁶ ADI-PEG20 treatment also modulates different autophagic
24 pathways involved in the cell survival. AMPK and ERK pathways are activated in ADI-
25 treated prostate cancer cells; while AKT, mTOR and S6K pathways are attenuated. ADI-

1 PEG20 treatment to CWR22Rv1 prostate cancer cells induced autophagy, as revealed by the
2 appearance of LC-II only after 30 minutes exposure [continues](#) its persistence after 24 hours
3 following ADI-PEG20 treatment.^{70,71} [Additionally](#), inhibition of autophagy by chloroquine, a
4 clinically approved anti-malarial agent which inactivates lysosomal functions, accelerates the
5 ADI-induced apoptotic cell death of prostate cancer^{70,71} and SCLCs.³⁹ Thus autophagy has
6 been proposed as a pro-survival mechanism of tumor cells during arginine deprivation.⁷¹

7 ADI-mediated arginine deprivation is also known to induce caspase-dependent
8 apoptotic pathways in many of the tumor cells types. ADI-PEG20 treatment activates
9 caspase-3 in ASS-methylated malignant lymphoma cells, whereas ASS-positive normal
10 lymphoblastoid cells are resistant to it.²⁶ Similarly, cell death has been attributed to caspases
11 activation in glioblastoma,⁵⁴ melanoma,^{38,72} leukemia⁷³ and pancreatic cancer cells.⁷⁴
12 Moreover, all these studies indicate that inhibition of autophagy leads to further advancement
13 in [the](#) ADI-PEG20-mediated demise of tumor cells, suggesting the induction of autophagy as
14 a mechanism of tumor resistance to ADI-PEG20 treatment.

15 Cumulative [pieces of](#) evidence suggest that [the](#) activation of caspases is not a sole
16 decisive phenomenon in programmed cell death pathways. Caspase-dependent apoptosis is a
17 major mode of cell death, but in its absence or failure, there are other pathways which can
18 also execute cell death.⁷⁵⁻⁷⁷ ADI-PEG20 treatment to SCLC, leukemia, retinoblastoma and
19 prostate cancer cells induces apoptotic cell death pathways, however, without activation of
20 caspases, suggesting the role of caspase-independent apoptosis as a cell death pathway.^{33,39,69,}
21 ^{70,78} The inter-membrane space of mitochondrion contains proteins such as apoptosis-
22 inducing factor (AIF) and endonuclease G (EndoG), which can induce apoptotic cell death in
23 a caspase-independent fashion.⁷⁹ EndoG is one of the predominant endonucleases that are
24 involved in the regulation of cellular functions such as mitochondrial biogenesis, DNA
25 synthesis and repair. AIF is an FAD-containing flavoprotein which plays an important role in

1 the stability of an electron transport chain.⁸⁰ Nutrient deficiency-mediated stress signals
2 induce mitochondrial outer membrane permeabilization (MOMP), which consequently
3 releases inter-membrane space proteins such as AIF, EndoG and cytochrome *c*. AIF plays a
4 role of central mediator in caspase-independent cell death pathway.⁸¹ AIF, once released into
5 the cytosol, interacts with EndoG and cyclophilin A prior to its translocation into the
6 nucleus.⁸² Subsequently after translocation into the nucleus, it triggers cell death either
7 directly, through interaction with DNA, or indirectly, through the production of reactive
8 oxygen species.^{73,74,79,80} MOMP promotes both, caspase-dependent and caspase-independent
9 apoptotic pathways, but with different kinetics.⁸³ Although, the upstream signaling stimulus
10 for both, a caspase-dependent and caspase-independent pathway is the same, *i.e.* via
11 induction of MOMP, their downstream pathways are different. Moreover, nuclear alterations
12 and the changes occurring in mitochondrial trans-membrane potential during caspase-
13 independent pathways are different than those observed in a caspase-dependent apoptotic
14 pathway.⁸⁴

15 To summarize, growing evidence suggests that autophagy is a prevailing cell survival
16 mechanism in tumor cells undergoing ADI-mediated arginine deprivation. The overall
17 cellular response to ADI-mediated arginine deprivation in different tumor cells operates
18 through a complex cascade, initiating with induction of autophagy and followed by the
19 activation of either caspase-dependent or caspase-independent cell death pathways. It is
20 worth emphasizing that the discrepancy of cellular responses of tumor cells to ADI-mediated
21 arginine depletion in activation of either caspases-dependent or caspases-independent cell
22 death pathways can vary depending on tumor cell type.^{38,39,70,71,74} As a result, the precise
23 mechanisms of tumor cell death- consequential of cellular response to ADI-mediated arginine
24 depletion- appear to be complex and variable, and need to be further elucidated.

25

1 ***1.4.2 Inhibition of de novo protein synthesis by ADI-mediated arginine deprivation***

2 Inhibition of *de novo* protein synthesis is another mechanism which can be attributed
3 to the anti-tumor potential of ADI. As extracellular arginine pool is responsible for 40% of *de*
4 *novo* protein synthesis, ADI treatment to human lung carcinoma cells results in an anti-
5 proliferative effect, mediated by inhibition of protein synthesis.⁸⁵ Arginine is present in
6 various compartments such as extracellular, intracellular and citrulline-arginine regeneration
7 *i.e.* cytosolic compartment and it is known to regulate various cellular pathways differently.
8 Protein synthesis mainly utilizes arginine either from the intracellular pool or the citrulline-
9 arginine regeneration mechanism, while polyamines synthesis largely utilizes arginine pool
10 from the intracellular origin.^{86,87} Polyamines are synthesized through the methionine salvage
11 pathway via decarboxylation of S-adenosylmethionine (SAM). SAM is a donor metabolite
12 necessary for the transfer of methyl group to DNA and proteins. Human colon cancer
13 (HCT116) cells treated with short hairpin CD44 RNA interference showed a decrease in the
14 total amount of methionine-pool metabolites including polyamines, suggesting the role of
15 polyamines in cancer proliferation.⁸⁸

16 ADI treatment towards human mammary adenocarcinoma and lung carcinoma cells
17 differently modulates polyamine synthesis and the global protein synthesis. Interestingly,
18 inhibition of protein synthesis has been correlated with the ASS-mediated regeneration of
19 arginine. Cells expressing low levels of ASS (A549) result in decreased protein synthesis
20 (without affecting polyamine synthesis) and those expressing higher ASS levels (MCF-7) are
21 resistant to ADI treatment, as the decreased arginine levels can be replaced by citrulline-
22 arginine regeneration pathway.⁸⁵

23 ***1.4.3 Anti-angiogenic effects of ADI-mediated arginine deprivation***

24 As a tumor grows beyond a certain size (2 mm in diameter for most solid tumors),
25 available vasculature within the tumor becomes inadequate to supply sufficient quantities of

1 essential nutrients for their growth.⁸⁹ This results in the generation of hypoxic tumor
2 microenvironment and leads to the development of new blood vessels (angiogenesis) as a
3 colossal requisite of the developing tumors.⁹⁰ Accordingly, neovascularization can be stated
4 as one of the decisive phenomena during tumor growth and metastasis.⁹¹ Emerging studies
5 now indicate that not only molecular signals but also metabolic mechanisms regulate
6 angiogenesis.⁹² Under stress conditions such as hypoxia, tumor cells secrete angiogenic
7 factors such as vascular endothelial growth factor (VEGF).⁹³ Increased levels of VEGF
8 activate VEGF receptor 2 (VEGFR2) signaling in the quiescent endothelial cells which in
9 turn initiate angiogenesis.⁹⁴⁻⁹⁶ Endothelial cells produce 85% of their total amount of ATP via
10 glycolysis. Addition of endothelial cells on anaerobic rather than aerobic pathway enables
11 them for the formation of vascular sprouts in hypoxic areas.^{97,98} Metabolism of tumor
12 endothelial cells resembles that of highly activated endothelial cells because of the tumor
13 induced switch from quiescence to proliferation due to metabolically regulated migration
14 during sprouting.^{99,100}

15 Besides ADI's role in modulation of apoptotic pathways, it has an anti-angiogenic
16 activity that contributes to its anti-tumor potential. The growth, migration and differentiation
17 of human umbilical vein endothelial cells (HUVECs) are strongly impaired in a medium
18 containing recombinant ADI.¹⁰¹ As a consequence; it results in decreased tube formation with
19 intermittent and incomplete microvascular network. Similarly, Park *et al.* found that *E. coli*
20 ADI inhibits angiogenesis by inhibiting tube formation of endothelial cells and
21 neovascularization in Chick Chorioallantoic Membrane (CAM) and Matrigel plug assay.¹⁰²

22 Suppression of nitric oxide (NO) generation is also another possible mechanism for
23 anti-angiogenic activity of ADI. Since L-arginine is required for nitric oxide synthases
24 (NOSs) to generate NO, the depletion of arginine by ADI suppresses NO synthesis.¹⁰²
25 Potential role of ADI-mediated arginine depletion in inhibition of NO synthesis has been

1 reported.^{103,104} We and others have previously reported that NO promotes tumor growth
2 through the stimulation of angiogenesis¹⁰⁵⁻¹⁰⁷ and regulates cellular interaction by controlling
3 adhesion molecule expression and ultimately cell adhesion.^{108,109} NO directly, or indirectly
4 through NO-mediated reactive nitrogen species (RNS), induces the activation of certain
5 angiogenic signaling pathways in the endothelial cells.¹¹⁰ NO acts as an autocrine mediator in
6 endothelial cell functioning and as a final modulator in VEGF stimulated angiogenesis.^{109,111}
7 NO not only mediates angiogenesis but also subsequent vessel maturation^{112,113} Moreover,
8 NO is known to inhibit angiostatin and thrombospondin-1, two main inhibitors of
9 angiogenesis.¹¹⁴ Owing to the important role of NO in angiogenesis, ADI inhibits tumor
10 growth not only by draining the supply of arginine, but also by its anti-angiogenic activity via
11 suppression of NO generation.

12 To summarize, certain tumor cell types such as, HCCs and metastatic melanomas are
13 invariably deficient in ASS expression and can be specifically targeted by ADI-mediated
14 ADT. It is worth noting that more than one pathway may be attributed to the cytotoxic
15 potential of ADI-mediated ADT [Figure 5]. The anti-tumor potential of ADI may not only be
16 simply accredited to its action as arginine degrading enzyme but also to several other
17 mechanisms important in the cellular functioning of tumor cells. Induction of apoptotic
18 pathways, inhibition of angiogenesis and inhibition of *de novo* protein synthesis are the
19 important mechanisms attributed to the cytotoxic potential of ADI. Moreover, studies have
20 revealed the ADI-mediated modulations in tumor cell-cycle. The fundamental difference of
21 cell cycle modulations in normal and malignant cells should be exploitable as a means of
22 selective demise of tumor cells and ADI, in combination with other anti-cancer
23 chemotherapeutic agents, which can be a potential strategy to improve chemo-sensitization
24 against tumor cells.¹¹⁵⁻¹¹⁸

25

1

2 **2. Arginase**

3 Arginase (E.C.3.5.3.1) is a mammalian enzyme which catalyzes the conversion of
4 arginine to ornithine and urea. Arginase is considered as an enzyme responsible for the cyclic
5 nature of urea cycle, since only the organisms containing arginase are able to carry out the
6 complete urea cycle.¹¹⁹ Two distinct isoforms of mammalian arginase have been identified
7 which are encoded by two separate genes.¹²⁰ Type I arginase (arginase I) is located in the
8 cytosol and is mainly expressed in liver. Type II arginase is located in the mitochondrial
9 matrix and is expressed in extra-hepatic tissues.^{121,122} Intracellular regulation of arginase
10 expression is of immense importance as it has crucial implications for the synthesis of
11 essential cellular metabolites,¹²³ For example, cytosolic co-localization of arginase I with
12 ornithine decarboxylase (ODC) preferentially utilizes ornithine for the biosynthesis of
13 polyamine. On the other hand, due to its co-localization with ornithine aminotransferase
14 (OAT) in the mitochondria, arginase II directs ornithine for the production of proline and
15 glutamine.^{124,125}

16 **2.1 PEGylated recombinant human arginase I**

17 Elevated requirements of arginine by tumor cells were first identified in 1947 and
18 preferential utilization of arginine by tumor bearing animals was revealed in 1953.^{126,127} The
19 use of bovine and murine arginase in arginine deprivation therapy was prevailing until the
20 advent of recombinant DNA technology,¹²⁸⁻¹³⁰ followed by the pervasive use of recombinant
21 human arginase in subsequent decades.^{131,132} Arginase from bovine and murine sources has
22 been extensively used for the arginine deprivation therapy *in vitro*. However, limited success
23 was achieved *in vivo* due to its alkaline optimum pH and very low affinity for the substrate.
24 Human arginase I also has a serious limitation of very short circulatory half-life (Approx. 30
25 minutes).

1 To extend plasma half-life of arginase, PEGylation has been applied successfully.
2 PEGylated recombinant human arginase I (rhArg-Peg_{5000mw}) had efficient catalytic activity at
3 physiological pH with improved *in vivo* half-life of 3 days. Furthermore, rhArg-Peg_{5000mw}
4 was found to have significant tumor inhibitory activity in BALB/c nude mice bearing HCC
5 xenografts.¹³¹ Notably, these results were consistent with those demonstrated by Tsui and co-
6 workers.¹³³ Recently, a bio-engineered form of human arginase I was developed by the co-
7 factor replacement, the replacement of two Mn²⁺ ions by Co²⁺ ions. The modified Co²⁺-
8 arginase I resulted in 10-fold increase in the catalytic activity and 5-fold greater stability at
9 the physiological pH. Nevertheless, IC₅₀ values for killing human HCC and melanoma cell
10 lines were lowered by 12-15 folds.¹³⁴ More recently, modifications in bioengineered Co²⁺-
11 arginase I were performed by conjugating 5-kDa PEG to enhance plasma half-life. This
12 modified version of bioengineered arginase I (Co-hArgI-PEG) was proven to be cytotoxic by
13 significantly increasing the expression of caspases-3 in HCC and pancreatic carcinoma (PC)
14 tumor xenografts.¹³⁵ Lately, the cytotoxic potential of Co-hArgI-PEG was identified in acute
15 myeloid leukemia (AML) and glioblastoma cells. AML cell lines were found sensitive
16 towards Co-hArgI-PEG-mediated arginine deprivation with very low (58-722 PM) IC₅₀
17 values, suggesting a very high potential of Co-hArgI-PEG-mediated arginine depletion in
18 AML cells.¹³⁶ Moreover, Co-hArgI-PEG-mediated arginine deprivation has been
19 demonstrated to induce caspase-independent, non-apoptotic cell death in human glioblastoma
20 cells.¹³⁷ Alternative method to extend the plasma half-life of recombinant human arginase
21 also has been established. Plasma half-life of a fusion protein form of a recombinant human
22 arginase (rhArg-Fc, constructed by linking rhArg to the Fc region of human immunoglobulin
23 IgG1), was evidenced to significantly extend up-to approx. 4 days.¹³⁸ In addition, rhArg-Fc
24 was confirmed to conspicuously inhibit the cell growth of human HCC cells *in vitro* and *in*
25 *vivo*.¹³⁸

1 Last decade has evidenced a prevalent use of recombinant human arginase-mediated
2 ADT in numerous cancer cell types, mainly metastatic HCC and melanomas.^{131,139,140}
3 Currently, PEGylated derivative of recombinant human arginase I is undergoing clinical trials
4 for the treatment of human HCC.^{141,142} Moreover, initiatives are now being taken to
5 overcome the possible problem of accumulation of PEGylated products in the liver by
6 impending approaches such as fusion proteins.¹³⁸

7 **2.2 Anti-tumor mechanisms of arginase-mediated arginine deprivation**

8 Selective starvation of L-arginine in tumor cells, which are auxotrophic for L-
9 arginine, is one of the most important anti-tumor mechanisms of ADT. Arginase can render
10 its cytostatic effect as a result of modulations in the cell cycle proteins, whereas, cytotoxic
11 effects rendered by arginase I-mediated arginine deprivation have been proposed as a result
12 of induction of potential cell death pathways namely apoptosis and probably by ‘autophagic
13 cell death’. Summarized below are the current understandings of the molecular mechanisms
14 of cytostatic and cytotoxic effects rendered by arginase-mediated ADT.

15 ***2.2.1 Role of autophagy in arginase-mediated arginine deprivation***

16 Autophagy is a key sensing and regulatory mechanism of cells in nutrient deprived
17 conditions. Under stress conditions, autophagy functions as a bio-energy management
18 system by recycling cell organelles and damaged and/or long-lived proteins.¹⁴³ Although
19 autophagy seems to be a survival mechanism of the cells, there is a growing evidence of
20 accumulation of autophagosomes and other autophagic markers in dying cells unable to
21 process apoptosis, raising the term ‘autophagic cell death’.¹⁴⁴⁻¹⁴⁷ However, the term
22 ‘autophagic cell death’ is based on morphological features rather than the causative role of
23 autophagy in cell death. New definition of ‘autophagic cell death’ has been proposed,

1 implying that cell death must occur without the involvement of apoptotic machinery,
2 (caspase activation) but **with** an increase in autophagic flux.^{148,149}

3 Mammalian target of rapamycin (mTOR) is a key regulator **of** coupling cell growth
4 and nutritional status of the cell.^{150,151} Autophagy is induced by the inhibition of mTOR
5 signaling pathway.¹⁵² During nutrient affluent conditions, mTOR is involved in the negative
6 regulation of Atg1 (autophagy related gene 1) **which** inhibits autophagy.^{153,154} Arginase-
7 mediated arginine deprivation leads to decreased levels of ATP, which in turn activates the
8 adenosine 5'-monophosphate-activated protein kinase (AMPK). Activated AMPK
9 eventually inhibits the mTOR-signaling pathway, manifested by the reduced
10 phosphorylation of key downstream molecules, such as 4E-BP1 (Eukaryotic translation
11 initiation factor 4E-binding protein-1). Dephosphorylation of 4E-BP1 is observed in Chinese
12 hamster ovary (CHO), human melanoma cells and human prostate cancer cells following
13 their exposure to recombinant human arginase I.^{65,155,156} Phagosome/lysosome activity is
14 also significantly increased following an incubation of human tumor cells in L-arginine
15 deficient medium.¹⁵⁷ Additionally, studies carried out by Hsueh *et al.*¹⁵⁶ evidenced no
16 significant induction of apoptotic mechanisms in prostate cells after their exposure to
17 rhArgI, suggesting the role of autophagic cell death, rather than apoptosis, as an alternative
18 cell death mechanism. In addition, autophagy has often accompanied damaged mitochondria
19 and higher levels of reactive oxygen species (ROS).^{158,159} Acute generation of ROS has been
20 attributed to causing severe damages to the cellular macromolecules, which in consequence,
21 leads to necrosis of the tumor cells.^{160,161} Overall, arginase leads to deprivation of arginine,
22 in consequence, it inhibits mTOR pathway during the deprivation and thus forcing tumor
23 cells to undergo 'autophagic cell death' pathway.¹⁶²

24 **SLC38A9, a member 9 of the solute carrier family 38, has been recently identified as**
25 **an integral component of the lysosomal machinery that controls amino acid-induced mTOR**

1 activation.^{163,164} Amino acid starvation in human embryonic kidney (HEK293T) cells with
2 stable expression of SLC38A9 has been shown to activate mTOR in a sustained manner.
3 Moreover, shRNA-mediated silencing of SLC38A9 results in a reduction of arginine-
4 induced mTOR activation. Also, depletion of SLC38A9 impaired mTOR activation induced
5 by cycloheximide (a protein synthesis inhibitor which induces accumulation of intracellular
6 amino acids), further suggests the role of SLC38A9 in mTOR activation at the lysosomal
7 rather than at the plasma membrane. These studies have demonstrated that SLC38A9 acts as
8 an upstream positive regulator for mTOR functioning and thereby modulating autophagy in
9 arginine-deprived tumor cells.

10 Although some studies have advocated autophagy as a cell death mechanism of
11 arginase-mediated ADT,^{156,157} many groups have explained it as a pro-survival mechanism;
12 mainly by postponing the activation of apoptosis.^{38,161} Thus, understanding the exact role of
13 autophagy in arginase-mediated cell death pathways is a complicated episode.^{162,165}
14 Therefore, much need to be elucidated about these new findings related to ‘autophagic cell
15 death’ and caution must be taken to assign autophagy as a cell death pathway in arginase-
16 mediated ADT.

17 ***2.2.2 Role of apoptosis in arginase-mediated arginine deprivation***

18 The role of autophagy, either in cell survival or in cell death, depends on many factors
19 such as cell type, nature and severity of the stimuli and so on.¹⁶⁶ If the attempt of the cells to
20 survive through autophagy fails, apoptotic pathways take over and ultimately cause cell
21 death.¹⁴³ Inhibition of autophagy in amino acid deficient conditions induces tumor cell
22 death, mainly because of further exacerbation of energy dearth.^{167,168} Also, longer
23 persistence of autophagy is proposed to eventually lead the activation of caspase-dependent
24 cell death pathways, as autophagy and apoptotic cell death pathways are interconnected and

1 also share some common pathways through the induction of the membrane permeability
2 transitions.¹⁶⁹⁻¹⁷¹ Induction of apoptotic pathways is another consequence of arginine
3 depletion and anti-tumor mechanism of arginase I-mediated arginine deprivation.

4 Involvement of apoptosis as a cell death mechanism in arginase-mediated ADT has
5 been illustrated in various literature reports. Annexin V is known to selectively stain the
6 cells, which are destined for apoptosis or in the process of apoptosis. 33% of human
7 melanoma cell population was destined for apoptotic cell death following rhArg
8 treatment.¹³⁹ Arginase I-mediated arginine deprivation led to the transcriptional up-
9 regulation of *caspase 3*, the intrinsic mitochondrial pathway of apoptosis, which is marked
10 by the change in mitochondrial membrane potential.¹⁷² Recently, an anti-leukemic potential
11 of PEGylated-arginase has been attributed to kinases general control nonderepressible 2
12 (GCN2)-mediated induction of apoptosis in T-ALL cells.¹⁷³

13 ***2.2.3 Cell cycle arrest by arginase-mediated arginine deprivation and combination*** 14 ***approaches***

15 rhArg-Peg_{5000mw}-mediated arginine deprivation in various HCC cells results in their
16 cell cycle arrest at G₂/M phase, by decreased expression levels of cyclin B1 and cdc2, or in S
17 phase, by a transcriptional up-regulation of cyclin A1 [Ref. 140]. rhArg-Peg_{5000mw}-mediated
18 arginine depletion was witnessed to impair the expression of cyclin D3 in T-ALL cells, which
19 was followed by an arrest of the cells in the G₀-G₁ phase of the cell cycle and induction of
20 apoptosis.¹⁷² Recent investigations of rhArg-Fc-mediated arginine deprivation in human HCC
21 cells exhibited cell cycle arrest at S phase.¹³⁸ The exact mechanisms of these findings are still
22 elusive, but the possible reasons seem to be the increased expression of cyclin A and declined
23 transcription levels of p27 and p21 (the key cyclin kinase inhibitors).

1 Owing to the evidence of cell cycle arrest, a combination of arginase and other cell-
2 cycle specific anti-cancer chemotherapeutics as potential anti-tumor approaches have been
3 established. Synergistic effects of rhArg-Peg_{5000mw} with 5-fluorouracil (5-FU, uracil analog
4 which interferes with RNA and DNA synthesis) and cytarabine (Ara-C, anti-metabolic
5 chemotherapeutic agent) have been investigated on the inhibition of proliferation of HCC and
6 T-ALL cells, respectively.^{131,172} Treatment of either rhArg-Peg_{5000mw} or Ara-C alone induces
7 a heterogeneous anti-tumor effect *in vivo*, whereas, combined treatment of rhArg-Peg_{5000mw}
8 and Ara-C induces a homogenous prevention of spleen growth, leading to the prolonged
9 survival in all of the T-ALL bearing mice.¹⁷² Moreover, combined treatment of PEGylated
10 recombinant human arginase I and oxaliplatin has been demonstrated to synergize the
11 inhibiting effect on tumor growth and enhanced overall survival probability as compared to
12 PEGylated recombinant human arginase I or oxaliplatin treatment alone.¹⁷⁴

13 Altogether, arginase has an advantage over ADI that it is efficacious in both ASS-
14 negative and OTC- negative tumors,⁵⁹ whereas ADI is efficacious only in ASS-negative
15 tumors. The tumor cell types expressing ASS are resistant to arginine deprivation treatment
16 by ADI.^{25,26,54,61,131} Even though arginase has been considered as a potential drug candidate
17 over a period of six decades, low substrate specificity (high k_m of 2-4 mM), short plasma life
18 and optimum alkaline pH (pH 9.3) limit *in vivo* applications of arginase.^{131,140} In addition,
19 robust homeostatic mechanisms in the body allow faster restoration of plasma free arginine,
20 making *in vivo* arginine deprivation by arginase more difficult. Most of the scientific efforts
21 nowadays pay attention to these limiting characteristics of arginase.^{134,175,176}

22 3. Arginine decarboxylase

23 Arginine decarboxylase (ADC) (E.C. 4.1.1.19) metabolizes arginine to agmatine, one
24 of the minor metabolic products of arginine. ADC is mainly found in plants, bacteria and
25 mammalian liver and brain membranes.^{177,178} The mammalian ADC is different from other

1 sources and distinct but related to ODC.¹⁷⁹ Although, arginine decarboxylation by ADC is a
2 minor metabolic route, its product i.e. agmatine has a significant role in numerous cellular
3 pathways.¹⁸⁰ Agmatine modulates the polyamine metabolism through its negative interaction
4 with ODC.¹⁸¹ Agmatine also confers an inhibitory effect on intracellular polyamine content
5 by inhibiting polyamine uptake¹⁸² and probably by increased polyamine catabolism.¹⁸³
6 Mayeur *et al.*,¹⁸⁴ has reported the effect of agmatine accumulation on polyamine metabolism,
7 cell proliferation and cell cycle distribution in human colon adenocarcinoma epithelial cell
8 lines. Due to the agmatine-mediated reduction in polyamine synthetic capacity of the cells,
9 agmatine markedly inhibits the cell proliferation of HT-29 and Caco-2 cells in a dose
10 dependent manner, without affecting cell membrane integrity. Moreover, agmatine modulates
11 the cell cycle progression by decreasing ODC activity and expression.^{181,185} As ODC plays an
12 important role in the G₁/S progression of the cells, agmatine-mediated modulations in ODC
13 expression lead to modifications in the cell cycle progression.¹⁸⁶ Additionally, agmatine also
14 has been shown to delay the expression of cyclins in tumor cells, leading to the modifications
15 in the cell cycle progression.¹⁸⁴

16 ADC has been investigated for the enzymatic degradation of arginine in normal and
17 malignant cell cultures.¹⁸⁷ Arginine deprivation in human diploid fibroblasts (normal cells),
18 achieved using human recombinant ADC, resulted in the cell cycle arrest at G₁/G₀. While
19 treatment of 0.1 unit ml⁻¹ ADC to HeLa (Human cervical cancer) cells resulted in cell cycle
20 arrest with an initiation of cell death after 2 days.¹⁸⁷ Similar results were evidenced in the
21 studies by Wheatley *et al.*,¹⁸⁸ where 5 units ml⁻¹ ADC was found as effective as arginase in
22 the inhibition of HeLa cells and cell cycle arrest at G₁ (quiescence) in fibroblasts.

23 Although some research groups have exhibited ADC as a potential anti-tumor
24 enzyme, only a few reports are available to support this fact [Table 1].^{187,188} Even though
25 ADC possesses low *K*_m and can degrade arginine very rapidly, the serious problem is related

1 to its product i.e. agmatine. Agmatine is toxic to normal cells when its concentration reaches
2 a millimolar level, particularly when free arginine levels are low. Additionally, agmatine is
3 not converted back to arginine under normal physiological conditions, which may lead to its
4 accumulation and toxicity to normal cells.¹⁸⁹ Though recombinant human ADC expressed in
5 *E. coli* has been evidenced more active than Sigma enzymes prepared from other sources, its
6 PEGylation has been shown to result in the loss of its entire activity.^{187,189} To consider [the](#)
7 further rational use of this prospective enzyme as potential anti-cancer modality, it clearly
8 warrants further evaluation [[Table 3](#)].

9 **Concluding remarks**

10 Sufficient evidence has been accumulated indicating that arginine catabolic enzymes-
11 based approaches may be an effective way to target malignant cells. These enzymes control
12 tumor cell proliferation as well as make them highly vulnerable to cell-cycle specific
13 chemotherapeutic agents. This combinatorial approach is one of the potential strategies to
14 maximize the efficacy to obliterate the tumor cells. Extensive research of the arginine
15 metabolic pathways led to the establishment of arginine-depriving enzymes as a potential
16 anti-cancer strategy against arginine auxotrophic tumors. However, many of these enzymes
17 can be co-expressed in the cells, which results in complex interactions. For example, arginine
18 is a common substrate for arginase as well as NOS. [The specific role of NO, either in](#)
19 [inhibition or induction of cell proliferation is dependent on numerous factors like its](#)
20 [interaction with other free radicals, cellular makeup, tumor milieu, proteins present the](#)
21 [cellular microenvironment and also upon the chemical and biological heterogeneity of NO.](#)
22 [NO has been known to demonstrate bipolar cellular effects and often termed as “double-](#)
23 [edged sword”.](#) Although, NOS remains a viable candidate for cancer treatment, the precise
24 [role of NO in the tumor microenvironment is extremely complex and conflicting.](#) Also, the
25 [preferential utilization of arginine by arginase and/or NOS pathway is not fully understood.](#)

1 Thus, many of these pathways warrant further research to understand the arginine metabolism
2 at cellular and molecular levels involving upstream and downstream pathways of the
3 enzymes involved.

4 It should be noted that modulation of the immunological responses is one of the major
5 roles of arginine availability. Arginine metabolism in myeloid-derived suppressor cells via
6 arginase and/or NOS markedly impairs the T-cell responses that would eradicate and remove
7 tumor cells.¹⁹⁰ Many excellent articles are available which focus on the role of arginine in
8 immunological aspects of the tumors.¹⁹¹⁻¹⁹⁴ It would suffice to say here that the arginine
9 deprivation therapy may have further anti-tumor effect through restoration of anti-tumor
10 immunity.

11 Arginine dependence of the tumor cells has been considered as the “*Achilles heel*” of
12 tumor cells.¹⁹⁵ Inability of tumor cells to proliferate in the absence of arginine can be targeted
13 for their selective destruction by arginine depriving enzymes. Large numbers of enzyme-
14 based anti-cancer therapies are currently undergoing clinical evaluation. It is encouraging that
15 arginase and arginine deiminase already have achieved considerable success, without causing
16 detrimental side effects and with high tolerability.^{51,63,141} The knowledge acquired about the
17 PEGylation has helped in the generation of adducts of potential value, overcoming the
18 serious limitations of the anti-cancer enzymes of the non-human origin. The approach of
19 enzyme-mediated arginine deprivation therapy is highly challenging, however rewarding
20 upon success due to the provision of overturning the cancer dogma.

21 **Acknowledgments**

22 MP gratefully acknowledges Department of Biotechnology (DBT), New Delhi, India
23 for the award of Senior Research Fellowship. Authors are also thankful to Prof. Rakesh K.
24 Jain, Department of Radiation Oncology, Massachusetts General Hospital, Harvard Medical

1 School, USA; Dr. Utpal Mohan, Department of Biotechnology, NIPER, Guwahati, India and
2 Dr. Umesh Patil, School of Chemical Sciences, North Maharashtra University, Maharashtra,
3 India for the assistance provided during preparation of the manuscript. We apologize to those
4 authors whose work could not be cited owing to space limitations.

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18

19

Figure legends

20

21 **Figure 1: Amino acid response (AAR) pathway**

22 Restriction of essential amino acids activates the general control nondepressible protein 2
23 (GCN2) kinase by increasing uncharged t-RNA pool.¹⁹⁶ Activated GCN2 kinase
24 phosphorylates the translation initiation factor eIF2 α . Phosphorylated eIF2 α binds more
25 tightly to eIF2 β , inhibiting the exchange of GDP for GTP. Inhibition of GDP exchange for
26 GTP further inhibits the binding of eIF2 complex to methionine aminoacyl tRNA, leading to
27 inhibition of translational initiation.¹⁹⁷ Recently, SLC38A9 has been identified as an

1 upstream positive regulator of the mTOR pathway. Amino acids activate the RAG GTPases,
2 which then recruit mTOR to the lysosomal surface. Rheb also localizes to lysosomal
3 membrane. mTOR activation occurs only when both RAG GTPases and Rheb are active.
4 Upon amino acid deprivation, tuberous sclerosis complex (TSC) translocates to lysosomal
5 surface and promotes GTP hydrolysis by Rheb and thereby inhibiting mTOR complex.¹⁶⁴

6

7 **Figure 2: Involvement of arginine in human physiology**

8 Arginine is a dibasic, cationic amino acid and is considered as ‘conditionally essential’
9 amino acid. Arginine plays a crucial role in innate and adaptive immunity. For example,
10 increased role of arginine in myeloid-derived suppressor cells results in the impairment of T-
11 cell proliferation and function.¹⁹⁰ Arginine has been identified as the sole physiological
12 precursor for nitric oxide (NO), a key performer in many cellular regulatory functions.
13 Arginine also is a precursor of two important amino acids, proline and glutamate.¹⁹⁸ One of
14 the most important roles of arginine is its implication in the synthesis of polyamines through
15 the diversion from NO synthesis pathway. Polyamines are known to promote tumor growth,
16 invasion and metastasis.¹⁹⁹ Arginine also plays a vital role in the synthesis of nucleotides,
17 creatine, agmatine and hormones such as insulin and prolactin.²⁰⁰

18 **Figure 3: Arginine synthesis and homeostasis pathways**

19 Arginine is synthesized as an intermediate in the urea cycle. Arginine homeostasis is mainly
20 achieved by catabolism. In neonates, the gene expression of arginine anabolic enzymes such
21 as 1-pyrroline-5-carboxylase, argininosuccinate synthetase (ASS) and argininosuccinate lyase
22 (ASL) is low. Thus, arginine is considered as an essential amino acid in neonates. After birth,
23 the expression of ASS and ASL increases and expression of arginase is found undetectable at
24 this stage.²⁰¹ Arginine can be degraded by arginase, ADC, ADI and NOSs (Please note that
25 ADI is not a mammalian enzyme). The products of arginine catabolism play important roles
26 in tumor cell biology. For example, ornithine, the product of arginase, is diverted to
27 polyamine synthesis via ornithine decarboxylase. NOSs degrade arginine into citrulline and
28 NO. Citrulline is recycled to urea cycle, while NO is as a modulator of important metabolic
29 and signaling cascades. Agmatine is synthesized by decarboxylation of arginine via ADC and
30 plays an important role in neurotransmission.

31

1 **Figure 4:** Timeline of important advancement in arginine deprivation therapy of cancer

2

3 **Figure 5:** Schematic representation of cytostatic and cytotoxic pathways involved in arginine
4 deprivation therapy

5 Arginine deprivation therapy (ADT) can potentially modulate numerous cellular and
6 signaling pathways rendering their cytotoxic and cytostatic pathways. Induction of apoptotic
7 pathways, inhibition of angiogenesis and inhibition of *de novo* protein synthesis are the
8 important mechanisms attributed to the cytotoxic potential of ADT. Moreover, ADT-
9 mediated modulations in tumor cell-cycle can be exploited as a means of tumor growth arrest.

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Figure 1:

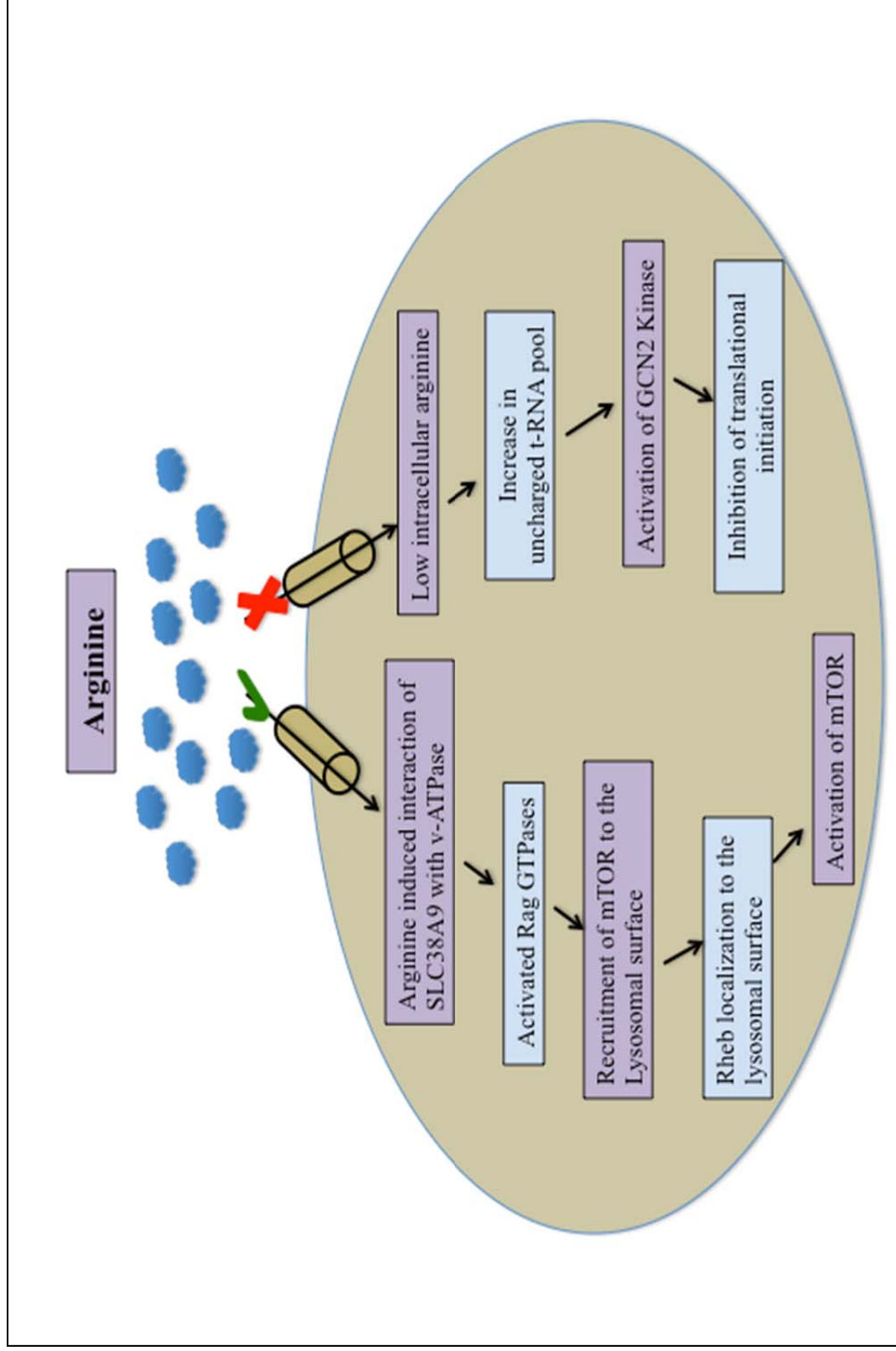


Figure 2

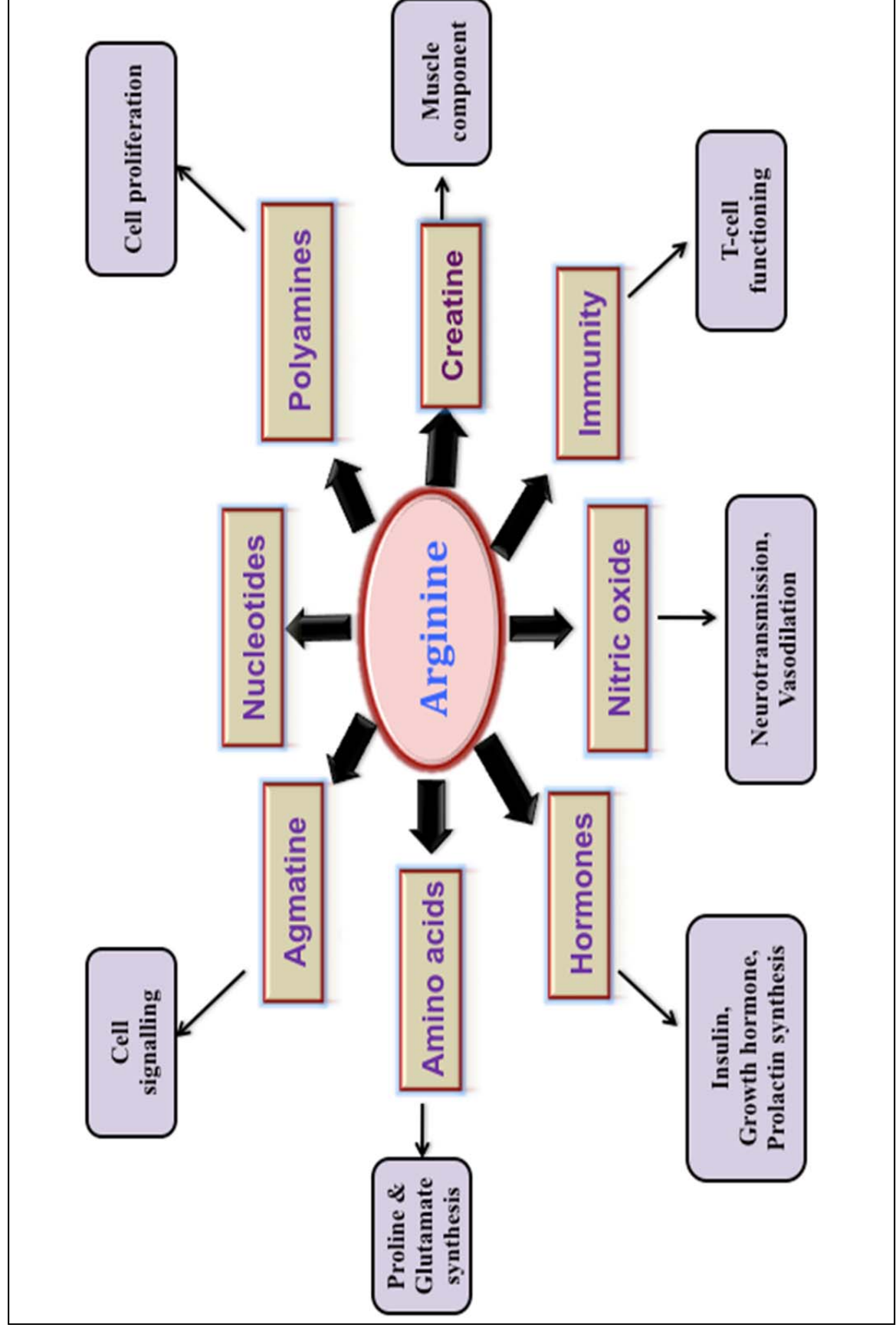


Figure 3

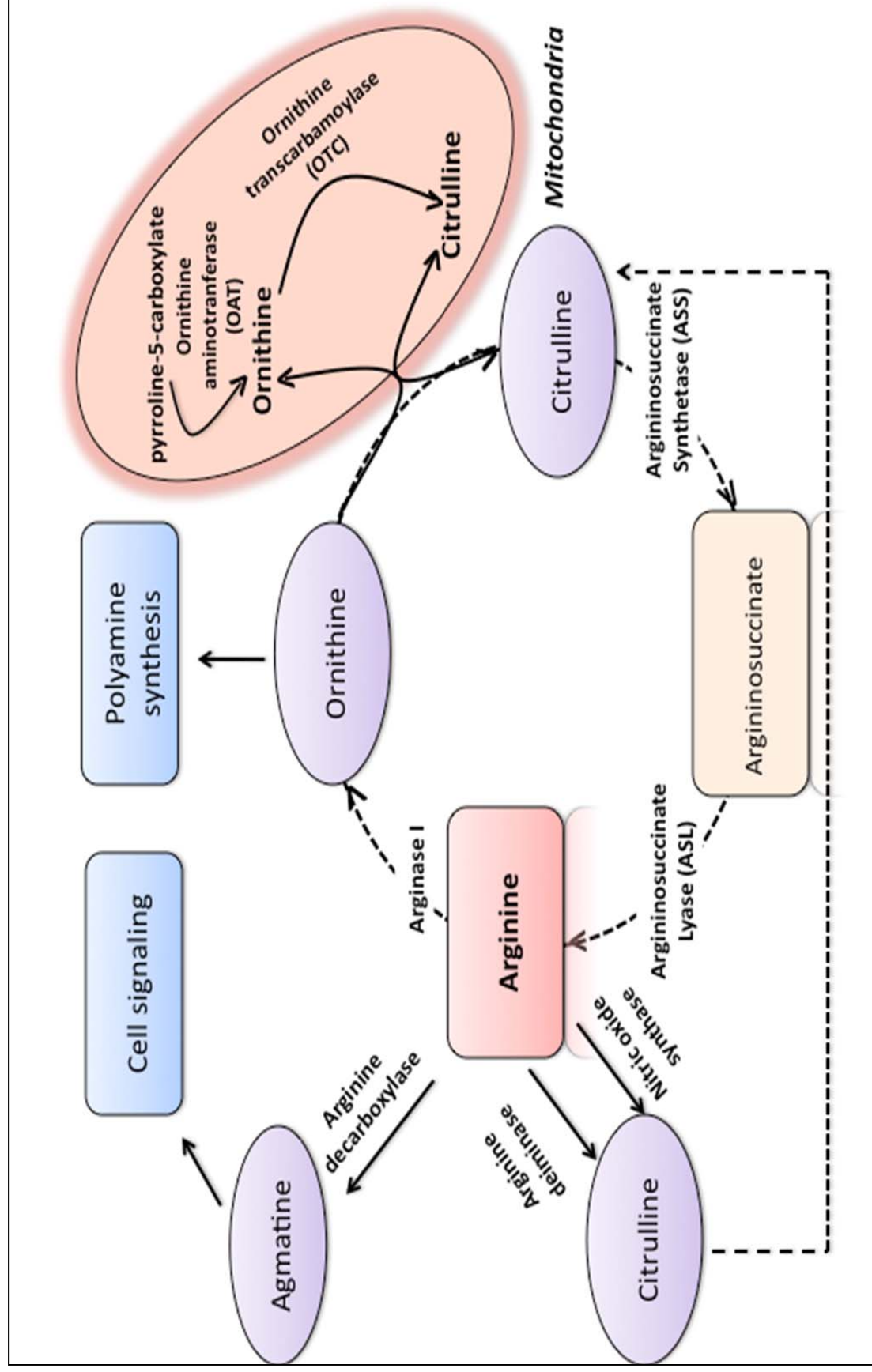


Figure 4

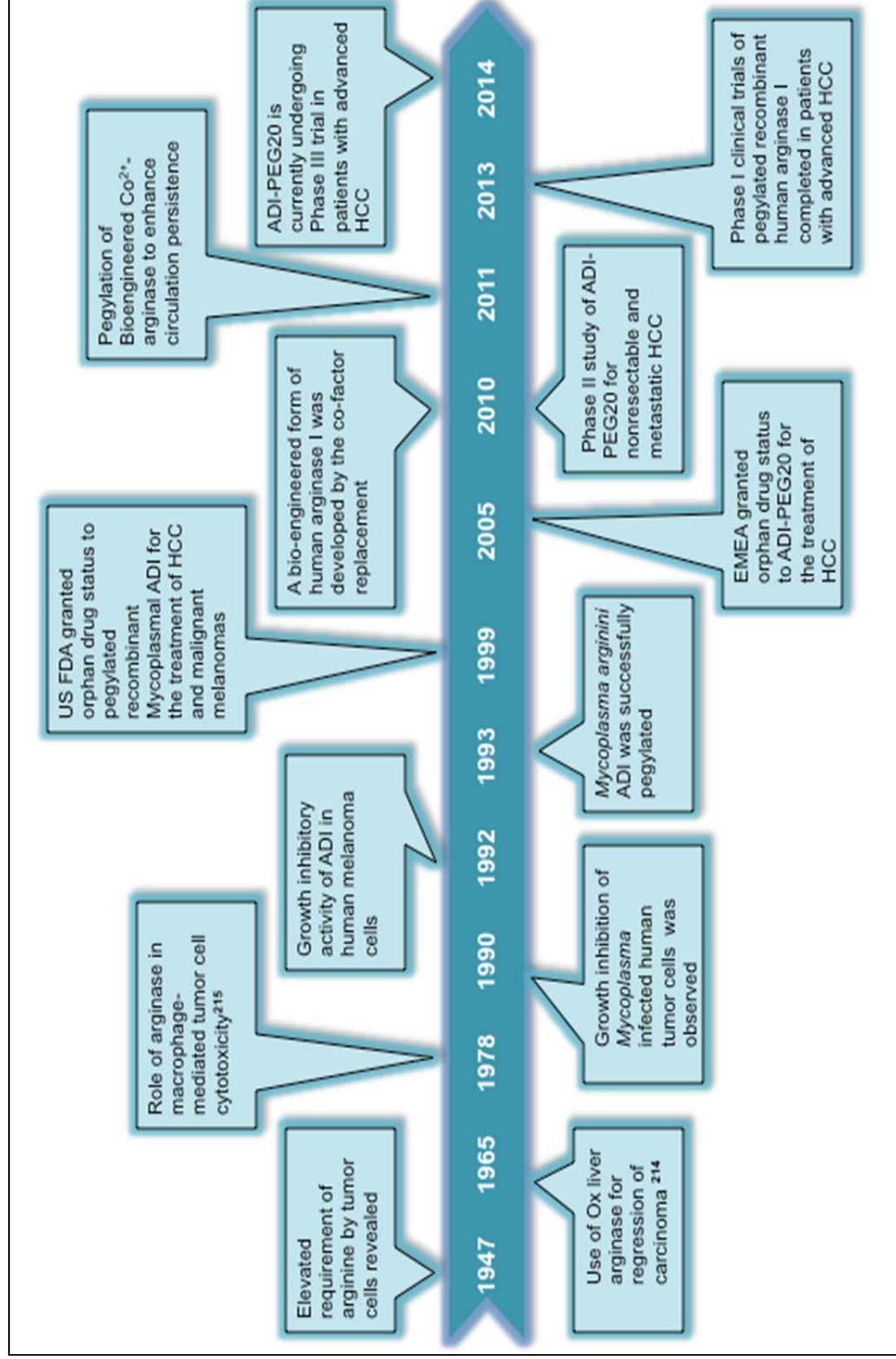
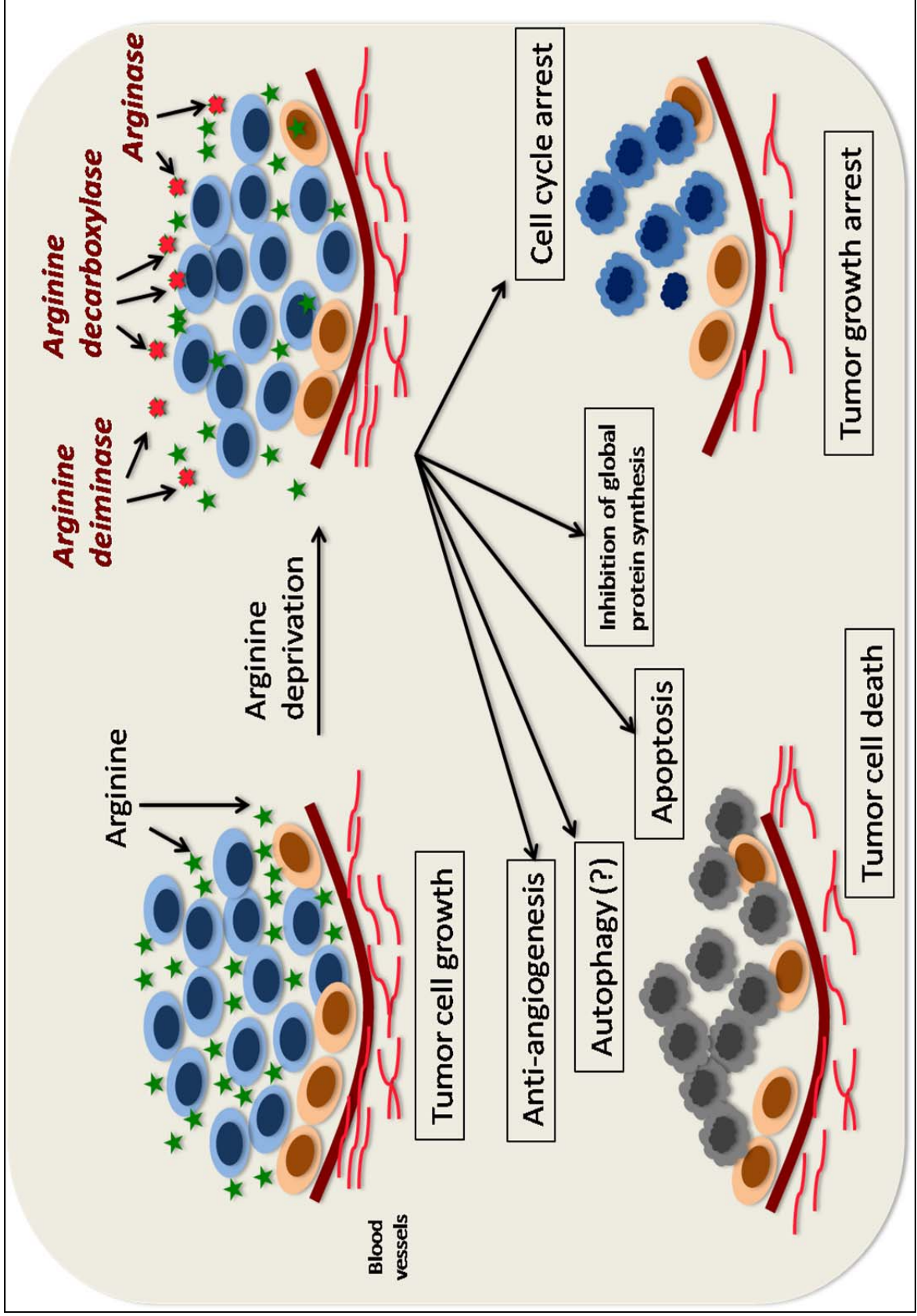


Figure 5



List of tables

Table 1: Use of arginine catabolizing enzymes in arginine deprivation therapy (experimental studies)

Table 2: Clinical investigations involving arginine depriving enzymes

Table 3: Properties of arginine depriving enzymes

Table 1: Use of arginine catabolizing enzymes in ADT (Experimental studies) [* indicates Tumor xenograft experiments]

Enzyme used for deprivation	Cell line	Source and Cell type	Studies carried out	Reference
ADI	HSC-3 HSC-4 CaSki C41 A549 SCC T98G	Human tongue squamous carcinoma Human cervix squamous carcinoma Human cervix squamous epithelium Human colon adenocarcinoma Human glioblastoma	Cell growth inhibitory effect of ADI (purified from <i>Mycoplasma</i> infected cell lines) in comparison with arginase	[43]
	HeLa CHO FF9 HUVEC	Human cervix Chinese hamster ovary Fetal foreskin fibroblast Human umbilical vein endothelium	Concentration dependent effect of ADI on cell proliferation	[102]
	SNU-1	Human stomach adenocarcinoma	Anti-angiogenesis effect of ADI by inhibiting capillary-like tube formation	[202]
	L5178Y MCF7 A549	Mouse lymphoblastic leukemia Human mammary adenocarcinoma Human lung carcinoma	Anti-proliferative effect and ADI induced cell cycle arrest and apoptosis Inhibition of cell division	[203] [85]
	SNUOT-Rb1 Y79	Human retinoblastoma Human retinoblastoma	Effect of ADI on the regulation of cellular protein and polyamine synthesis	[204]
	CWR22Rv1* A2058 SK-Mel-2 HUVE SaOS WAC2 Y-79 Meth AC14	Human prostate Human melanoma Human melanoma Human umbilical vein endothelium Human osteosarcoma Human neuroblastoma Human retinoblastoma Human sarcoma	ASS expression related sensitivity of cells towards ADI	[71] [66]
			Autophagy and caspase independent apoptosis Combination effect of ADI and TRAIL,	[78]
			Cell cycle progression and apoptotis Inhibition of NO using Pegylated ADI	[103]
			Effect of ADI-PEG20-mediated deprivation on the production of NO	[39]
			ASS expression related sensitivity of cells towards PEG-ADI, Induction of autophagy and caspase-independent	

<p>Bovine liver arginase</p> <p>rh-Arginase I</p>	<p>NCI-H82</p> <p>A375</p> <p>SK-mel-2*</p> <p>SK-mel-28*</p> <p>SK-hep 2*</p> <p>SK-hep 3*</p> <p>HEP3B</p> <p>A2058*</p> <p>SK-MEL-2</p> <p>MDA-MB-231</p> <p>Karpas-422</p> <p>MyLa</p> <p>SeaX</p> <p>OEC-M1</p> <p>SCC-15</p> <p>HONE-1</p> <p>A375</p> <p>Sk-Mel2</p> <p>A2058</p> <p>MEL-1220</p> <p>MIA-PaCa-2*</p> <p>PANC-1</p> <p>Capan-1</p> <p>HPAF II</p> <p>L1210</p> <p>HeLa</p> <p>A375</p> <p>MEWO</p> <p>SAos-2</p> <p>IPEC-1</p>	<p>Human small cell lung</p> <p>Human melanoma</p> <p>Human melanoma</p> <p>Human melanoma</p> <p>Human HCC</p> <p>Human HCC</p> <p>Human HCC</p> <p>Human melanoma</p> <p>Human melanoma</p> <p>Human breast</p> <p>Human B-cell lymphoma</p> <p>Human T-cell lymphoma</p> <p>Human T-cell lymphoma</p> <p>Human head and neck cancer</p> <p>Human head and neck cancer</p> <p>Human head and neck cancer</p> <p>Human melanoma</p> <p>Human melanoma</p> <p>Human melanoma</p> <p>Human melanoma</p> <p>Human pancreatic cancer</p> <p>Human pancreatic cancer</p> <p>Human pancreatic cancer</p> <p>Human pancreatic cancer</p> <p>Murine lymphocytic leukemia</p> <p>Human cervical adenocarcinoma</p> <p>Human melanoma</p> <p>Human melanoma</p> <p>Human osteogenic sarcoma</p> <p>Pig intestinal porcine epithelial cells-1</p>	<p>apoptosis</p> <p>Specificity of ADI for degradation of arginine and other amino acids; ASS expression dependent sensitivity of HCC and melanomas towards ADI</p> <p>Involvement of Ras/PI3K/ERK pathway in induction of c-Myc stabilization and up-regulation of ASS</p> <p>Correlation between ASS methylation status and sensitivity of the cells towards ADI</p> <p>Potential clinical correlation between ASS expression and tumor prognosis</p> <p>The role of ASS gene expression in ADI response/resistance</p> <p>The role of ASS gene expression in ADI response/resistance</p> <p>Cell proliferation and non-recoverable cell death of malignant cells on restoration of arginine</p> <p>Cell proliferation and ASS expression dependent recycling of citrulline to arginine</p> <p>LPS- induced cell damage involving mTOR and TLR4 pathways</p>	<p>[47]</p> <p>[61]</p> <p>[26]</p> <p>[205]</p> <p>[72]</p> <p>[74]</p> <p>[187]</p> <p>[132]</p> <p>[206]</p>
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Table 2: Clinical investigations involving arginine depriving enzymes

Enzyme	Cancer type	Phase of a clinical trial	Number of patients	Clinical outcomes	Common side effects	Post-treatment levels of plasma arginine#	Reference
ADI-PEG20	HCC	II	71	SD:31% (22/71) DCR: 31% (22/71)	Hypersensitivity/skin rash, local tissue reaction at injection site, hyperuricemia, pruritus, fatigue, hyperammonemia, fever, diarrhea	< 2 µM	[63]
	ASS (-) melanoma	I	17	PR: 23.5 % (4/17) SD: 29.4 % (5/17) CBR: 52.9 % (9/17)	Mild/moderate discomfort at the intramuscular injection site, neutropenia and thrombocytopenia, anaemia, fatigue	Undetectable	[25]
	HCC	I/II	19	CR: 11% (2/19) PR: 37 % (7/19) SD: 37% (7/19)	Occasional elevation in serum lipase, bilirubin and amylase levels, hyperuricemia, mild pain at the site of injection, increase in fibrinogen	< 2 µM	[208]
	MM	I/II	24	OR: 25 % (6/24) SD: 25 % (6/24)	Mild pain at the site of injection, hyperuricemia, elevated serum lipase, bilirubin, amylase and LDH, decreased hemoglobin, platelet and WBC count	< 2 µM	[52]
	HCC	II	76	OR: 3% (2/76) SD: 61% (50/76)	Transient and reversible encephalopathy, skin irritation, or discomfort at the site of injection combined with low-grade fever, decreased serum sodium, hemoglobin, albumin, fibrinogen levels, increased Potassium levels, uric acid and lipase	Undetectable	[51]
	MM	II	36	OR+SD: 28 % (10/36)	Discomfort at the injection site		[209]
	Melanoma	I/II	31	SD:31% (9/29) PMR: 27% (8/29)	Pain and rash at injection site, nausea, anorexia, pruritus, arthralgia	Undetectable	[210]

ADl-PEG20 plus Cisplatin	MPM	II	39	PMR: 46% (18/39) SD: 31% (12/39)	Skin injection site reactions, neutropenia, anaphylactoid reactions, serum sickness	2 µM ^{\$}	[64,211]
ADl-PEG20 plus Cisplatin and Pemetrexed	HCC	III		Ongoing (NCT01287585)			
	Non-Hodgkin's Lymphoma	II		Ongoing (NCT01910025)			
	SLCL	II		Ongoing (NCT01266018)			
	MM	I		Ongoing (NCT01665183)			
	Arginine auxotrophic tumors such as MPM and NSCLC	I		Ongoing (NCT02029690)			
ADl-PEG20 plus Docetaxel	Solid Prostate and NSCLC tumors	I	18	PR: 6% (1/18) SD: 33% (6/18)		Undetectable	[212,213]
ADl-PEG20 Plus Doxorubicin	HER2 (-) Breast Cancer	I		Ongoing (NCT01948843)			
Peg-rhArgI	HCC	I	15	SD:26.7% (4/15)	Abdominal pain, diarrhea, nausea, elevated ALT, AST, GGT & bilirubin	< 8 µM	[141]
Peg-rhArgI plus Oxaliplatin and Capecitabine	HCC	II		Ongoing (NCT02089633)			

Peg-rhArgI (the second-line therapy after sorafenib)	HCC	II	Ongoing (NCT02089763)	
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Basal (Pre-treatment) level of arginine was ~ 130 µM

\$ Basal (Pre-treatment) level of arginine was ~ 63 µM

DCR- Disease-control rate (complete/partial response + stable disease)

SD- Stable disease

OS- Overall survival

PR- Partial response

CBR- Clinical benefit rate

MM- Metastatic melanoma

OR-Overall response (Complete + partial response)

CR- Complete response

PMR- partial metabolic response

MPM- Malignant Pleural Mesothelioma

Peg-rhArgI - Pegylated recombinant human arginase 1

ALT - Alanine Transaminase

AST - Aspartate Transaminase

GGT - Gamma-glutamyl transferase

SLCL- Small Cell Lung Cancer

NSCLC- Non-Small Cell Lung Cancer

HER2- Human epidermal growth factor receptor 2

Table 3: Properties of arginine depriving enzymes

Arginine deiminase (E.C. 3.5.3.6)	Arginase (E.C.3.5.3.1)	Arginine decarboxylase (E.C.4.1.1.19)
Main products are citrulline and NH ₃	Main products are ornithine and urea	Main products are agmatine and CO ₂
At physiological pH, Mycoplasma ADI is 300x more effective than arginase at depleting arginine	Very high alkaline pH optimum (pH 9.3) and has little enzymic activity at physiological pH	Mammalian ADC has a basic pH optimum (pH 8.23)
Circulatory half-life of ~ 4 h	Very short circulatory half-life (Approx. 30 minutes)	Not reported
Very high affinity for arginine (K _m of 0.1-1 mM)	Low affinity for arginine (K _m of 2-4 mM)	High affinity for arginine (K _m of ~ 1mM)
Most normal cells and tissues are able to take up citrulline from the circulation	Ornithine can only be reconverted back into arginine in the liver and can cause toxicity to extra-hepatic tissues by inhibiting protein synthesis	Agmatine is not converted back to arginine under normal physiological conditions, may lead to its accumulation and toxicity to normal cells
Only found in microorganisms and is strongly antigenic in mammals	Human enzyme, non-immunogenic	Found in plants, microbes and human brain
Tumor sensitivity to ADI is dependent on ASS expression	The sensitivity of tumors to rhArg is independent of ASS expression	Studied only in human cervical cancer (HeLa) cell lines
Efficacious only in ASS-negative tumors	Efficacious in both ASS-negative and OTC-negative tumors	
No cofactor requirement	Mn ²⁺ is essential for catalytic activity	Pyridoxal phosphate is a cofactor
Pegylation improves catalytic activity at physiological pH	Pegylation improves catalytic activity at physiological pH	PEGylation results in the total loss of catalytic activity