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REVIEW

Amino acid deprivation using enzymes as a targeted therapy for cancer and viral infections

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ABSTRACT

Introduction: Amino acid depletion in the blood serum is currently being exploited and explored for therapies in tumors or viral infections that are auxotrophic for a certain amino acid or have a metabolic defect and cannot produce it. The success of these treatments is because normal cells remain unaltered since they are less demanding and/or can synthesize these compounds in sufficient amounts for their needs by other mechanisms.

Areas covered: This review is focused on amino acid depriving enzymes and their formulations that have been successfully used in the treatment of several types of cancer and viral infections. Particular attention will be given to the enzymes *L*-asparaginase, *L*-arginase, *L*-arginine deiminase, and *L*-methionine-γ-lyase.

Expert opinion: The immunogenicity and other toxic effects are perhaps the major limitations of these therapies, but they have been successfully decreased either through the expression of these enzymes from other organisms, recombination processes, pegylation of the selected enzymes or by specific mutations in the proteins. In 2006, FDA has already approved the use of *L*-asparaginase in the treatment of acute lymphoblastic leukemia. Other enzymes and in particular *L*-arginase, *L*-arginine deiminase, and *L*-methioninase have been showing promising results *in vitro* and *in vivo* studies.

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1. Introduction

According to the World Health Organization, cancer figures among the leading causes of morbidity and mortality in the world, with approximately 14 million new cases and 8.2 million cancer-related deaths in 2014. In 2016, this trend has still not been reversed, and it is expected an increase of 70% of new cases during the next two decades [1].

During the last five decades, the field of oncology has witnessed a steady flow of exciting developments. A whole new range of molecules have been developed, which can be efficiently used to treat tumors, or significantly extend the survival of patients who previously had no effective treatment options. At the same time, the understanding of tumor biology is continuously evolving, allowing a better evaluation of tumor growth during and after treatments, which also has a direct impact on the efficacy of the treatment, and at the same time, improves the life quality of the patients.

Alterations to cellular metabolism seem to constitute a nearly universal feature in many types of cancers. For instance, many tumors exhibit deficiencies in their enzymatic armamentarium and cannot biosynthesize one or more amino acids that are essential for its development, survival, and proliferation (Figure 1(a)). In order to overcome these problems, tumors rely on the extracellular pool of these amino acids to satisfy protein biosynthesis demands and continue to grow without being affected (Figure 1(b)). This means that if the concentration of those amino acids is decreased, the development of tumor cells can be impaired or even annihilated. At the same time, normal cells would remain unchanged since they are less demanding or they can synthesize these compounds in sufficient amounts by alternative pathways (Figure 1(c)). This key difference between the nutritional needs of tumors and normal tissues creates a metabolic vulnerability of tumors that can be exploited to preclude their survival or proliferation. This vulnerability is the base of the amino acid depletion therapies.

Amino acid depletion therapy involves the degradation of a specific amino acid in the bloodstream and is fundamental for the survival and/or proliferation of a tumor (Figure 1(d–g)). This type of therapy has a long history in both clinical practice and experimental therapeutic settings but only now is gaining popularity because of the advances in protein engineering technology and on more sophisticated approaches that enable the study of genetic and metabolic differences between tumor and normal cells [2].

The studies made so far indicate that a simple diet restriction is not enough to achieve the required level of amino acid depletion for therapeutical purposes. The best results are obtained with the introduction of specific enzymes into the bloodstream of the patients that can decrease the concentration of a certain amino acid in the plasma (e.g. converting it into another molecule) that has been shown to be essential for the development of the tumor (Figure 1(d–g)). Unfortunately, the human genome does not encode enzymes with the pharmacological or catalytic requisites that are

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Article highlights

- Amino acid deprivation therapy constitutes a valuable approach to treat cancer, infection and inflammation.
- FDA has already approved the use of L-asparaginase in anti-leukemic treatments in 2006 and 2011.
- *L*-arginine deiminase is used in the therapy of hepatocellular carcinomas and melanomas
- *L*-methioninase is a novel niche that is currently being explored in the treatment of breast, colon, lung and renal cancers.
- The main limitation of these therapies is the immunogenic effect of enzymes from bacterial sources.
- Several modifications to these enzymes have been conducted aiming to decrease these undesirable effects, such as the expression of these enzymes from other organisms, recombination or pegylation processes, or even by specific mutations on the active site of the enzymes.

This box summarizes key points contained in the article.

essential for the pretended therapeutic purpose. For this reason, heterologous enzymes (from other organisms), recombinant, and/or engineered human enzymes are used instead.

The potential of the amino acid depletion therapies is often illustrated by the treatment of childhood leukemia with the bacterial enzyme, L-asparaginase (Figure 1(g)). In 1950, this enzyme was used essentially as a monotherapy and could be successfully used to treat 5% of the diagnosed cases. However, nowadays, with the advent of multimodality chemotherapy regimens containing L-asparaginase, the cure rate for acute lymphoblastic leukemia (ALL) increased from 5% in the 1950s to 90% in the present days. Other successful examples include the treatment of metastatic melanomas with arginine (ARG)-depleting enzymes with low toxicity and high-efficacy results (Figure 1(e,f)). Methionine auxotrophy is also a novel niche, currently under exploration for targeting central nervous system cancers [3] and ALL (Figure 1(d)).

The success obtained so far with the amino acid depletion therapies is currently being exploited and explored in the treatment of several types of cancer that are auxotrophic for a certain type of amino acid. In addition, the same type of treatment is also being used in the treatment of viral infections (Figure 1(h)). Some viruses are also auxotrophic for some specific amino acids, and therefore, their depletion can be used to prevent the pathogens growth (Figure 1(i)).

In this review, the therapeutic potential, function, and the intellectual property related to the use of amino acid depriving enzymes and their formulations in the treatment of several types of cancer and other diseases will be revised. Particular attention will be given to the heterologous enzymes L-arginase, L-arginine deiminase (ADI), L-methioninase, and L-asparaginase taking into account their current therapeutic potential.

2. Amino acid depletion therapies

Humans and most mammals have long ago ceased the capability to produce 9 of the 20 amino acids that are needed for the biosynthesis of proteins. This means that they need to be obtained from the diet and/or microbiota. These amino acids are normally denominated as essential and include L-phenylalanine, L-valine, L-

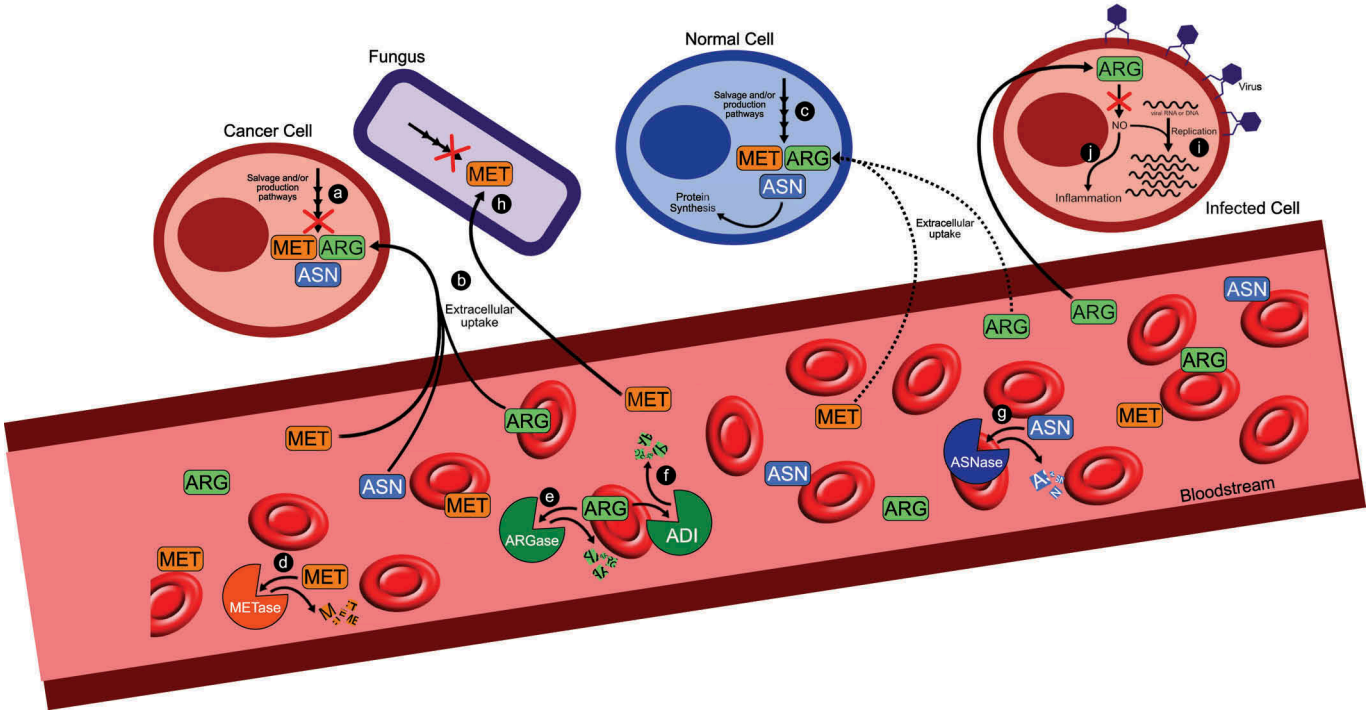

Figure 1. Schematic overview of the metabolic mechanisms involving the amino acid depletion therapies. Cancer cells (a) have some genetic modifications that compromise the *de novo* and/or salvage pathway synthesis of a key amino acid such as methionine (MET), arginine (ARG) or asparagine (ASN) and therefore require their uptake from the bloodstream (b). Normal cells are capable to synthesize endogenously MET, ARG, and ASN under normal conditions(c). The enzymes that have been tested for amino acid depletion therapy are methionine-γ-lyase (METase) (d), whose substrate is MET arginase (ARGase) (e) and L-arginine deiminase (ADI) (f) that metabolizes ARG, and asparaginase (ASNase) (g) that metabolizes ASN. Additionally, the depletion of MET can also be used as antifungal since fungus are auxotrophic for MET (h), and the depletion of ARG inhibits viral replication, also acting as anti-inflammatory (i).

threonine, L-tryptophan, L-methionine (MET), L-leucine, L-isoleucine, L-lysine, and L-histidine. Six other amino acids are termed conditionally essential; this means that their synthesis can be carried out by humans although limited by a variety of factors, such as prematurity in the infant or individuals in severe catabolic distress. These amino acids are L-arginine, L-cysteine, L-glycine, L-glutamine, L-proline, and L-tyrosine. The remaining five amino acids are dispensable from the human diet (also denoted as non-essential), meaning that human cells have the required enzymatic machinery to synthesize them, e.g. L-aspartic acid, L-asparagine (ASN), L-glutamic acid, L-alanine, and L-serine.

The optimal conditions for amino acid depletion therapies occur when the tumors are sensitive to one of the nonessential or conditionally essential amino acids. This will ensure that only tumor cells will be affected by the therapy whereas the normal cells remain unaltered since they can synthesize these compounds in sufficient amounts by their own means. The most notable and successful example of an amino acid depletion therapy is illustrated by the use of L-asparaginase (Figure 1(g)) in the treatment of childhood ALL and, to a lesser extent, non-Hodgkins lymphoma. Other examples involve the use of ADI (Figure 1(f)) in the hepatocellular carcinoma (HCC), melanoma, and other urea cycle-deficient cancer cells that have a high demand for L-arginine. The essential amino acids can also be used in amino acid depletion therapies, but it is not very common. This only happens when the normal metabolism of one essential amino acid in tumors is disrupted or when there is some sort of defect in the ability to use a certain amino acid. This is, for example, the case of MET that has been shown to be detrimental to the survival of a variety of tumor tissues, including colon, breast, prostate, ovary, lung, brain, kidney, stomach, and bladder cancers as well as larynx melanoma, sarcoma, leukemia, and lymphomas. In this case, the action of methionine-γ-lyase (Figure 1(d)) has been shown to be successfully used in the treatment of these types of cancers.

Taking into account the therapeutic potential of these enzymes in cancer therapies, they have been deeply studied in the last two decades. These therapies have been showing promising results, in spite of some side effects that were observed during clinical trials. In the following sections, a detailed analysis of each of these enzymes is done based on the amino acid that they metabolize.

2.1. Methionine

MET is a sulfur-containing amino acid that plays several important roles in the cell [4,5]. MET is crucial for protein synthesis, and it is also a precursor of several compounds, such as glutathione, polyamines spermine, and spermidine [6–9]. MET is also required for the synthesis of *S*-adenosylmethionine that it is essential in DNA methylation, and therefore, in genomic imprinting, X-chromosome inactivation, repression of repetitive elements, and carcinogenesis [10,11].

MET is a peculiar essential amino acid because its long-term deprivation does not inevitably compromise the life of the organism. Indeed, in normal cells, MET can be recycled by re-methylation of homocysteine (*de novo* synthesis), either by MET synthase or in the liver by betaine-homocysteine methyltransferase [6,12,13] (Figure 1(c)). In addition, MET can also be obtained by a salvage pathway in which it is generated from 5′-methylthioadenosine toward methylthioadenosine phosphorylase (Figure 1(c)). These two alternative sources of MET are available for normal cells from eukaryotes. However, numerous malignant cell lines, such as lung, breast, colon, kidney, and bladder cancers, melanoma, and glioblastoma [14,15] do not present an intact and functional mechanism capable of synthesizing MET (Figure 1(a)). In these conditions, cells only survive under an exogenous supply of MET (Figure 1(b)). As these cells divide more quickly than normal cells, their requirements for MET is very high [16,17], making them optimal for MET-depletion therapies.

Bacteria and fungi require a huge supply of MET (Figure 1(h)) which is required for the synthesis of *N*-formylmethionine (fMET) by the methionyl-tRNA formyltransferase [18,19]. fMET is required for the initiation of the protein synthesis in bacteria and fungus, and therefore, the depletion of MET can be used to impair their development or even to promote their destruction [20]. In addition, in these organisms, MET is required for the production of pathogenic agents, such as methanethiol [21], which turns MET depletion therapies very interesting for the development of new antibiotics.

During the last two decades, MET-restricted diets revealed a reasonable efficacy to prevent several diseases, such as aging [22–24], cancer [25,26], obesity, insulin resistance [27], and bacterial infection [21]. However, an MET-restricted diet implicates the consumption of a narrow range of nutrients that culminate in the emergence of some side effects, such as low bone density [27] and weight loss [28]. The usage of enzymes, like L-methionine-γ-lyase, has thus become a better alternative to efficiently decrease MET levels in the bloodstream of the patients having these diseases (Figure 1(d)).

L-methionine-γ-lyase (EC 4.4.1.11, METase), is an important enzyme involved in the catalysis of α- and γ-elimination of MET to α-ketobutyrate, ammonia, and methanethiol (Figure 2) [21]. METase is present intracellularly in bacteria and extracellularly in fungi, being absent in mammals [29]. Currently, the administration of this enzyme has been used as an attractive pharmacological approach to lower the MET levels, targeting the treatment of either cancer cells or other infections [30].

Indeed, the intravenous administration of recombinant and PEGylated form of METase was tested in phase I clinical trials. Patients in these trials experienced no significant toxicity, and MET levels significantly fell in plasma [31,32]. Nevertheless, the antitumor activity of this treatment was not tested, and to date, no other clinical trials have been published. Based on the results of these toxicological tests, some therapeutic applications have been assigned to METase, and they are given below.

2.1.1. Anticancer

It has been known for nearly a half century that human tumors, including those derived from the nervous system such as glioblastomas, medulloblastoma, and neuroblastomas, are much more sensitive to MET starvation than normal tissues.

Most studies on the antitumor action of MET depletion have used the METase from the potentially pathogen

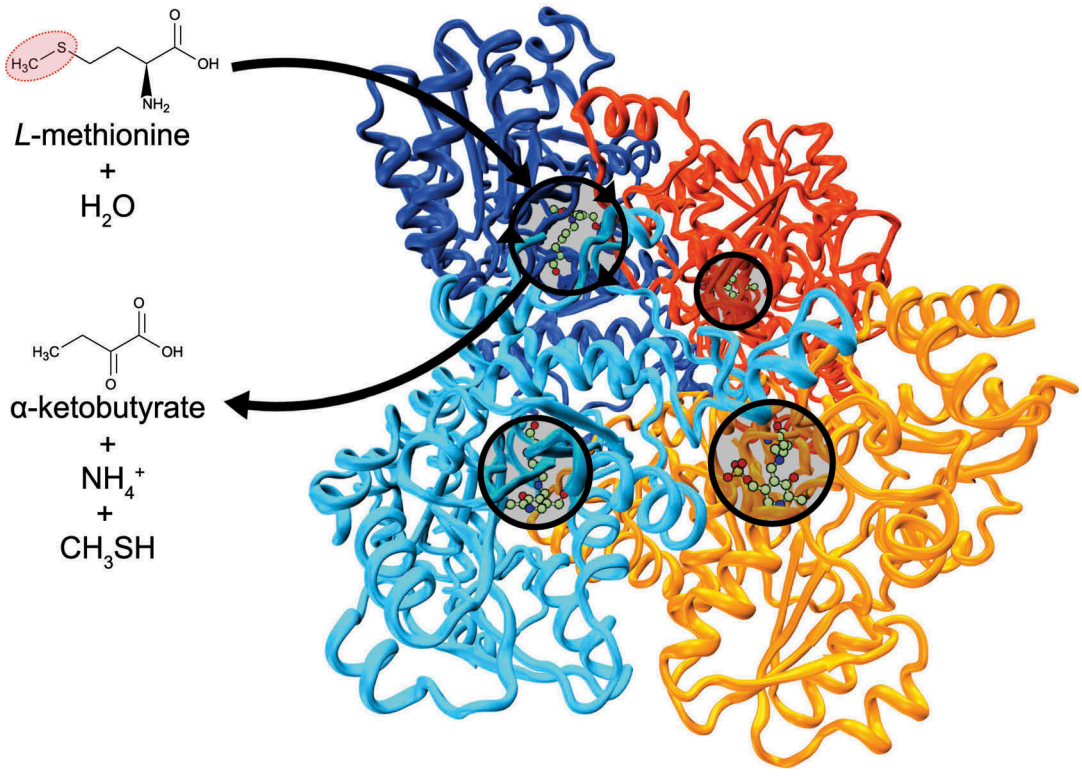

Figure 2. New Cartoon representation of the co-crystalized homotetramer structure of methionine-γ-lyase homotetramer with the PLP cofactor (PDB code: 2O7 C). The four subunits were represented with different colors (cyan, orange, blue, yellow). In the close-up of the active site, the PLP cofactor is depicted in ball and sticks. The reaction catalyzed by each active site is also represented with a highlight for the chemical group under catalytic attack. Full color available online.

pseudomonade, *Pseudomonas putida* (*Pp*-METase). Accordingly to patent US 6231854 B1, METase can be easily isolated and purified with less than a 1 ng of endotoxin per each mg of isolated, using a recombinant system, such as *Escherichia coli*, and the METase encoding gene from *Pseudomonas putida*. The purified recombinant METase could, then, be used *in vivo* in anticancer MET depletion therapies by direct intravascular administration. Besides the METase role in the cancer treatment, this enzyme could also be used to perform a global MET depletion in the organisms, followed by the radiolabeled MET administration in order to assess its distribution on the organism of patients and create a cancer location body map [28].

Various METases have also been produced from several microorganisms or by the recombination of genes encoding the enzyme originating from other bacterial species and protozoa. For example, the patent US 20140140978 A1 describes the isolation of a protein with METase and homocysteinase activities from *Brevibacterium aurantiacum*. This turns the MET deprivation in cancer therapies more efficient because homocysteine is a precursor of MET synthesis, enhancing, by this way, the desired therapeutic effect [33].

In spite of the success obtained using METase in several types of cancer in *in vitro* studies, its use *in vivo* reveals several problems. One of the main drawbacks of bacterial-derived METase is its poor stability in serum and highly immunogenicity in humans [34]. To overcome these issues, different strategies have been followed. One solution is presented in the patent US 6524571 B1 that describes a gene therapy in which the encoding gene of METase is inserted into cancer cells, and therefore, it is only produced inside the tumors. This presents several advantages since it leads to a local and more controlled MET deprivation and overcomes the instability of METase in the blood serum. In addition, the fusion of a fluorescent protein into the gene, such as GFP (Green Fluorescent Protein), can enable the control of the treatment [35]. The non-desirable immunogenicity of METase can be solved through the use of pegylated enzyme (5-kDa PEG). This posttranslational chemical modification to METase also increases the serum half-life of the enzyme, according to the registered patents US 7799549 B2, US 8465734 B2, US 20130252306 A1, US 20140205583 A1, US 6231854 B1, US 6461851 B1, and US 5891704 A [28,36–40].

Currently, a different pathway is being followed to overcome the disadvantages of METase from bacterial sources [41]. This includes the use of modified eukaryote enzymes with METase capabilities. The recent patent US 20150064159 A1 disclosed a primate modified cystathionine-γ-lyase, with amino acid substitution at positions 59, 63, 91, 119, 268, 331, 339, and/or 353 that induces a similar methionine-γ-lyase activity of the ones found on bacterial sources. This modified pegylated enzyme is currently being tested in several clinical trials in different types of cancers that have high requirements of MET. So far, the results are very promising since the immunogenicity effects and instability of the enzyme in the blood serum are not affecting the treatments [42].

2.1.2. Antiviral

The MET depletion therapies can also be expanded to the treatment of pneumonia, in particular to the one caused by *Pneumocystis carinii* (US 7264819 B2). *P. carinii* is a fungus that

causes pneumonia in patients in which the immune system is compromised, such as AIDS patients, patients undergoing chemotherapy, or patients being treated with immunosuppressants.

The use of METase is an interesting approach to treat this infection because *P. carinii* has a high MET exogenous dependency [43,44].

The patent US 7264819 B2 describes the successful use of METase in the treatment of this infection and proposes its administration with the conventional therapeutics in order to reduce their doses and minimize by this way the severe side effects from which results high mortality rates.

2.2. Arginine

ARG is a fundamental building block for protein synthesis in any organism, but in mammals, it is also an important precursor/mediator of a series of important biological pathways, such as nitrogen metabolism [45], creatine, agmatine, and polyamine synthesis [46], nitric oxide (NO) production [47], and anabolic hormone release. Additionally, it has immunostimulatory and thymotrophic effects [48,49]. ARG is classified as a semi or a conditionally essential amino acid. The term 'conditionally' is used because in healthy adults, the level of endogenous synthesis of ARG is sufficient to make it a non-essential amino acid [50]. However, under catabolic stress (e.g. inflammation, infection, etc.), the levels of endogenous synthesis may not be sufficient to ensure the metabolic demands of ARG, and in these cases, this amino acid becomes essential, requiring an exogenous supply.

The *de novo* biosynthetic pathway for ARG in mammals involves the conversion of citrulline to arginine catalyzed by argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) (Figure 3(a,b)). ASS catalyzes the condensation of citrulline and aspartate to form argininosuccinate (Figure 3(a)). ASL then splits argininosuccinate to release fumarate and ARG (Figure 3(a)). First identified in liver, as a rate-limiting enzyme in urea synthesis, ASS is now recognized as a ubiquitous enzyme in mammalian tissues [51].

In the last two decades, it was found that several types of tumors have a deficient expression of ASS (Figure 3(c)), a condition that causes ARG auxotrophy in those types of cancer. This deficiency was identified in a multitude of human cancers including metastatic melanoma, prostate carcinomas, HCC, cervical carcinoma, squamous cell carcinoma, breast carcinoma, ovarian carcinoma, prostate carcinoma, colon carcinoma, lung carcinoma, osteosarcoma, glioma/astrocytoma, glioblastoma, premyelocytic leukemia and lymphoblastic leukemia [52], non-Hodgkin's lymphoma, Hodgkin's lymphoma, pancreatic carcinoma, osteosarcoma, and malignant pleural mesothelioma [53].

Moreover, there are also tumors that have, additionally or alternatively, a deficient expression of ornithine transcarbamyl transferase (OCT) (Figure 3(d)), blocking the salvage pathway to ARG production from L-ornithine. It has been suggested for more than a decade that the lack of OTC expression was a typical feature of human epithelial cells of different organs of origin [54]. Later, Bobak and colleagues corroborated this hypothesis and reported negative OTC expression in several human cell lines, such as keratinocytic carcinoma A431, lung adenocarcinoma A549, HCC HepG2, breast adenocarcinoma MCF7, keratinocytes HaCaT, cervical carcinoma HeLa, ovarian carcinoma SKOV3, pancreatic carcinoma MIA PaCa-2, and melanomas MeWo, WM115, WM45 [55].

Deprivation of this amino acid is therefore investigated as a novel strategy for the treatment of these tumors [56–58].

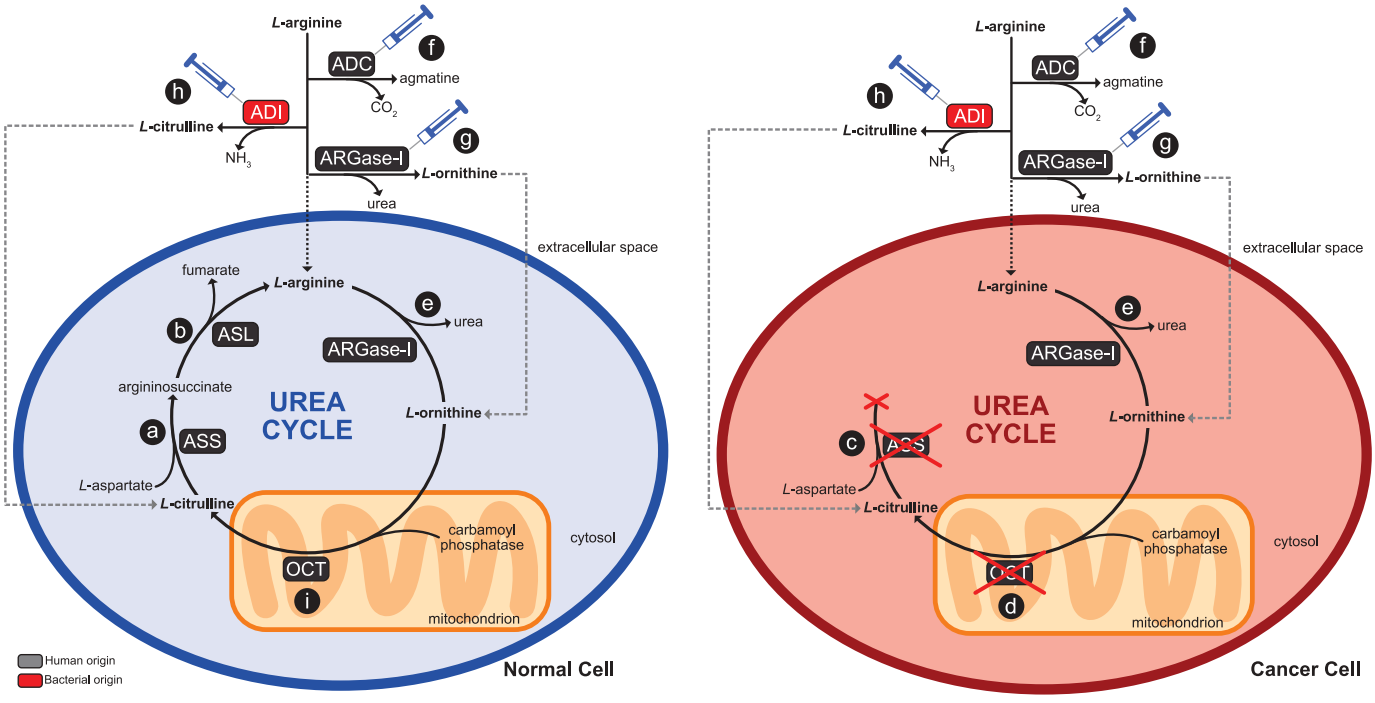

Figure 3. Schematic representation of the enzymes and metabolic pathways involved in ARG metabolism and degradation in normal cells in comparison with the genetically modified cancer cells. The enzymes with human origin are represented with a grey background while enzymes from bacterial sources are represented with red background. The enzymes engineered for amino acid depletion therapies are marked with a syringe. Full color available online.

The first step to implement an amino acid deprivation therapy is the choice of an enzyme that could metabolize ARG into another molecule, and at the same time, have optimal pharmacological characteristics and minimum side effects. ARG can be degraded by several mechanisms, either using enzymes available on the mammal cells, such as L-arginase (ARGase) (Figure 3(e)) or L-arginine decarboxylase (ADC) (Figure 3(f)), using human recombinant ARGase-I (Figure 3(g)), or using parasite–enzymes from a bacterial source such as ADI (Figure 3(h)) [59]. From these enzymes, ARGase-I and ADI are the most promising ones since they have therapeutic potential. ADC is relatively toxic to normal cells, and therefore, it is not suitable for therapeutic application [60].

ARG is also important in many viruses, where it has been shown to be essential for their replication [61–65]. The mechanism by which the ARG levels influence virus replication is still poorly understood and more studies are required [66–68]. However, several virus infections have been treated with ARG depletion therapies, using ARGase-I and ADI, with great success.

Despite the promising results in cancer and virus therapies, it must be noted that prolonged *in vivo* administration of arginine-depleting enzymes may impair NO synthesis by both constitutive and inducible nitric oxide synthase (NOS). Since NO participates in virtually every cellular and organ function in the body, it is the major endothelium-derived relaxing factor, a mediator of the immune response, a neurotransmitter, a cytotoxic free radical, and a widespread signaling molecule [69]. Its limitation may bring serious deleterious effects on the patient health.

2.2.1. Arginase

L-Arginase (EC 3.5.3.1, ARGase) is an enzyme that converts ARG into L-ornithine (Figure 4(a)). The isolation, purification, formulation, and production of a recombinant ARGase is described in the US 20100041101 A1 patent [70].

The use of this enzyme for amino acid depletion therapies in tumors (Figure 1: 1) or viral infections (Figure 1(i)) that have high demands of ARG would present many advantages. First, it is not immunogenic to humans and second, it has good serum stability. However, the overexpression of ARGase, increases considerably the concentration of L-ornithine in cells (product of the reaction of ARGase) (Figure 3(e)), which triggers a feedback mechanism in which of L-ornithine is converted back into L-citrulline by OCT (Figure 3(i)) and then recycle back to ARG by ASS/ASL (Figure 1(a,b)). This amino acid homeostatic mechanism keeps the various amino acid levels at constant ranges, and it is the main reason why normal cells and some tumors are resistant to therapies involving ARGase [71].

2.2.1.1. Anticancer.

The use of ARGase in the treatment of breast, rectal, and colon cancers has been shown with great success in *in vitro* studies; however, *in vivo* attempts were not always successful [56,72–75].

In order to improve ARGase activity *in vivo*, a pegylated variant of the enzyme was developed (US 8679479 B2 and US 20150010522 A1 patents) [76,77]. Other attempts to improve ARGase activity involved the mutation of a single amino acid in the enzyme structure as described in the US 20140242060 A1 patent [78]. However, the *in vivo* results indicate that no significant differences in activity of ARGase were obtained.

The amino acid homeostatic feedback mechanism is perhaps the main reason that limits the applicability of ARGase in the treatment of tumors in *in vivo* conditions. Several ways were developed in order to overcome this limitation. The US 6261557 B1 patent [79] describes a therapeutic composition in which ARGase is used combined with protein breakdown inhibitors, such as insulin, in order to prevent the muscles of the body from replenishing the depleted ARG. In the US 20080292609 A1 patent [80], it is described a combination therapy where the ARG depletion is achieved by a pegylated-ARGase administration together with an antineoplastic compound such as 5-fluorouracil or an alkylating agent [80,81]. In the WO 2014001956 A2 patent, a synergic therapy is described in which a recombinant ARGase with a mammalian target of rapamycin inhibitor, an alkylating agent, a mitotic inhibitor, or a small molecular inhibitor of tyrosine protein kinases and Raf kinases are used. The results of this combinatory therapy showed an interesting efficiency to treat liver and prostate cancers [82]. The patent CN 103402537 A describes the combination of a recombinant and pegylated-ARGase-I with doxorubicin in the treatment of patients suffering from lymphocytic leukemia and/or myeloid leukemia with great success [83].

A composition comprising an isolated human ARGase-I and a nonnative metal cofactor, wherein the nonnative metal cofactor is Co^{2+} was also patented [78]. The authors claimed that the substitution of the Mn^{2+} cofactor with Co^{2+} resulted in marked increase in catalytic activity and a drastic reduction in K_m at physiological pH.

The same author publicized a study showing that the substitution of the Mn(II) metal center in h-ARGase-I by Co(II) (Co-r-h-ARGase-I) results in an enzyme that displays 10-fold higher catalytic efficiency for ARG hydrolysis and a 12–15-fold reduction in the IC50 toward a variety of malignant cell lines, including melanomas and HCCs *in vitro* assays [84]. They also demonstrated that the modification of Co-r-h-ARGase-I with PEG-5K esters has shown an increase retention of the enzyme in circulation by about two orders of magnitude. The use of PEG(5K)-Co-h-ARGase-I was later on found to be effective in the treatment of hepatocellular and pancreatic carcinomas in xenograft models [85].

2.2.1.2. Antiviral.

ARG deprivation is also pointed as a therapeutic approach to treat infections, namely ocular lesions, hepatitis, and other inflammatory diseases.

In 2014, the eye lesions and/or infections gained a new type of treatment by the ocular administration of a pegylated-ARGase-I formulation in order to attenuate inflammation and neovascularization (US 8877183 B2 and WO 2014003850 A2). This treatment shows a good alternative to treat an eye that has been infected by a drug-resistant infectious agent [86,87]. Moreover, it can also be applied to an ocular trauma resulting from a medical procedure or from an accidentally caused injury (US 20130344049 A1) [86]. The attenuation of the inflammation is a consequence of the decrease of the NO production, which is a product of the NOS that uses ARG as

substrate. Consequently, if the ARG availability decreases, a lower amount of NO is produced, and the inflammatory process is attenuated (Figure 1(j)) [88].

The ARG-degrading enzyme, ARGase-I, can also be used to treat hepatitis B when it is used in its pegylated form, according to the patent WO 2006026915 A1. Actually, ARGase can be used in this type of treatment in the pegylated form in order to increase their serum half-life time up to 3 days. The pharmacological formulation could include a solution, a solid, an emulsion, a micelle, a dispersion, or a liposome, suitable for oral use or injection [89]. The usage of ARGase-I to treat hepatitis happens through a double-role mediated by the inhibition of the NO production similarly to the previous case. On one hand, the decrease of the NO levels attenuates the inflammation (Figure 1(j)), and on the other hand, the viral replication (Figure 1(i)) is also inhibited because viral replication requires NO to be successfully accomplished.

A worldwide patent WO 2015165374 A1 also describes an invention wherein a purified human pegylated-ARGase-I or a functional fragment is used in inflammatory diseases treatment, such as rheumatoid arthritis or multiple sclerosis. The procedure starts with an intravenous administration of the pharmacological formulation containing recombinant ARGase-I leading to a decrease of the ARG levels. The treatment can initiate an inhibition of T-cell polarization as well as modulation of cytokine release, namely IL-6 and IFN-γ. Furthermore, this treatment can also be taken by patients under immunosuppression in cases of cell, tissue or organ transplant. Osteoporosis associated with an osteoclast dysfunction was also reported as a healthy condition wherein the ARGase administration can be used as a treatment [90].

2.2.2. Arginine deiminase

ADI (EC 3.5.3.6) (Figure 4(b)) is an enzyme widely distributed among prokaryotic organisms and some anaerobic eukaryotes, which use it to convert ARG to L-citrulline and ammonia [91]. L-Citrulline is further degraded, forming ATP (adenosine triphosphate), carbon dioxide, and L-ornithine [92]. The so-called ADI pathway is used to generate energy, carbon, and nitrogen and also to protect some bacteria from acidic conditions, through the production of ammonia [93].

2.2.2.1. Anticancer.

The interest in ADI for therapeutic purposes began in the 90s when Miyazaki et al. observed a decrease in tumor development in Rous sarcoma virus-transformed buffalo–rat liver cells. They identified the mycoplasmal ADI as the growth inhibitory activity agent and suggested the possibility of its chemotherapeutic use for human cancers that have high demands of ARG such as hepatoma, cervix squamous cell carcinoma, and melanoma [94].

Soon after the identification of the ADI potential, a mycoplasmal proteinase K-resistant ADI, presenting optimum pH at physiological conditions and high stability, was developed and patented (US5372942 A). When applied to leukemia cell lines *in vitro*, this enzyme is 100% toxic and does not present any signs of toxicity to mice when administered intraperitoneally at much higher doses [95]. From there on, several studies, including the patent US5474928 A, confirmed the potential of ADI purified from *Mycoplasma arginine* (Figure 1(f)) as an antitumor agent in different types of human cancers, such as hepatoma, malignant fibrosarcoma, squamous cell carcinoma, malignant melanoma, nasopharyngeal carcinoma, and lung carcinoma [96–98].

Some years later, it was also found that beyond the antitumor activity, ADI has anti-angiogenic activity via suppression of NO generation [99–101]. In line with these studies, ADI was patented (US5474928 A) to be used as an active component of a pharmaceutical composition to inhibit angiogenesis. In that patent, the inventors use a pharmaceutical composition comprising ADI obtained from *M. arginini* or prepared by a genetic recombination technique, with an activated polymer to lower its immunogenicity and increase its life time [102].

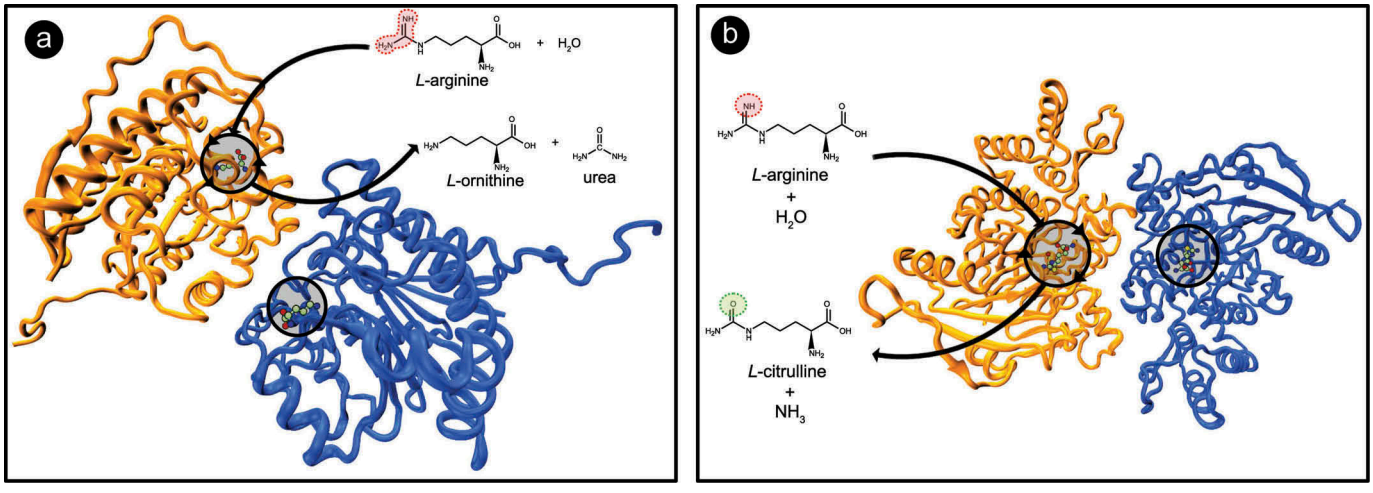

Figure 4. (a) New Cartoon representation of the co-crystalized structure of the human arginase I with L-ornithine (PDB code: 3GMZ). The two subunits were represented with different colors (orange and blue). In the close-up of the active site, it is shown, using the ball and sticks representation, the product L-ornithine. The reaction catalyzed by each active site is also represented with a highlight for the chemical group under catalytic attack. (b) New Cartoon representation of the co-crystalized structure of arginine deiminase from Mycoplasma arginini with a tetrahedral reaction intermediate (PDB Code: 1S9R).The four subunits were represented with different colors (cyan, orange, blue, yellow). In the close-up of the active site, it is shown, using the ball and sticks representation, the enzyme substrate, ASN. The reaction catalyzed by each active site is also represented with a highlight for the chemical group under catalytic attack. Full color available online.

The cooperative antiproliferative and antiangiogenic activities of ADI from *M. arginini* are believed today, to be the key assets that turn ADI very effective in the treatment of several tumors and other diseases. To date, cancers that present a sub-expression of ASS (Figure 3(c)) have already been shown to be sensitive to ARG deprivation via ADI, such as melanoma, lung cancer, renal cell carcinomas, and HCC [103].

In spite of the promising results, the application of ADI in the treatment of several cancers presents some limitations. The therapeutic efficiency of ADI is limited to tumors that do not express ASS (Figure 3(c)) and/or have an inactivated L-citrulline to ARG recycling pathway [104,105]. In addition, ADI has a short serum half-life and it is also highly immunogenic when used in prolonged treatments. In order to reduce this effect, several solutions were patented (US5372942 A; US5804183 A; US5916793 A; US6132713 A) proposing the attachment of ADI trough a covalent bond to a polyethylene glycol (pegylated-ADI) or similar water-soluble polymers [95,106–108]. In patent US6183738 B1, it was shown that pegylated-ADI is far less immunogenic, and has a greatly extended circulating half-life, and it is much more efficacious in the treatment of tumors [109]. Particular success has been obtained with the PEG(20)-ADI (molecular weight: 20 kDa) that has a remarkable efficiency *in vivo* assays with melanomas and HCC, comparing with native ADI [104,105,110–116]. The only limitation of ADI pegylated-modification is a partial decline in the activity of the modified proteins in relation to the native one, apparently because polyethylene glycol (PEG) attaches to sites on the enzyme, which interfere with the reaction that is catalyzed by the enzyme. Another problem is the formation of non-homogenous products during the pegylation process due to the random attachment of PEG on the protein surface. This makes the enzyme difficult to characterize and perform quality control during the manufacturing process. In order to overcome these issues, several modifications to pegylated-ADI have been proposed. First, in US6635462 B1 patent, it was proposed that one or more amino acid mutations in ADI structure could facilitate both the renaturation and formulation (i.e. pegylation) of the enzyme, thereby improving its manufacturing processes while retaining the ability to convert ARG to L-citrulline. These modified pegylated-ADI provided excellent results in the treatment of certain types of cancer, inhibiting the metastasis of cancer, and treating other disease states [117]. Good results have also been published in patent US8663967 B2 with the mutation of lysine residues present on the protein surface. The authors of this invention claim that when compared with pegylated-ADI, the modified pegylated-ADI has better bioactivity. Since the quantity of lysine in ADI is reduced, the PEG-modified products are more uniform and can be applied to the clinical treatment of various cancers such as leukemia, pancreatic, head and neck, colorectal, lung, breast, liver, nasopharyngeal, esophageal, prostate, stomach, and brain cancers [118].

Some studies have also shown that after the intravenous injection of pegylated-ADI *in vivo*, leakage or detachment of free PEG is observed and the ADI (without PEG) can elicit the immunogenicity problem. In an attempt to overcome this situation, in patent US9255262 B2, it is proposed the fusion of albumin to the ADI protein. This does not appear to influence the catalytic activity of the enzyme and seems to increase the circulating half-life, and at the same time, decrease the immunogenicity [119].

To date, the therapeutic efficacy of PEG(20)-ADI has already been validated through phase I/II clinical trials in advanced HCC [120–122] and melanoma patients [112,123,124]. Additionally, there are several ongoing phase I and/or II clinical trials using PEG20-ADI in patients with other tumors such as advanced non-Hodgkin's lymphoma, acute myeloid leukaemia, malignant pleural mesothelioma, prostate and non-small cell lung, advanced gastrointestinal, HER2 (Human Epidermal growth factor Receptor 2) negative metastatic breast, advanced pancreatic, and small-cell lung cancers.

2.2.2.2. Antiviral. In spite of some controversy, it is known that the elimination of extracellular ARG will inhibit the production of NO (Figure 1(i,j)), whose absence has been shown to protect mammals from the lethal effects of several viri such as influenza [125,126], polio virus [127], rabies virus [128], and flavivirus [129].

In this context, in patent US7204980 B2, it is shown that the administration of a therapeutically or prophylactically effective amount of a composition comprising modified pegylated-ADI inhibits Hepatitis C virus replication *in vitro*. Contrary to other existing inhibitors that preclude NO production and are toxic to animal and humans, ADI-PEG 20 is safer and more effective to inhibit NO production and can be used to gather a better role of this biomediator in protection against viral infections [130].

2.3. Asparaginase

ASN is an important amino acid that is involved in the synthesis of proteins. It also plays an important role in the biosynthesis of glycoproteins where it provides key sites for N-linked glycosylation and in the metabolic control of many cell functions in nerve and brain tissue.

ASN is a nonessential amino acid in humans, since it can be synthesized endogenously. The precursor in the biosynthesis of ASN is oxaloacetate, which reacts with L-glutamate to form L-aspartate and α-ketoglutarate. In the presence of a transaminase enzyme, L-aspartate then reacts with L-glutamine to form ASN in an ATP-dependent reaction that is catalyzed by ASN synthetase (EC 6.3.5.4, ASNS).

Certain types of tumors, such as leukemic cells, cannot synthesize ASN because they lack or have a deficient expression of the enzyme ASNS (Figure 1(a)). This means that the proliferation and survival of these tumor cells are dependent on the external supplies of this amino acid (Figure 1(b)). As normal cells can synthesize ASN (Figure 1(c)), this means that ASN deprivation therapies would mainly interfere with tumor cells that are ASN deficient.

L-Asparaginase (EC 3.5.1.1, ASNase) (Figure 5) is a relatively wide-spread enzyme found in many microorganisms (mostly in bacteria) that has been used with great success in leukemia treatment regimens since 1950. This enzyme performs the opposite reaction of ASNS, hydrolyzing ASN to L-aspartic acid

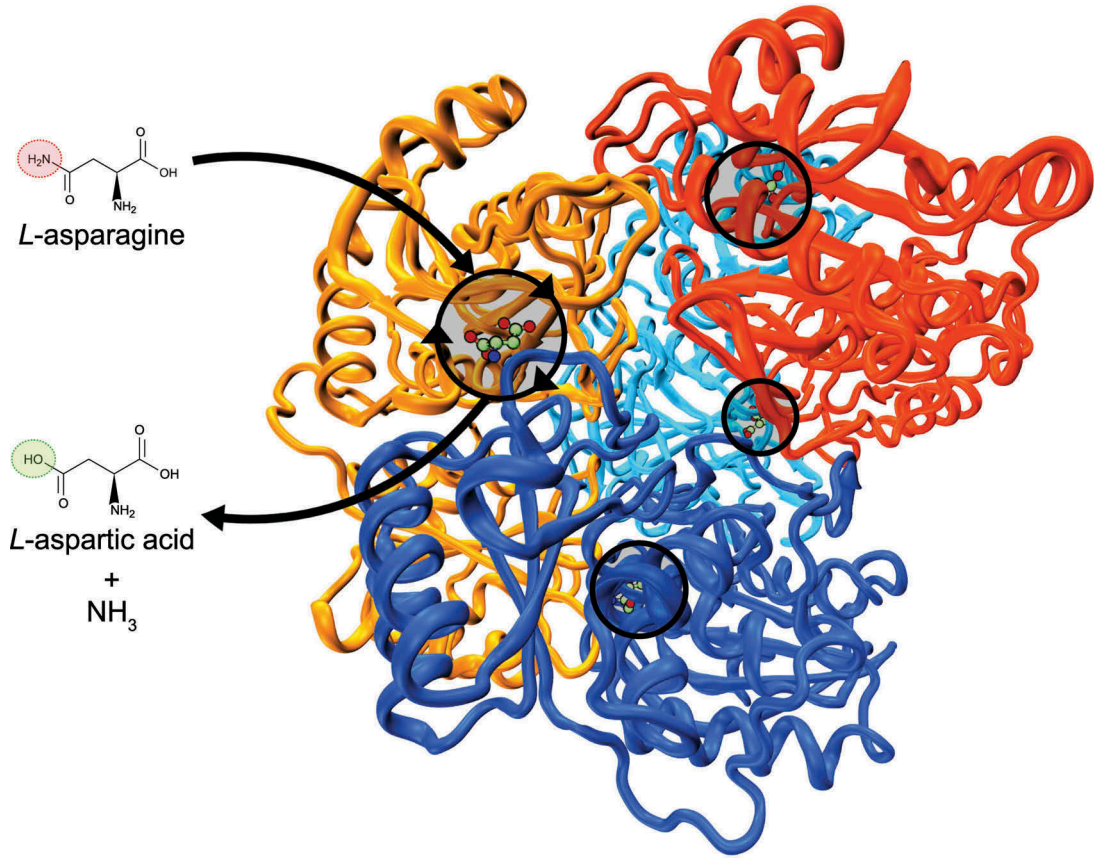

Figure 5. New Cartoon representation of the co-crystalized structure of asparaginase II from Escherichia coli with L-asparagine (PDB code: 3ECA). The two subunits were represented with different colors (orange and blue). In the close-up of the active site it is shown, using the ball and sticks representation, the product L-aspartic acid. The reaction catalyzed by each active site is also represented with a highlight for the chemical group under catalytic attack. Full color available online.

and ammonia [131]. By reducing the levels of plasmatic ASN (Figure 1(g)), ASNase starves leukemic cells and promotes tumor cell apoptosis. ASNase has also been proposed for the treatment of NK tumors, subtypes of myeloid leukemia, and T-cell lymphomas respond to ASNase, ovarian carcinomas, and other solid tumors [132]. ASNase has also been proposed in the treatment of AIDS.

2.3.1. Anticancer

ASNases derived from *E. coli* (Ecoli-ASNase) and *Erwinia chrysanthemi* (Echry-ASNase) are the ones that are commonly used in cancer treatments. For a large number of years, only the native Ecoli-ASNase (Elspar®, Lundbeck Inc.) was commercially available. This happened because this enzyme could be easily produced in large amounts as described in patents US3597323 A [133] and US3511755A [134], and efficiently used in the treatment of ALL. However, some patients showed hypersensitivity reactions and other toxicities (e.g. hepatic, renal, splenic, pancreatic dysfunction, and blood coagulation), which limited the application of Elspar® [135]. In 2006, Food and Drug Administration (FDA) approved the use of a pegylated form of ASNase from *E. coli* (Ecoli-PEG-ASNase), manufactured by Enzon Pharmaceuticals, Inc. (Oncaspar®) for the first-line treatment of patients with ALL. This new form of the ASNase (Oncaspar®) is much more stable and more efficient than Elspar®, requiring only a single dose to obtain a similar antileukemic activity of Elspar® that requires 6–9 doses of native Ecoli-ASNase.

Echry-ASNase is often used in the event of hypersensitivity to Ecoli-ASNase in the pegylated or not pegylated forms [136]. It is therefore used as a second- or third-line treatment protocol. In 2011, the FDA approved the usage of Echry-ASNase (Erwinaze, EUSA Pharma) as a component of a multi-agent chemotherapeutic regimen for the treatment of patients with ALL who have developed hypersensitivity to Ecoli-ASNase. A pegylated form of Echry-PEG-ASNase has also been claimed and it is described in patent WO 2011003886 A1 [137].

A number of comparative clinical studies have been conducted with Echry-ASNase and Ecoli-ASNase [138,139]. The dose and schedules of ASNase in these studies have been inconsistent and outcomes have been variable. However, the efficacy of Erwinia asparaginase following hypersensitivity to *E. coli*-asparaginase preparations has been well demonstrated [140]. The Echry-PEG-ASNase is currently under preclinical studies, and so far, it is showing promising results, similar to the ones obtained with Ecoli-PEG-ASNase but with less immunogenic effects [141,142].

In spite of the advantages promoted by the use of EColi-PEG-ASNase and Echry-ASNase, some patients continue to present adverse side effects, such as acute pancreatitis, thrombotic complications, and immunosuppression. This happens

because the main therapeutic effect of some forms of recombinant ASNases is also accompanied by glutamine deamination [143]. In an attempt to overcome this issue, new ASNases are currently being developed. The idea is to mutate some amino acids on the active site of ASNase in order to turn the enzyme more thermostable and specific to the substrate and, at the same time, preclude the reverse reaction that involves the conversion of L-glutamine into ASN [144,145]. One of such examples is described in the US 20130330316 A1 patent [146] where several mutations on the three-dimensional structure ASN isolated from *Pyrococcus furiosus* are conducted. Some of the mutated enzymes present high thermostability, pH stability, and no glutaminase activity that is important either for therapeutics or also for the process of preparing the same. These mutated enzymes also show high cytotoxicity on the leukemic cell lines making them promising candidates to help the treatment of leukemia [147].

2.3.2. Antiviral

The use of an EColi-PEG-ASN has also been proposed to inhibit or treat human immunodeficiency virus (HIV) infection. The patent WO 1999039732 A1 [148] describes a new pharmaceutically composition comprising a PEG-ASNase compound and optionally at least one compound selected from the group consisting of a protease inhibitor, a ribonucleotide reductase inhibitor compounds and an HIV reverse transcriptase inhibitor.

3. Conclusions

A prerequisite for making an effective medication for the treatment of cancer is that some fundamental difference between normal cells and cancer cells must be defined. An optimal chemotherapeutic agent must thus exploit this cellular difference in such a way that normal cells are spared and only cancer cells are injured. In this context, the amino acid depriving enzymes are promising anticancer drugs that have proven to be active and very specific against various types of cancers. Their mode of action is simple: they decrease the concentration of certain amino acids in the bloodstream and thus impair the development or even destroy tumor cells that are auxotrophic for those amino acids. Normal cells remain unaltered since they are less demanding and/or can synthesize these compounds in sufficient amounts by other mechanisms.

ASNase is probably the best known example of amino acid depletion chemotherapies using enzymes. The application of this enzyme in the treatment of childhood ALL has already been approved by FDA and shows very good results. ARG deprivation by ADI is a novel approach to target tumors that lack ASS expression. ADI showed promising results during initial clinical trials for the therapy of HCC and melanomas [149]. Some studies also suggest that ADI has potential to become a better therapeutic agent for the treatment of leukemia than ASNase [150,151].

Currently, the major drawback of ADI and ASNase when used for therapeutic purposes is their heterologous origin. As these enzymes are derived from a microorganism, they are immunogenic in humans and have a very short circulating half-life. The chemotherapeutic treatments using these enzymes thus require multiple injections to produce the desired effect and from which results in a variety of adverse effects including hypersensitivity reactions, anaphylactic shock, and the inactivation and clearance of the enzyme itself [152]. In order to overcome these problems and allow a safer application in cancer treatments, these enzymes have been linked to PEG to improve the pharmacokinetic and pharmacodynamic properties. The therapeutic results of these modified enzymes are much safer and have prompted the use of ADI in many clinical trials.

METase is another heterologous enzyme discussed in this review. Similarly to ASNase and ADI, this enzyme presents several immunogenic effects, but contrarily to those enzymes, the pegylated form does not seem to overcome this problem. Several attempts were made to decrease the toxicity of this enzyme by the coadministration of pyridoxal-5-phosphate and oleic acid or dithiothreitol [153,154]. However, several side effects persist and its utilization in cancer therapies continues to be inappropriate [155,156].

From the discussed enzymes, ARGase is the only enzyme that is from human origin. This means that it presents low immunogenicity, a great advantage over ADI in the treatment of cancer that is auxotrophic for ARG. Indeed, several authors consider ARGase a better chemotherapeutic agent for cancer treatment than ADI because, apart from its low immunogenicity, this enzyme is effective in tumors that are OCT-deficient and it is independent of ASS gene expression. Since most tumors are OCT-deficient, and ADI is ineffective in ASS-positive tumors, ARGase has a clear advantage over ADI to be used in therapeutic. Its major disadvantage when compared with ADI is its high K_m and high-optimum pH, which requires large quantities of enzyme to be included in the treatment in order to become effective. Actually this problem may be overcome by the utilization of cobalt-substituted ARGase, since the replacement of the two manganese ions at the active site by cobalt (II) leads to a 10-fold increase in enzymatic activity under biological conditions [84].

Despite the problems arising from the use of amino acid depleting, the recent progresses made in this field of research will undoubtedly facilitate the pursuit of new treatments to combat cancers and virus infections. ASNase was the first enzyme approved by FDA for the chemotherapeutic treatment of leukemia with remarkable success. Taking into account the on-going clinical trials made with other enzymes such as, ARGase or ADI, and the promising results that are being obtained, one might expect that the clinical approval of these enzymes should not be too distant in the future and will have undoubtedly a profound influence on human health.

4. Expert opinion

Cancer therapy has long relied on the rapid proliferation of tumor cells for an effective treatment. However, the lack of specificity in this approach often leads to undesirable side effects. Many reports have described various 'metabolic transformation' events that enable cancer cells to survive, suggesting that metabolic pathways might be good targets.

The amino acid depletion therapies using enzymes uses the uncommon metabolism of certain types of cancer that are auxotrophy for a certain amino acid or have a metabolic

defect and cannot produce it. This metabolic vulnerability has been used with great success to prevent the development or even the survival of some types of cancer. At the same time, the normal cells are spared because they are less demanding and/or can synthesize these compounds in sufficient amounts for their needs by other mechanisms.

The benefits of enzymatic therapies in amino acid depletion are well established in the literature and FDA has already approved the use of different ASNases in antileukemic treatments. Based on the ASNase paradigm, several ARG catabolizing enzymes have also been developed, including mycoplasma-derived ADI and human recombinant ARGase, for cancer therapy. Although the data are preliminary, early signs suggest that ARG deprivation has the potential to make an impact across a broad range of human malignancies. METase is another enzyme that is being efficiently employed in tumors that are autotrophic for methionine.

In spite of the success of amino acid depletion therapies using enzymes in auxotrophy tumors, they present many side effects, such as high immunogenic effects and other toxicities that preclude a generalized administration to patients. This is particularly evident in the treatments of tumors employing METase, ARGase, and ADI, but also in certain cases when ASNase is used.

Several modifications to these enzymes have been conducted aiming to decrease these undesirable effects, such as the expression of these enzymes from other organisms, recombination or pegylation processes, or even by specific mutations in the active site of the enzymes. In spite of the recent developments in the field, further drug optimizations are still needed to gain traction in the clinic. This is particularly true in the case of the enzymes are used to decrease the plasma levels of ARG and MET.

It is our opinion that the current limitations on the amino acid depletion therapies lie in the insufficient understanding about the pharmacokinetic and pharmacodynamic effects of these enzymes when they are applied to patients. Nowadays, this problem has been partially solved by the conjugation of PEG to the enzyme. However, while this approach can delay the formation of antibodies to the enzyme, it does not completely eliminate the eventual production of neutralizing antibodies, and it compromises enzyme activity. Furthermore, a precisely defined and reproducible conjugation of PEG to proteins is a laborious and expensive process. An alternative solution has been recently proposed through the use of fusion proteins containing albumin-binding proteins but more studies are required to test its effectiveness.

Despite all of these limitations of the amino acid therapies using enzymes, these treatments continue to be very attractive because it is less aggressive and more innocuous (apart of some immunogenicity associated with the enzymes that are not from human origin) when compared with other anticancer therapies. In addition, the promising results that are being obtained with the use of the same type of therapies in the treatment of several viral infections may turn them into the next antiviral generation of medications.

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Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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