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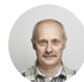

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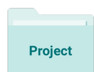

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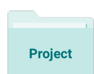

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MINI-REVIEW

Recombinant arginine-degrading enzymes in metabolic anticancer therapy and bioanalytics

Oleh V. Stasyk[1]*, Yuriy R. Boretsky[1,2], Mykhailo V. Gonchar[1,3] and Andriy A. Sibirny[1,4]

1 Institute of Cell Biology, National Academy of Sciences of Ukraine, Drahomanov St. 14/16, Lviv 79005, Ukraine
2 Department of Biochemistry and Hygiene, Lviv State University of Physical Culture, Kosciuszko St. 11, Lviv 79000, Ukraine
3 Institute of Applied Biotechnology and Basic Sciences, Rzeszow University, Sokolowska Str. 26, Kolbuszowa 36-100, Poland
4 Department of Biotechnology and Microbiology, Rzeszow University, Cwiklinskiej 2, Rzeszow 35-601, Poland

Abstract

Tumor cells often exhibit specific metabolic defects due to the aberrations in oncogene-dependent regulatory and/or signaling pathways that distinguish them from normal cells. Among others, many malignant cells are deficient in biosynthesis of certain amino acids and concomitantly exhibit elevated sensitivity to deprivation of these amino acids. Although the underlying causes of such metabolic changes are still not fully understood, this feature of malignant cells is exploited in metabolic enzymotherapies based on single amino acid, e.g., arginine, deprivation. To achieve efficient arginine depletion in vivo, two recombinant enzymes, bacterial arginine deiminase and human arginase I have been evaluated and are undergoing further development. This review is aimed to summarize the current knowledge on the application of arginine-degrading enzymes as anticancer agents and as bioanalytical tools for arginine assays. The problems that have to be solved to optimize this therapy for clinical application are discussed.

Keywords: arginine deprivation; cancer; metabolic therapy

Introduction

Arginine is a semi-essential amino acid in humans. Its physiological level is maintained through food intake, protein degradation, and endogenous synthesis in the so-called "intestinal-renal axis". Arginine is an important signaling molecule, a key intermediate in urea cycle and a direct precursor for the biosynthesis of polyamines and nitric oxide. The demand for arginine increases for a growing organism and upon certain pathologies (reviewed in Morris, 2006). A growing number of tumor types is being identified as deficient in the key arginine anabolic enzyme of urea cycle, argininosuccinate synthetase (ASS, see Figure 1), and thus, fully dependent on its exogenous supply (for review: Wheatley, 2004; Kuo et al., 2010; Delage et al., 2010; Phillips et al., 2013 and references thereof). It has been established that ASS deficiency in tumor cells is often due to its transcriptional silencing evoked by hypermethylation (Delage et al., 2012; Huang et al., 2013). Tumor cells in vitro were also shown to be deficient in ornithine transcabamylase (OTC, Figure 1), thus being not able to convert exogenous ornithine to arginine (Wheatley et al., 2005; Cheng et al., 2007; Bobak et al., 2010). Therefore, arginine has to be considered as an essential amino acid for tumor cells cultured in standard media, as well as for many tumors in vivo.

Although arginine deprivation-based enzymotherapy is at the developmental stage, it clearly bears significant potential as an efficient, selective, and relatively non-toxic approach against highly malignant ASS-negative tumors, e.g., melanoma, pancreatic, prostate, renal and hepatocarcinomas, mesotheliomas, lymphomas, and others (see Phillips et al., 2013 and references thereof), which are often refractory to conventional therapies.

ASS deficiency of tumor cells is a main prognostic marker of tumor cells sensitivity to arginine deprivation in vivo. Growth of ASS-deficient tumors can be potentially controlled with recombinant arginine degrading enzymes, arginase, arginine deiminase, or arginine decarboxylase (Figure 1). Below we provide their short characterization and describe positives and shortcomings of each enzyme as anticancer therapeutics.

*Corresponding author: e-mail: sibirny@cellbiol.lviv.ua

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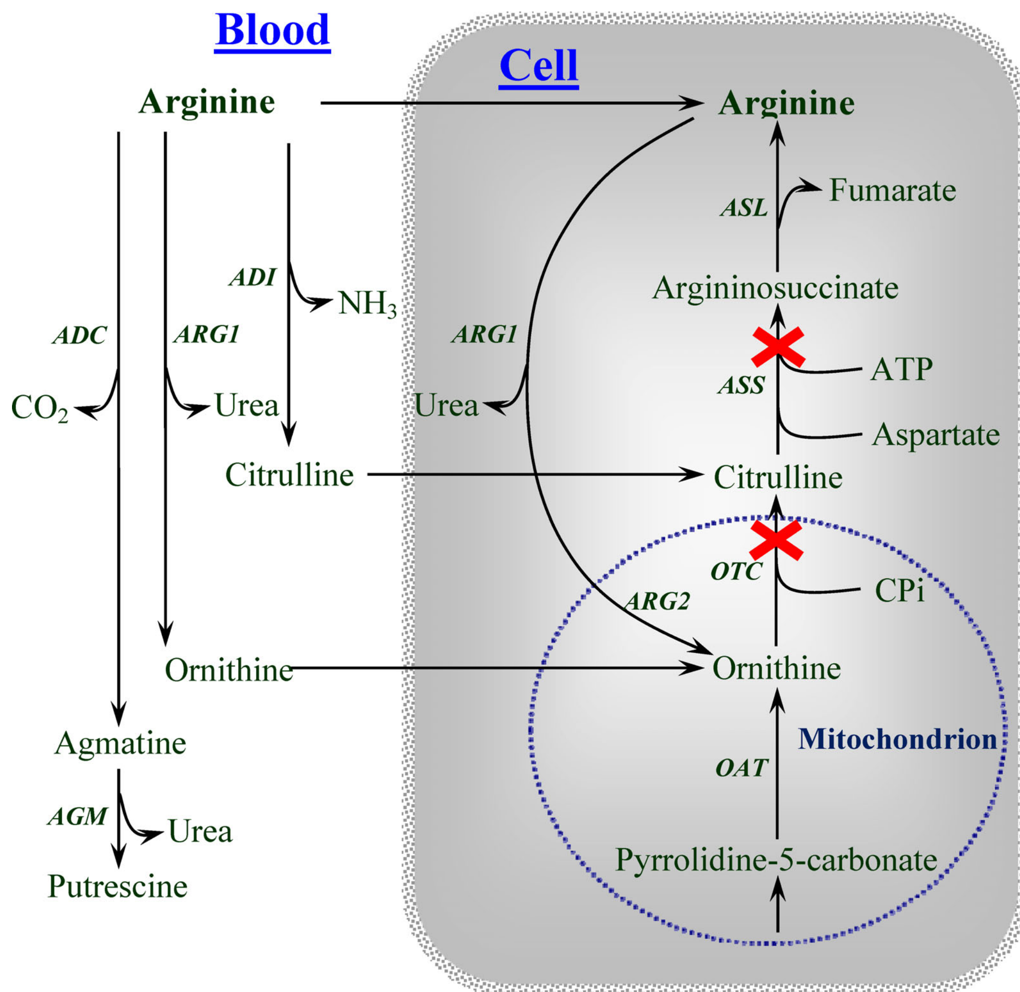

Figure 1 **Scheme of arginine metabolism and action of recombinant enzymes used for arginine deprivation in blood stream.** RG, arginase; ADI, arginine deiminase; ADC, arginine decarboxylase; AGM, agmatinase; ASS, argininosuccinate synthetase; ASL, argininosuccinate lyase; OTC, ornithine transcarbamylase; OAT, ornithine aminotransferase; CPi, carbamoylphosphate. X: Enzymatic defects in tumors auxotrophic for arginine.

Recombinant arginine-degrading enzymes as anticancer therapeutics

Two recombinant arginine-degrading enzymes have been evaluated and proposed as anticancer therapeutics in vitro and in clinical trials, bacterial arginine deiminase from *Mycoplasma hominis* (ADI) and human arginase I (ARG1). Their short characterizations are summarized in Table 1. It has to be mentioned that arginine can also be potentially degraded via consecutive action of two other enzymes, arginine decarboxylase (ADC), which produces CO_2 and toxic agmatine, which can potentially evoke its own anticancer effect (Piletz et al., 2013), and agmatinase (AGM), which decomposes the latter to urea and putrescine (Figure 1). However, this combinational approach and the mentioned enzymes as recombinant products have not been evaluated so far and will not be discussed here.

Arginine deiminase (ADI)

ADI (L-arginine iminohydrolase, EC 3.5.3.6) catalyzes the conversion of L-arginine to L-citrulline and ammonia (Figure 1). It is absent in mammals; however, is quite common in prokaryotic microorganisms: *Mycoplasma, Pseudomonas, Lactobacillus* etc. The physiological function of ADI in host organisms, in addition to arginine catabolism, is generation of ATP from arginine as a major non-glycolytic energy source in so-called “ADI pathway”. Its predicted advantages and shortcomings with respect to alternative arginine-degrading enzyme, arginase, are listed in Table 1.

ADI genes from different species of *Mycoplasma* and *Pseudomonas* were heterologously expressed in *Escherichia coli* and *Bacillus subtilis* and proved to be efficient in lowering arginine levels both in vitro and in vivo (Feun et al., 2008 and references thereof). ADI expressed in *E. coli*

Table 1 Comparative analysis of arginine-degrading enzymes as anticancer therapeutics.

Human Arginase I (ARG1)	Bacterial Arginine deiminase (ADI)
Advantages	
• Low immunogenicity (human enzyme) • Nontoxic products and deep conversion of arginine (to ornithine and urea) • Potentially wider spectrum of sensitive tumors (OTC and ASS deficient) • Available in pegylated form for human use • New mutant and modified forms (as cobalt-substituted ARG1) with catalytic properties optimized for clinical use	• Low Km (in micromolar range); as a result, less enzyme is needed for the therapy • Available in pegylated form for human use • New mutant forms optimized for clinical use • FDA-approved drug
Shortcomings	
• Non-optimal kinetic properties of native ARG1 when administered into blood stream (high Km, in millimolar range, pH optimum ~9.5); as a result, large quantities of the enzyme needed for therapy. However, application of cobalt-substituted ARG1 essentially solves this problem. • High price of recombinant ARG1 produced in bacteria (multistep purification protocol) and high cost of pegylation	• Immunogenicity even in pegylated form (bacterial enzyme) that often leads to inactivation by neutralizing antibodies • Arginine conversion to citrulline and ammonia (may be toxic in a long term) • Only ASS-deficient tumors are sensitive to ADI treatment (those unable to convert citrulline to arginine). ASS-deficiency in tumors is often reversible.

on LB medium using pET expression platform produced inclusion bodies and inactive enzyme had to be solubilized and reactivated. These procedures increase the costs of the enzyme preparation, but save costs for enzyme purification as ADI in inclusion bodies is practically homogenous. In general, ADI production costs are high due to use of the expensive inducer isopropyl β-D-1-thiogalactopyranoside (IPTG) and an extraction buffer, as well as LB medium for the enzyme induction (Misawa et al., 1994).

We developed a new advanced protocol for ADI expression in *E. coli*, its extraction and renaturation. The main improvements included: utilization of the dense suspensions of *E. coli* in cheap synthetic medium, use of inexpensive lactose as an inducer instead of IPTG, and a cheaper but not less efficient buffer for solubilization of ADI inclusion bodies (Fayura et al., 2013). Besides, methods for stabilization of the producer and the purified enzyme have also been developed (unpublished data).

Immunogenicity of bacterial ADI in humans can be reduced by covalent binding to polyethylene glycol (PEG) of molecular weight of 20,000, thus protecting its immunogenic determinants (ADI-PEG20, Polaris Group). It was reported that the response rate to ADI-PEG20 in the completed Phase I/II trials performed on hepatocarcinoma and melanoma patients was encouraging (summarized in Phillips et al., 2013). In clinical trials, serum arginine levels were generally not detectable after ADI-PEG20 dosing ($<\sim 1\ \mu M$). However, there was also an evidence of the appearance of the enzyme neutralizing antibodies that limit the duration of arginine depletion and lead to rebound in arginine plasma levels. It was also reported that insensitivity to ADI-PEG20 treatment is related to reappearance of ASS activity in tumor cells apparently triggered by arginine deprivation.

It is of note that ADI-PEG20 is a FDA-approved drug and so far is considered a favorite arginine degrading enzyme for clinical applications.

Human arginase I (ARG1)

Arginase (L-arginine amidinohydrolase, EC 3.5.3.1) catalyzes the conversion of L-arginine to L-ornithine and urea (Figure 1). Arginases are found in variety of organisms from bacteria to mammals. There are two arginase enzymes in humans: arginase I (cytosolic, so-called “liver-type”) and arginase II (mitochondrial, so-called “kidney type”). Arginase I is abundantly expressed in liver and is a key enzyme of urea cycle, but arginases I and II are also found in cells and tissues that lack a complete urea cycle, in which case these isozymes regulate the nitric oxide synthesis and vascular function. Only arginase I (ARG1) has been evaluated as anticancer drug and its predicted advantages and limitations as an anti-tumor agent are listed in Table 1.

ARG1 was heterologously expressed in *E. coli*, *B. subtilis* as (His) 6-tagged enzyme for efficient affinity purification (Cheng et al., 2007). Expression in bacterial hosts, despite its efficiency (~100–200 mg of the purified protein per liter of culture), has some limitations as bacterial expression systems are potentially sensitive to phage infections and product contamination with highly toxic bacterial lipopolysaccharides and lipoproteins can pose a danger for ARG1 use in humans.

As an alternative, we constructed the recombinant strain of the yeast *Saccharomyces cerevisiae* overproducing (His) 6-tagged ARG1 with a higher specific activity (up to $1600\ \mu mol\ min^{-1}\ mg^{-1}$ protein) as compared to the reported for ARG1 derived from bacterial cells (Zakalskiy et al., 2012). In addition, the methylotrophic yeast *Hansenula polymorpha*, one of the best known efficient

eukaryotic expression platforms, was utilized by us for secretory overexpression of human ARG1. By creating the strains that harbor multiple copies of ARG1 expression cassettes and designing optimal cultivation conditions, we achieved considerably high yield of the recombinant product (approx. 15,000 IU (up to15 mg) from 1 L of culture) (O.V. Stasyk, unpublished data). Preparations of ARG1 were successfully tested in laboratory tumor models (2D and 3D cultures) as tumor suppressors. These results suggest that the developed yeast expression platform may be a useful alternative for bacterial expression systems, especially for the production of ARG1 as anticancer agent for human use.

Similarly to ADI, ARG1 was obtained in a pegylated form (peg-rhArg, BCT, Hong Kong, China) for in vivo use (Tsui et al., 2009).

To overcome limitations of ARG1 non-optimal pH range, recently the substitution of Mn^{2+} for Co^{2+} in ARG1 active center that lowers pH optimum and also stabilizes enzyme at physiological pH was reported. Introduction of certain point mutations into the second-shell metal ligands of ARG1 led to a remarkable optimization of its characteristics for in vivo use (Stone et al., 2010, 2012). Our preliminary data indicate that it is possible to express cobalt-containing ARG1 directly from the yeast producers (unpublished data).

ARG1 is currently evaluated in laboratory, animal and clinical trials as an anticancer enzyme, for instance for the treatment of hepatocellular carcinoma (see Phillips et al., 2013 and references thereof). Importantly, it was proposed that sensitivity to arginine depletion by ARG in vivo is not strictly dependent on ASS status (as in the case with ADI), but also on the status of OTC (ornithine transcarbamylase, Figure 1), shown to be absent in many tumors. This notion, however, has to be experimentally confirmed. Currently ARG1 is not a FDA approved drug.

Arginine-degrading enzymes in bioanalytics

Development and clinical application of anticancer therapy based on arginine-degrading enzymes requires efficient monitoring of blood plasma arginine levels. Besides, arginine monitoring is also important as this amino acid is used as food supplement for building body mass, and in wines and wine products. It is known that arginine, due to conversion to urea by microbial arginases and chemical reaction of urea with ethanol, generates a dangerous carcinogen, ethylcarbamate (urethane).

A variety of detection procedures for L-arginine have been developed. The majority of them are time-consuming, expensive, and require skilful laboratory techniques, such as the high performance liquid chromatography, ion-exchange chromatography, spectrophotometry, fluorometry, atom absorption spectrometry, catalytic–thermometric titrametry, flow-injection techniques, polarography, capillary electrophoresis, or mass-spectrometry. Most of currently existing methods for arginine detection in solutions (except flow-injection chemiluminescence method) are not sufficiently selective and sensitive (the linear concentration range for arginine determination in the final reaction mixture varies from 1 to 100 mM), and therefore problematic when applied to biological samples (free arginine blood plasma levels are around 0.1 mM).

Recently we reported the development of bi-enzyme arginine-selective sensors based on ARG1 and urease with potentiometric (Stasyuk et al., 2011a) and amperometric signal registration (Stasyuk et al., 2012a). The first biosensor exhibits selective response to L-arginine with the linear range from 0.12 to 40 mM with a detection limit below 0.1 mM. The amperometric biosensor demonstrates a good sensitivity ($110\ A\ M^{-1}\ m^{-2}$ with a linear frame of 0.07–0.6 mM). Its sensitivity could be further improved with a bigger area of the working electrode and nanomatherials with a high electron transfer activity. Amperometric biosensor, based on the use of recombinant ADI immobilized by cross-linking with bovine albumin using glutaraldehyde, reveals a liner range 0.005–0.100 mM with apparent Michaelis constant of 0.35 mM, and possesses a higher sensitivity and better storage stability (Zhylak et al., unpublished data).

ARG1 was immobilized on gold and silver nanoparticles and used for constructing arginine biosensors and for developing enzymatic methods of arginine assay with spectrophotometric and fluorometric modes of reaction product detection (Stasyuk et al., 2011b, 2013). Fluorometric detection allows a very high sensitivity: linear range is 0.2–250 μM with a detection limit of 0.16 μM.

In summary, two bioanalytical approaches look the most promising to monitor arginine level during arginine deprivation anticancer therapy: arginase/urease-based amperometric biosensor and arginase-based enzymatic method with fluorometric detection of the product.

Problems and future perspectives of arginine-deprivation therapy

Despite of the recent significant progress in developing anticancer therapy based on arginine deprivation, to bring it into viable clinical use, several problems have to be addressed and solved. Initial clinical trials with arginine-degrading enzymes demonstrated this monotherapy as considerably efficient in controlling proliferation of ASS-negative tumors but less efficient, than had been expected, as a curative approach (see Phillips et al., 2013).

We suggest that the two arginine-degrading enzymes currently undergoing phase I/II clinical trials, ADI and ARG1, have to be compared side-by-side in a single trial to evaluate which enzyme is more effective, and also more cost-efficient, in maintaining arginine deficiency in patients.

Also, the question has to be experimentally addressed how plasma citrulline level fluctuates under ADI relative to ARG1 administration, and whether ASS-negative and ASS-semi-deficient tumors respond differently to these enzymes in vivo. It may be also possible to develop protocols for simultaneous or sequential application of both ADI and ARG1 as each enzyme has its own advantages (Table 1), and drawbacks of one enzyme could be compensated by the presence of another one.

The molecular mechanisms that determine the sensitivity of tumor cells to arginine starvation in vitro and in vivo are been actively studied but have to be further deciphered in detail. It was originally proposed that the elevated tumor cell sensitivity to arginine deprivation may result from the complex nature of its metabolism and impact of arginine and its catabolites on a number of signaling pathways. We recently reported however that in vitro the levels of intrinsic arginine metabolic enzymes do not determine tumor cell response to arginine-deprivation stress and that sensitivity to arginine deprivation often correlates with cells response to a deficit of alternative essential amino acids (Bobak et al, 2010; Vynnytska-Myronovska et al., 2013).

Cultured tumor cells are highly variable in their sensitivity to arginine deprivation and those sensitive often exhibit signs of apoptosis (Wheatley, 2004; Bobak et al, 2010). However, the molecular markers of such differential sensitivity are not well understood. It remains to be elucidated what are the molecular triggers of apoptosis and how it can be augmented given the block in protein biosynthesis evoked by arginine starvation. Nevertheless, we observed that downregulation of apoptosis by pancaspase inhibitor does not rescue tumor cells viability in monolayer culture (Vynnytska-Myronovska et al., 2013). We have also found that p53-deficiency sensitizes cells to arginine deprivation by altering cell cycle regulation (unpublished data).

We recently reported that sensitivity of tumor cells of different organ of origin to arginine starvation dramatically decreases in 3D spheroid relative to corresponding monolayer cell cultures. Although a fraction of tumor cells exhibited apoptosis, spheroids were still able to resume growth upon arginine re-supplementation after several weeks of arginine starvation. Aberrant arginine metabolism apparently is not involved in such a differential sensitivity, as 3D spheroid cultures became also remarkably less sensitive to deprivation of alternative essential amino acids, i.e., methionine (Vynnytska-Myronovska et al., 2012, 2013). Interestingly, simultaneous starvation for two essential amino acids did not augment the effect on cell viability (unpublished data). We propose, therefore, that 3D tumor cultures have to be included as an invaluable informative tool, more closely mimicking tumor physiology than conventional monolayer cell culture, for amino acid deprivation studies (Vynnytska-Myronovska et al., 2012, 2013).

For further optimization and development of rational combinational approaches, signaling mechanisms governing tumor cell response to arginine deprivation have to be elucidated (reviewed in Phillips et al., 2013). The frequent appearance of therapy-resistant tumor relapses due to ASS-derepression and intracellular arginine re-synthesis (from exogenous citrulline) poses an important problem. ASS-positive tumor relapses have to be studied in details to evaluate what combinational approaches, especially those based on "synthetic lethality" may be more efficient for the particular tumor types. For instance, it was reported that ASS upregulation in response to arginine deprivation in melanoma cells is evoked by HIF-1alpha downregulation and C-Myc upregulation (Kuo et al., 2010). Availability of FDA-approved drugs targeting these proteins opens the possibility for the design of new combinational treatments. One may assume that the simultaneous use of efficient ASS inhibitors together with arginine-degrading enzymes can address this problem as ASS-positive relapses will not have a selective advantage.

It was observed by several groups that cultured tumor cells often exhibit upregulation of pro-survival autophagic protein degradation (process of lysosomal protein recycling) in response to arginine deprivation stress (Kim et al., 2009; Shuvayeva et al., 2014). Given the availability of autophagy downregulating drugs (such as chloroquine), these findings may lead to new more efficient combinational treatments.

It was also reported that ADI-PEG20 suppresses target enzymes of the antifolate drug pemetrexed, thymidylate synthase (TS) and dihydrofolate reductase (DHFR) in several types of cancers (Philips et al., 2013), which may lead to new combinational treatments as well. We also demonstrated that toxic arginine analogue of plant origin, canavanine, strongly augments effect of ARG1 (but not ADI) in 2D and 3D cultured tumor cells and leads to their radiosensitization (Vynnytska et al., 2011; Vynnytska-Myronovska et al., 2012). The combined regimen of ARG1 and canavanine is currently evaluated in animal studies.

Other therapeutically highly-relevant questions are whether and how arginine deprivation affects tumor cells motility and invasiveness, and how concomitant limitation of nitric oxide (arginine catabolite) affects functioning of immune cells. With this respect, we recently observed that deficit of arginine specifically destabilizes actine cytoskeleton in glioma cells and decreases their motility and clonogenic potential (Pavlyk et al., 2014). It was also found that supplementation of exogenous nitric oxide donor, as a potential attenuating therapeutic compound, does not rescue tumor cells from the effects of arginine deprivation stress (Chen et al., 2011). Very recent reports also point at a possible ASS role as tumor suppressor (Huang et al., 2013; Allen et al., 2014), opening yet new possibilities and

stimulating the development of arginine deprivation as an efficient anticancer therapy.

Conclusions

Metabolic antitumor enzymotherapy based on arginine deprivation draws a renewed interest during recent years and gains momentum in its development. Recombinant arginine-degrading enzymes, ADI, and ARG1, have been substantially improved for the clinical use and preliminary results of clinical trials are encouraging. Further research on molecular mechanisms that determine tumor cells sensitivity to arginine deprivation, especially in vivo, and development of rationally designed multimodality therapies against particular types of tumors is anticipated.

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