REVIEW ARTICLE

Arginine-depleting Enzymes, A Potential Treatment Option for Tumors With Arginine Auxotrophy : A Review

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ABSTRACT

The World Health Organization reports that one of the top global causes of illness and mortality is cancer, with nearly 10 million deaths in 2020. Changes in cellular metabolism are common characteristics of a wide variety of malignancies. Enzymatic deficits cause many tumors to lose the ability to synthesize amino acids required for their growth, survival, or proliferation. Thus, some tumors depend on the extra-cellular supply of specific amino acids to meet their needs, allowing them to survive. Amino acid depletion as a targeted therapy takes advantage of these tumor traits by depleting certain amino acids in the body that is required for the tumor to survive. This review aims to discuss the potential and challenges of arginine-depleting enzymes as a means in treating arginine auxotrophic cancers. Previously, arginine deiminase (ADI) of bacterial origin has been studied for the in vivo arginine auxotrophic tumour therapy. However, it has been hampered by drawbacks, including immunogenicity and toxicity issues. Thus, human arginase I (hARGI) has been considered a better candidate due to its low immunogenicity and toxicity effects. However, hARGI's application as an anti-cancer drug is hindered by its low activity towards arginine owing to its high Km values indicating the enzyme's low substrate affinity. Thus, it is necessary to improve the enzyme catalytic capability and stability for more practical application in therapeutic cancer treatment. With the advancement of bioinformatics tools, more studies are anticipated to rationally engineer the enzyme for more practical clinical application in the treatment of arginine auxotrophic cancers.
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INTRODUCTION

Cancer is a set of diseases characterized as cell proliferation, invasion, and metastasis that is uncontrolled. According to World Health Organization (WHO), cancer has constituted an enormous burden on society and is appointed as the second leading cause of death globally and is responsible for about 10 million deaths in the 2020 (1).

Cancer surgery, radiation, and chemotherapy are conventional cancer treatments. Radiation therapy uses ionizing radiation delivers directly to the diseased tumour meanwhile, chemotherapy is the cancer treatment with agents that promptly eliminate all dividing cells. Depending on the type of treatment, these two types of treatments are frequently used in conjunction with each other, for instance neoadjuvant therapy (pre-surgical), adjuvant therapy (post-surgical), and concomitant therapy (radiotherapy and chemotherapy without surgical intervention) (2). Combining these treatments allows cancer to be addressed from various angles, potentially preventing cancer cells from progressing into resistant to one or both treatments. Conventional treatments have yielded some remarkable results, but there is a growing resistance to these treatments, which will eventually lead to more aggressive cancer. As a result, there is a greater mortality rate. According to WHO

in 2021, survival after diagnosis takes 3 to 6 months on average, with a 5-year survival rate of less than 5% (3). The mechanisms behind conventional cancer therapies are that all somatic cells have the same malignant potential. These techniques are inefficient in providing long-term cancer protection due to their lack of specificity. Besides, cancer cells are reported to develop unique mechanisms by which they can protect themselves from harmful xenobiotic agents, in the same way, normal stem cells work. As a result, the admission of these toxic agents kills both proliferating cancer cells and the normal cells, resulting in serious side effects and, in some cases, patient death (4). Thus, the development of a therapeutic method based on the cellular physiological difference between the tumour and healthy cells is essential and essential to enhancing life quality for the patients.

This review discusses the cellular physiology of tumour tissues which are auxotrophic to specific amino acids and also the past, present, and future recommendations for arginine-depleting enzymes as a potential treatment option for tumours with arginine auxotrophy.

AMINO ACID DEPLETION THERAPY

Changes in cellular metabolism seem to be common characteristics of a wide variety of malignancies. Several cancers display impairments in their enzymatic armamentarium and are unable to synthesize one or more essential amino acids for their development, survival, and expansion. They are known to be auxotrophic to specific amino acids, requiring high amino acid concentrations to survive (5). Therefore, these tumors rely on the extracellular pool of amino acids to satisfy protein production requirements and continue to develop unimpeded. (6). This situation suggests that by lowering the concentration of particular amino acids, tumor cell proliferation can be inhibited or destroyed. Theoretically, normal cells should be unaffected since they are able to generate sufficient amounts of these amino acids via other mechanisms. This fundamental divergence in the nutritional demands of tumors and healthy tissues generates a metabolic vulnerability of tumors can be utilized to prevent their survival and proliferation. Moreover, the auxotrophic mechanism of the cancer cells to different amino acids could be excellent targets since they make a cancer type susceptible to specific amino acid starvation treatments (7).

The remarkable success of asparaginase in the treatment of childhood acute lymphoblastic leukemia (ALL) epitomizes the therapeutic potential of amino acid depletion. Normal cells can synthesize the non-essential amino acid, asparagine through the enzyme asparagine synthase (AS), but leukemic cells have low concentrations or activity of this asparagine-producing enzyme. Hence, the tumour cells proliferate and survived with an external supply of this amino acid from serum. Asparaginase catalyses the hydrolysis of asparagine to aspartic acid and ammonia, resulting in systemic asparagine depletion (8, 9, 10) and death in ALL lymphoblasts (11, 12). This mechanism has prompted research venture on asparaginase deprivation therapies that interfere with asparagine-deprivation tumour cells. Although there are no reports for anti-tumour activity in asparaginase in various types of cancer, this enzyme is clinically effective against ALL and some lymphomas but not others. In 1950, this enzyme was employed as a mono-therapy with a 5% success rate in treating diagnosed cases. Practically all standard childhood ALL chemotherapy protocols involve a combination of asparaginase and vincristine drugs, with a cure rate of 90%.

At present, asparaginase available for clinical use is derived from either Escherichia coli or Erwinia chrysanthemi (13). Asparaginase has been employed for many years as an effective drug in the treatment of ALL (14). Nonetheless, they rarely generate a therapeutic response without some indications of toxicity (15). A complete therapeutic regimen with the commercial E.coli asparaginase (EcAII) (ElsparT, Merck) that can be utilized as a single agent has shown nearly complete remission in 40–60% of patients. In comparison, a combination treatment of vincristine and prednisone results in a remission rate increases up to 95% (16). It is well established that some patients still develop adverse immune responses derived from asparaginase treatments. The antibodies produced by naıve E. coli asparaginase may cross-react with the pegylated version of the enzyme, resulting in an allergic reaction (17).

Besides asparagine autotrophic, there are other amino acids with dysfunctional metabolism in cancer cells and could be helpful in the targeted therapies for auxotrophic cancers. Recent studies on amino acid deprivation have discovered that glutamine deficiency effectively treats ovarian, pancreatic, and breast cancer (18, 19, 20). Methionine auxotrophy is also a novel niche and currently under exploration to target central nervous system cancers (21) and ALL (22). Other successful examples include the use of arginine-depleting enzymes to treat metastatic melanomas, which provided remarkably low toxicity and high efficacy results (6).

This review focuses on the therapeutic potential and functions associated with arginine depletion. Therefore, the next section of this review will cover this ground.

ARGININE AUXOTROPH IN TUMOR CELLS

In humans, arginine is one of the amino acids that is employed as a fundamental building block in the synthesis of ribosomal proteins. It has been used as a precursor of various biological pathways and indispensable for important cellular functions such as the synthesis of proteins, urea, polyamines, agmatine, and amino acids like glutamate and proline (23, 24). Arginine is a committed substrate in a urea cycle, which is catalysed by the arginase (ARG) enzyme to ornithine and urea. This process removes urea and regenerates ornithine, ensuring its availability for cycle repetition (23, 24).

Throughout the previous two decades, it has been reported that numerous types of tumours show a lack of ASS expression. This condition causes arginine auxotrophy in the respective types of cancer. Cells have lost the ability to synthesize their arginine and require massive amounts of this amino acid for malignant proliferation and metastasis (25). This theory has its roots in Gilroy's early 1930s research, which showed that mice fed an arginine-enriched diet developed tumors more quickly and visibly than mice fed a standard diet. On the other hand, an arginine deficient diet reduces tumor incidence and growth. Numerous human cancers, such as metastatic melanoma, prostate carcinomas, hepatocellular carcinoma (HCC), cervical carcinoma, breast carcinoma, ovarian carcinoma, squamous cell carcinoma, pancreatic carcinoma, prostate carcinoma, colon carcinoma, lung carcinoma, osteosarcoma, glioma astrocytoma, glioblastoma, promyelocytic leukemia, Hodgkin's lymphoma, osteosarcoma, and malignant pleural mesothelioma has been reported with such deficiency (26). Hence, the deprivation of arginine is being investigated as a novel approach to treating these malignant tumors. (27).

ARGININE-DEPLETING ENZYMES AS ANTICANCER THERAPEUTIC AGENT

Later in 2015, amino acid depletion therapy has discovered the arginine metabolism pathway as one of the approaches for treating arginine auxotrophic cancer cells. Arginine is known as semi-essential amino acid, indicates its derivation by de novo biosynthesis (27). The ability to produce arginine is influenced by the amount or activity of 2 enzymes, namely argininosuccinate synthetase (ASS) and argininosuccinate lyase (ASL) (28). Nevertheless, previous studies reported that arginine auxotrophy occurs in cancer cells with a lack expression of these two key enzymes. This incompetency has contributed to higher arginine demand than normal cells and results in higher sensitivity towards arginine deprivation.

Providing the function of arginine in multiple metabolic pathways, the depletion of this versatile amino acid is well accepted and has been used for suppressing the growth and proliferation of arginine auxotrophic cancers, especially hepatocellular carcinoma (HCC) as well as melanoma. Depletion of arginine in cells is usually rendered by the enzymatic approach. Enzymes will catabolize arginine to produce sub-products hence, limiting the arginine availability for cancer cell growth and proliferation. Studies have been conducted on few arginine-degrading enzymes, including arginase, arginase deiminase, and their pegylated form. Compared with arginase deiminase isolated from bacteria, arginase from humans has low immunogenicity for use in vivo. Human arginase I (hARGI) is involved in the final step of the urea cycle, which functions to hydrolyzes L-arginine to produce L-ornithine and urea. The enzyme is beneficial since the urea cycle protects cells from excessive ammonia, while L-ornithine is required for the growth of new cells, the production of collagen, and other physiological processes (29). hARGI has been reported to successfully deplete arginine from tissue culture medium in vitro and exhibit cytotoxicity to various cancer cells lines (30). Unfortunately, the therapeutic application of hARGI as a targeted cancer therapy encounters considerable challenges, such as a short circulating half-life (<30 minutes), the low affinity of the enzyme towards arginine, and slightly higher optimal pH (pH 9.6) (31).

POTENTIAL AND CHALLENGES OF HUMAN ARGINASE I (ARGI) IN TREATING ARGININE AUXOTROPHIC CANCER

The initial step in implementing amino acid depletion therapy is the selection of enzymes that can metabolize arginine into another molecule while maintaining optimal pharmacological properties with minimal side. Arginine can be catabolized by several mechanisms, using native enzymes found in mammalian cells such as arginase and arginine decarboxylase (ADC), and human recombinant arginase. There is also foreign enzyme isolated from bacterial source such as arginine deiminase (ADI) (32). Several factors are considered, including arginine depletion efficacy, immunogenicity, stability, and potential by-products; hence, only recombinant arginase and ADI are applied for the treatment of arginine auxotrophic tumors (33) while ADC is not ideal for therapeutic use because it is relatively harmful to normal cells.

On the other hand, arginase is a manganese metalloenzyme with a binuclear structure that convert arginine to ornithine and urea (34). In the mammalian liver, it is a crucial enzyme of the urea

cycle that catalyzes the process of urea production. Since the 1950s, there have been reports of the use of ARGI for the treatment of tumors (35). Despite the fact that the in vitro experiments were very promising, the in vivo studies were discouraging due to the low affinity of the enzyme towards arginine (Km 6 mmol/L at physiological pH), its alkaline pH optimum (pH 9.6), and very short half-life (approximately 30 minutes) (36). Fortunately, the ARGI administration can be improved by using recombinant technology. Since then, the use of recombinant human arginase I (rhARGI), which was successfully cloned and expressed in Bacillus subtilis and E. coli have been used to examine the function of arginase in cancer therapy (37). Various studies have been conducted on rhARGI against arginine auxotrophic cancers for instance, hepatocellular carcinoma and melanoma (39), pancreatic cancer (40), prostate cancer (41), leukemia (42) glioblastoma (43), breast cancer (44), and non-Hodgkin's lymphoma (45). Notably, the treatment of arginine auxotrophic cancer cells using rhARGI would present advantages over ADI. Clinical trials with arginine degrading enzyme arginine deiminase (ADI) from Mycoplasma arginii have been successful. Despite the exciting development, ADI enzyme originated from bacteria results in adverse immune response and high toxicity after repeated administration, a significant liability for prolonged treatment. rhARGI however has impressive serum stability and low antigenicity compared to ADI. Studies indicate this enzyme as a better candidate for targeted cancer treatment, aside from being less immunogenic (31, 46). Despite that, the properties of rhARGI were not really improving from the naïve enzyme, hence limit its usefulness as a drug candidate. According to a study, rhARGI has a very short hal-life (<30 min) with a low affinity towards arginine, necessitating the use of a significant amount of the enzyme (31). The optimal pH for rhARGI is also high (pH 9.6), and the Km value is 10.5 mM which is ineffective in cancer therapy in humans. Besides, cells expressing OCT produces intermediary metabolites such as ornithine, which can be converted to arginine in order to avoid intracellular arginine depletion (46).

MODIFICATIONS OF ARGINASE FOR IMPROVED THERAPEUTIC POTENTIAL

Since the discovery of the therapeutic potential of arginase in treating arginine auxotrophic cancer cells, several studies have been reported to increase the effectiveness and stability of this therapeutic enzyme, as listed in Table I.

The modification of the recombinant hARGI mainly focuses on the surface immobilization with either pegylation or engineered ice nucleation as demonstrated by several research groups (46-50). Such strategy aimed at improving the bio-availability and half-life of the enzymes in vivo. These strategies have been proven successful as the half-life of the enzyme has been extended significantly after being administrated into the rats. On the other hand, improving the catalytic activity of the enzyme by various mutagenesis methods is another strategy that can be employed to increase the therapeutic potential of hARGI.

Enzyme engineering strategies and the creation of novel target enzymes employ gene modification and recombinant technology. These strategies are necessary to improve an enzyme's catalytic activity, which includes eliminating allosteric restrictions, improving substrate and reactant specificity, improving thermostability, altering optimal pH, and appropriateness for organic solvents usage, among other things (51). The success of the rational design of a protein or enzyme depends mainly on the availability of crucial biochemical information in regard to its structure. The crystal structure of human arginase I has been reported at 1.29-E resolution (52). The exact process by which human arginase I catalysed the conversion of arginine to ornithine was explained in their study. They suggested that the protonation of the amino leaving group of ornithine by the conformationally flexible imidazolium group of general acid H141. This residue of H141 rotates 90 degrees to transfer the proton from the NE atom of the imidazolium ring to the NE atom of the formed intermediates. D128 may also donate a proton to ornithine before product release. In the final step of catalysis, the H141 imidazole may serve as a general base to abstract a proton from the metal-bridging water molecule (possibly through an intervening solvent molecule). In shorts, the amino acids at the position 141, 128 and 277 are known as the catalytic triad of the enzyme active site. The catalytic triad plays an important role in substrate binding, catalysis of the hydrolysis reaction, and the dissociation of products from the enzyme (52).

Rational engineering relies on gathering extensive structure-function relationships of enzymes and the availability of information rendering to the enzyme of interest. Bioinformatics-driven and rational engineering of hARGI can be developed by manipulating the amino acid residues at these three locations. Enzyme with improved functional properties can be assessed on several aspects, including the protein binding affinity towards ligand, stability, and total energy (53). Thus, rationally engineered hARGI with higher catalytic activity at physiological pH designed via bioinformatics-driven strategies can be anticipated soon. Together with a suitable surface immobilization method, hARGI could be improved successfully for better application in therapeutic cancer treatment.

Table I : Modifications towards hARGI from previous studies

Modification	Findings	References
Recombinant hARGI expressed in *E. coli*	*E. coli* expressed hARGI had chemical, immunological, and catalytic properties that are distinguishable from the native enzyme.	38
Pegylation of native hARGI	No degradation and immunogenicity effects. Retain >90% of its native catalytic activity. Remained efficacious in depleting arginine in rats after a single ip injection of 1,500 U of the conjugate as the native enzyme, plasma arginine falling to >0.05 μM from ~170 μM within 20 min and lasting 6 days	47, 46
Pegylated of hARGI and substitution of Mn^{2+} cofactor with Co^{2+} at the active sites (Co-rhArgI-PEG)	10-fold increase in overall catalytic activity (K_{cat}/K_m) at pH 7.4, close to the pH of human serum. pKa shifted from 8.5 to 7.5, improving catalytic activity at physiological pH.	48
Saturation mutagenesis of the second-shell metal ligands hARGI, focusing on cysteine residues at site 45, 168, and 303	Improved metabolic activity of pegylated arginase for substrate (K_m 6 mmol/l)	48
Recombinant hARGI expressed in *Bacillus subtilis*	rhARGI induced remarkable growth inhibition, cell cycle arrest, and caspase-dependent apoptosis in Raji and Daudi non-Hodgkin's lymphoma (NHL) cells through arginine deprivation.	45
Surface immobilization of hARGI with engineered ice nucleation protein	Up to 95% conversion rate of arginine to ornithine in 16 hours.	49
Site-selective single isoform of pegylated, Co^{2+} chelated hARGI by mutation of two of the three cysteine residues to serine	Extended half-life of the enzyme up until 72 hours after drug administration in rats	50
Rational design, engineer, and characterization of a novel pegylated single isomer human arginase for arginine depriving anti-cancer treatment	A novel single isoform of PEGylated human arginase (PT01) is created. Intravenous administration of PT01 maintains circulating arginine at low levels for 120 hours in rats, exhibits potent cytotoxicity at sub-nM levels against cancer cell lines of breast, prostate or pancreatic origin	53

CONCLUSION

Arginine-depleting strategies provide hope for the treatment of several malignancies. Nevertheless, there are still challenges that limit the clinical application of these enzymes in treating arginine auxotrophic cancer cells. Extensive studies were carried out to overcome these obstacles. The bioavailability in vivo, enzymatic activity, half-life and immunogenetic resistance to these enzymes poses a significant challenge and adds to a long list of examples in which experimentally proven substances face clinical application limitations. Recent development in nanotechnology and nano conjugation reveals some novel aspects and potential strategies for overcoming these challenges. Aside from the modification of the recombinant enzymes with advanced technology to improve their bioavailability in vivo, the rational engineering of the enzymes for better enzymatic activity and functionality at physiological pH has seen a rapid improvement of the enzymes for clinical application. With the advancement of various bioinformatics tools and studies, the arginine-depleting enzymes exhibit exciting potential for the treatment of arginine auxotrophic cancers.

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