CHAPTER

CANCER THERAPIES BASED ON ENZYMATIC AMINO ACID DEPLETION

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CHAPTER OUTLINE

1 INTRODUCTION

Cancer has become the leading cause of death in the developed world and has remained one of the most difficult diseases to treat. During the past 5 decades, cancer therapy has relied mainly on low molecular weight molecules. However, this class of compounds, very potent in their action, unfortunately lacks tumor specificity and often has unacceptable toxicities toward normal cells. All of these factors are often followed by side effects, which often undermine the effectiveness of the therapeutic treatment in patients (Cantor et al., 2012b). For this reason, the demand for new and improved therapeutics against cancer remains high, and at the same time a deeper knowledge regarding the development of tumors is required.

The development of tumor cells is still not fully understood, but many advances have been made in this area. 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 spread. 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. 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 remain unchanged because they are less demanding or can synthesize these compounds in sufficient amounts by alternative pathways.

These observations are the basis of the amino acid deprivation cancer therapy that involves the systemic depletion of tumor-essential amino acid. This knowledge is not new and was initially suggested

over 50 years ago, but only now is it gaining popularity with the advances in protein engineering technology and in more sophisticated approaches that enable the study of genetic and metabolic differences between tumor and normal cells (Cantor et al., 2012a).

In these tumors, diet is not enough to achieve a therapeutically relevant level for amino acid depletion, and therefore enzymes are used instead. Regrettably, the human genome does not encode enzymes with the pharmacological or catalytic requisites that are essential for the pretended therapeutic purpose. For this reason heterologous enzymes (from other organisms), recombinant, and/or engineered human enzymes are used both in animal studies and in clinical trials. Currently the enzymes of bacterial origin are the most frequently used.

The main function of heterologous or engineered human enzymes used in amino acid depletion therapies is to decrease the concentration of a certain amino acid in the bloodstream (e.g., converting it into another molecule) that is essential for the development of a tumor. Some examples include a large fraction of hepatocellular carcinomas (HCC) and metastatic melanomas that become apoptotic under conditions where the nonessential amino acid L-arginine (L-ARG) in serum is depleted (Ni et al., 2008), central nervous system cancers that respond to L-methionine (L-MET) deprivation (Tan et al., 2010), and acute lymphoblastic leukemia (ALL) for which depletion of L-asparagine (L-ASN) has been shown to significantly reduce cancer growth (Rytting, 2010).

In this review, the structure, function, and catalytic mechanism of therapeutic heterologous enzymes involved in L-ARG, L-MET, and L-ASP degradation will be revised. Particular attention will be given to the heterologous enzymes L-arginase, L-arginine deiminase, L-methioninase, and L-asparaginase, taking into account their current therapeutic potential.

2 AMINO ACID DEPRIVATION ENZYMES

At least 20 amino acids have been identified in cells and among these are 9 that the human body cannot manufacture de novo (from scratch), and consequently these must be obtained from the diet. These amino acids are normally denominated as essential and include L-phenylalanine, L-valine, L-threonine, L-tryptophan, L-methionine, 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 but their synthesis can be 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 in humans (also denoted as nonessential), meaning they can be synthesized in the body, for example, L-aspartic acid, L-asparagine, L-glutamic acid, L-alanine, and L-serine.

The optimal conditions for amino acid depletion therapies occur when 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 because they can synthesize these compounds in sufficient amounts by their own means. The most notable example of target enzymatic amino acid depletion in cancer therapy is illustrated by the remarkable success of L-asparaginase in the treatment of childhood ALL and, to a lesser extent, in non-Hodgkins lymphoma. Other examples involve the use of arginine deiminase in the 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 in tumors where the normal metabolism of

one essential amino acid is disrupted or when there is some sort of defect in the ability to use a certain amino acid. For example, this is the case of L-methionine, which has been shown to be detrimental for 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 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 past 2 decades. These therapies have been showing promising results, but also some problems in 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 L-ASPARAGINE

L-ASN is a nonessential amino acid that is involved in the metabolic control of cell functions in nerve and brain tissue. This amino acid is important in the synthesis of a large number of proteins and plays an important role in the biosynthesis of glycoproteins where it provides key sites for *N*-linked glycosylation. ASN also plays an important role in the metabolism of ammonia, which is toxic in the human body.

Since L-asparagine is a nonessential amino acid, it can be easily synthesized by the cells. 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. The L-aspartate then reacts with L-glutamine to form L-asparagine in an ATP-dependent reaction that is catalyzed by L-asparagine synthetase (Richards and Kilberg, 2006).

Certain types of tumors, such as leukemic cells, cannot synthesize ASN because they lack or have deficient expression of the enzyme L-Asparaginase synthase. This means that the proliferation and survival of these tumor cells are dependent on the external supplies of this amino acid. As normal cells can synthesise ASN, this means that L-asparagine deprivation therapies would mainly interfere with tumor cells that are ASN deficient.

L-Asparaginase (ASNase) is a bacterial enzyme that has been used with great success in leukemia treatment regimens for 4 decades. This enzyme performs the opposite reaction of L-asparagine synthetase by hydrolyzing ASN to L-aspartic acid and ammonia. By reducing the levels of plasmatic L-asparagine, ASNase starves leukemic cells and promotes tumor cell apoptosis.

The therapeutic role of ASNase was first noted by Kidd (1953), who found that guinea pig serum had antitumor activity against two kinds of lymphoma in a murine model and lymphosarcoma in rats. This fact was later attributed to the enzyme ASNase that was present in the serum (Broome, 1961, 1963). Later on, in 1966, DeLowrey and coworkers using a purified guinea pig serum treated a boy with acute lymphoblastic leukemia, and obtained an objective response (Dolowy et al., 1966). This turned ASNase into a promising anticancer drug, and therefore large amounts of the enzyme were required to start new studies and large-scale clinical trials. However, this was not an easy task at that time since the isolation and purification of the enzyme was difficult and not all the ASNases possess antitumor activity.

L-Asparaginases (EC 3.5.1.1) is a relatively widespread enzyme, found in many microorganisms, such as *Aerobacter*, *Bacillus*, *Pseudomonas*, *Serratia*, *Xanthomonas*, *Photobacterium* (Peterson and Ciegler, 1969), *Streptomyces* (Dejong, 1972), *Proteus* (Tosa et al., 1971), *Vibrio* (Kafkewitz and

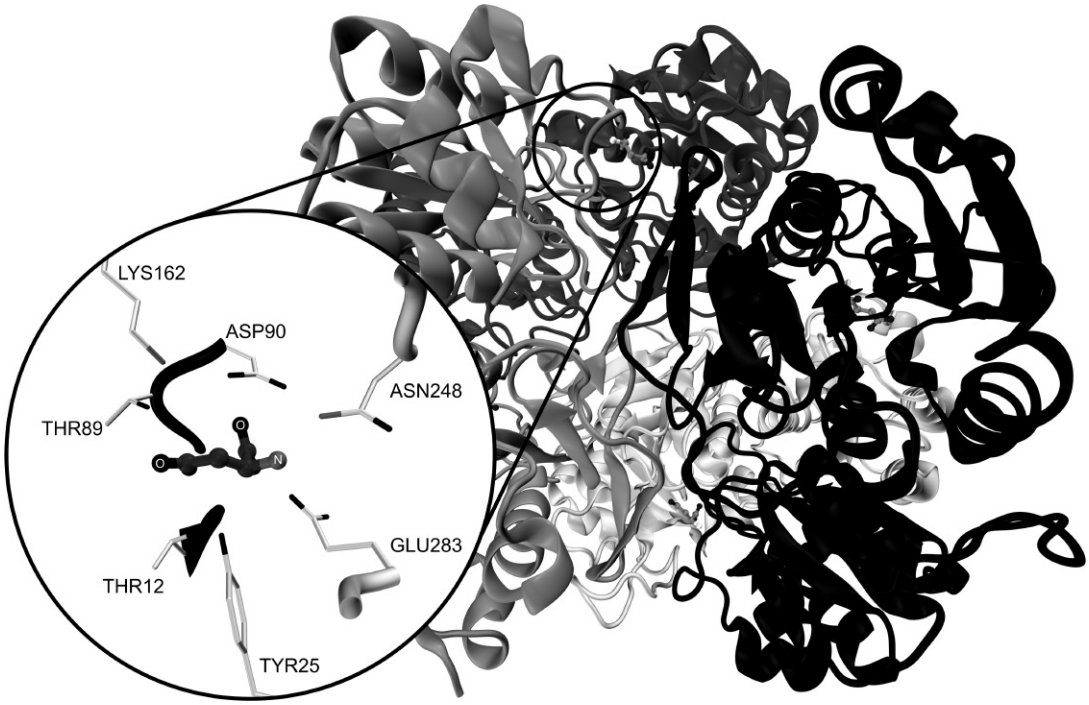

FIGURE 24.1 New Cartoon Representation of the Cocrystalized Ctructure of ASNase With L-Asparagine (PDB Code: 3ECA)

Each subunit is represented with a different black tonality and the substrate in ball and sticks and colored by CPK. In the close-up of the active site the residues of the active site that are important for the catalytic process, as well as the substrate are represented in licorice.

Goodman, 1974), and *Aspergillus* (Sarquis et al., 2004). After intense research ASNases derived from *Escherichia coli* (Ecoli-ASNase) and *Erwinia chrysanthemi* (Echry-ASNase) turned out to have the best anticancer capabilities. Ecoli-ASNase has thereby become the main source of ASNase because it is easy to produce in large amounts. Echry-ASNase is typically reserved for cases of Ecoli-ASNase hypersensitivity.

The *E. coli* expresses two ASNases denoted as Ecoli-ASNase-I and Ecoli-ASNase-II that are found in different intra- or extracellular localizations. Ecoli-ASNase-I (cytosolic) has a lower affinity for ASN, while Ecoli-ASNase-II (periplasmic) has a high ASN affinity. Due to the higher affinity of Ecoli-ASNase-II toward L-asparagine, this enzyme is the mostly studied enzyme and the one that is used for oncologic therapeutic purposes.

Ecoli-ASNase-II is a tetrameric protein that is composed of four identical subunits, each one containing 326 amino acid residues. The binding pocket of this enzyme involves residues from both subunits of each dimer, namely, THR12, TYR25, SER58, GLN59, THR89, ASP90, and LYS162 from one subunit (Fig. 24.1—black chain), and ASN248 and GLU283 from the other subunit (Fig. 24.1—light gray

chain). All of these residues are interconnected by a strong hydrogen bond network, including a water molecule that is structurally conserved. The position of the amino acid residues in the active site suggests several possible pathways for the catalytic reaction, but the almost symmetric location of two threonine residues, THR12 and THR89, observed in the X-ray structure 3ECA above and below carbon C2 of the substrate, suggests that one of them must be directly involved in the reaction (Fig. 24.1) (Swain et al., 1993).

The first step of the mechanism involves the formation of a tetrahedral intermediate (Fig. 24.2a) that results from the nucleophilic attack of the water molecule to α-carbon of the substrate. In the course of this reaction, LYS162 receives the proton from the water molecule and becomes positively charged. The second step (Step 2) of the reaction involves a concerted double proton transfer from LYS162 to THR89 and from THR89 to the substrate, from which results the formation of ammonia and aspartic acid. However, the ammonia molecule is still weakly bound to the substrate at this stage and its dissociation requires an additional step (Step 3). At the end of this process (Step 4), the enzyme is ready for the next turnover (Gesto et al., 2013).

Ecoli-ASNase-II and Echry-ASNase have been established as a general treatment for acute lymphoblastic leukemia for nearly 40 years. It is used for remission induction and intensification treatment in all pediatric regimens and in the majority of adult treatment protocols. Currently, it is estimated that these enzymes have contributed to significant improvements of therapy outcomes and to achieve complete remission of leukemia in about 90% of patients.

Notwithstanding its high therapeutic efficacy, the administration of Ecoli-ASNase-II for long periods produces acute allergic reactions and silent immunity, a condition that is characterized by circulating antibodies and rapid clearance of the enzyme from the blood (Avramis et al., 2002; Henriksen et al., 2015; Woo et al., 1998). In order to overcome this problem, a modification of Ecoli-ASNase-II with 5-kDa units of monomethoxypolyethylene glycol was introduced (PEG-Ecoli-ASNase-II) (Rytting, 2010). The modified enzyme preserves enzyme activity, but decreases immunogenicity of the protein and extends elimination half-life (5 times longer than native enzyme) (Keating et al., 1993).

Currently, three L-asparaginase agents are available. These are either derived from *E. coli* in its native form (Ecoli-ASNase-II) or as a pegylated enzyme (PEG-Ecoli-ASNase-II), or extracted from *Erwinia chrysanthemi* (Echry-ASNase). Due to the unique anticancer mechanism of action, PEG-Ecoli-ASNase-II and Echry-ASNase have already been approved by US Food and Drug Administration (FDA) for the treatment of several cancers, such as acute lymphoblastic leukemia (Hill et al., 1967), lymphosarcoma (Story et al., 1993), and a few subtypes of non-Hodgkin's lymphoma (Kobrinsky et al., 2001). Ovarian carcinomas and other solid tumors have also been proposed as additional targets for ASNase, with a potential role for its glutaminase activity (Covini et al., 2012).

2.2 L-ARGININE

L-ARG is a precursor/mediator of a series of biological pathways, some of them involved in important cellular functions, such as in nitrogen metabolism (Rogers and Visek, 1985), creatine, agmatine, and polyamine synthesis (Klein and Morris, 1978). In addition, ARG is the major substrate for the production of nitric oxide (Billiar, 1995), stimulates anabolic hormone release, and is immunostimulatory and thymotrophic (Barbul et al., 1977, 1983).

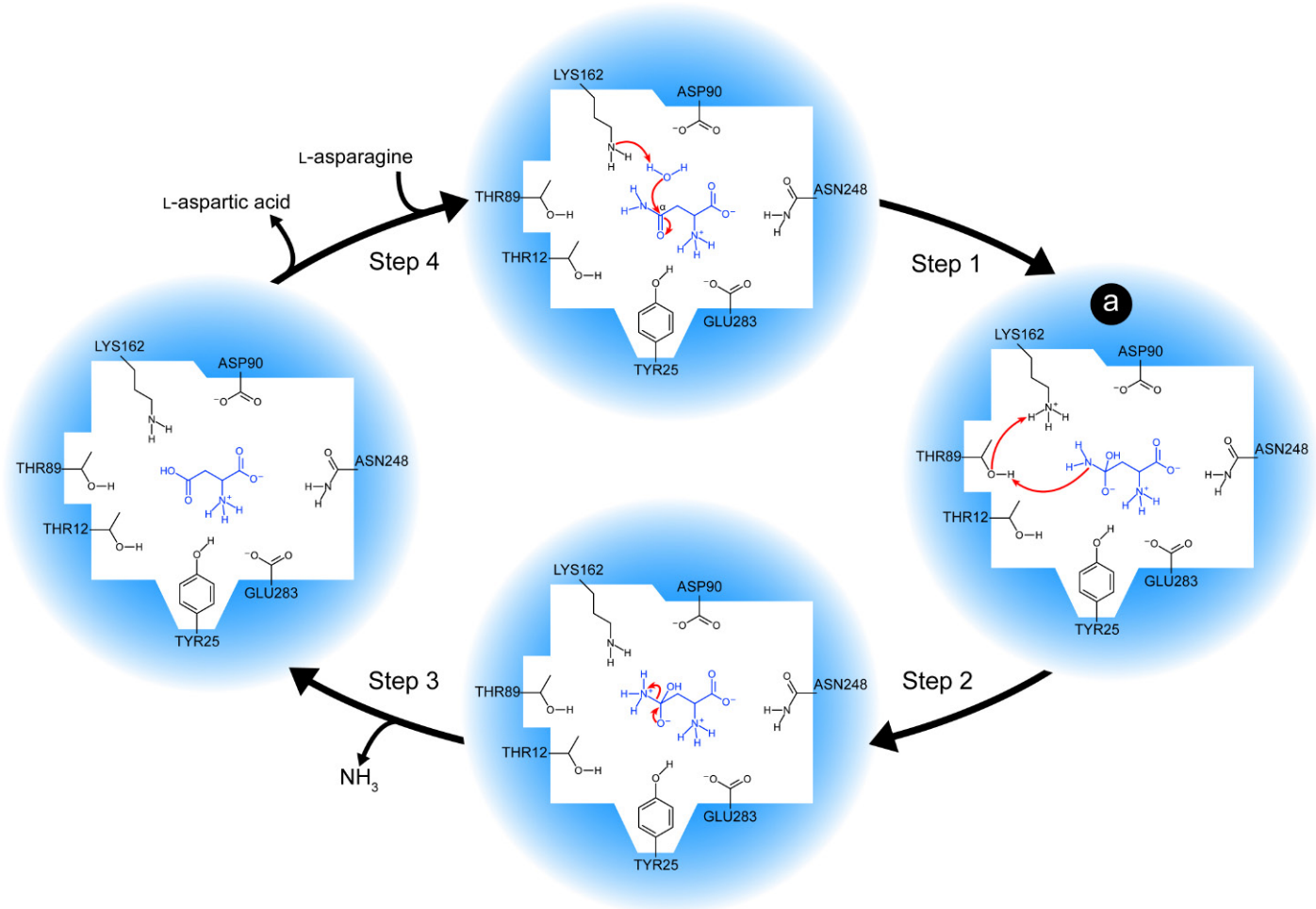

FIGURE 24.2 Schematic Representation of the ASNase Catalytic Mechanism

Each circle represents a different reaction intermediate. The important reaction intermediates are labeled with labels (a).

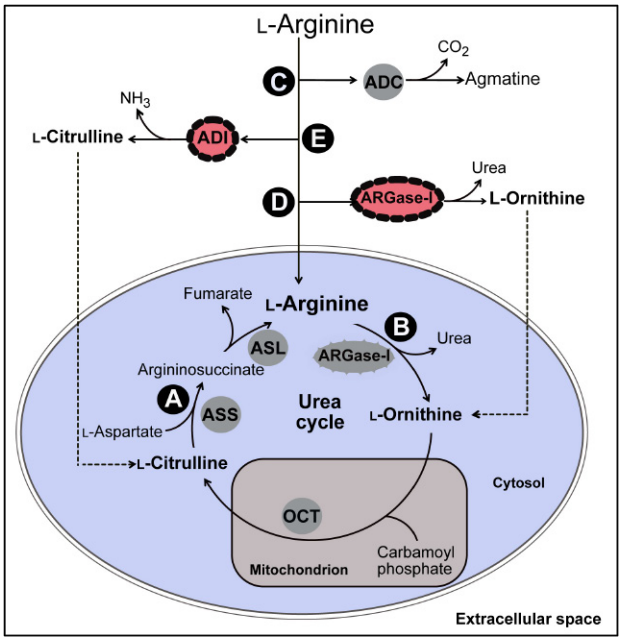

FIGURE 24.3 Schematic Representation of the Enzymes Involved in ARG Metabolism and Degradation

The enzymes available on the cell have grey background. Recombinant enzymes or from bacterial sources are represented with dashed outline.

The enzyme argininosuccinate synthetase (ASS) catalyzes the condensation of L-Citrulline and L-aspartic acid to argininosuccinate which is subsequently converted to ARG and fumaric acid by argininosuccinate lyase (ASL) (Fig. 24.3A) (Haines et al., 2011).

In healthy adults the level of endogenous synthesis is sufficient to make ARG a nonessential dietary amino acid (Castillo et al., 1993). However under catabolic stress (e.g., inflammation, infection, etc.), the levels of endogenous synthesis may not be sufficient to meet metabolic demands and ARG becomes an essential amino acid. For this reason, this amino acid is often denoted as a semiessential amino acid instead of nonessential (Reeds, 2000).

Certain tumor cells, such as metastatic melanoma, prostate carcinomas, HCC (Dillon et al., 2004), non-Hodgkin's lymphoma, Hodgkin's lymphoma, pancreatic carcinoma, osteosarcoma, and malignant pleural mesothelioma and some breast tumors (Qiu et al., 2015) have an elevated requirement for ARG due to the deficient expression of ASS. This causes ARG autotrophy and therefore these tumors rely only on exogenous sources of ARG for growth and proliferation. Deprivation of this amino acid is, for this reason, investigated as a novel strategy for cancer therapy and has been showing promising efficacy against the ARG-auxotrophic tumors mentioned earlier (Feun et al., 2015; Shen et al., 2003; Wheatley and Campbell, 2003).

ARG can be degraded by several mechanisms, either using enzymes available on the cell, such as L-arginase (ARGase) (Fig. 24.3B) or L-arginine decarboxylase (ADC) (Fig. 24.3C), using human recombinant ARGase-I (Fig. 24.3D), or using parasite-enzymes from a bacterial source, such as arginine

deiminase (ADI, Fig. 24.3E). From these enzymes, ARGase-I and ADI are the most promising ones since they have therapeutic potential. L-arginine decarboxylase is relatively toxic to normal cells and therefore it is not used for therapeutic purposes (Savaraj et al., 2010).

2.2.1 L-Arginase

L-ARG can be degraded directly to L-ornithine by ARGase (EC 3.5.3.1), an enzyme of the urea cycle (Fig. 24.3B). Thus the insertion of ARGase on the extracellular moiety could be used to decrease the levels of ARG in cells. However, under salvage conditions, higher concentration of L-ornithine (product of the reaction of ARGase) can be converted back to L-citrulline by ornithine transcarbamyl transferase and then recycle back to ARG by ASS/ASL (Fig. 24.3A). This feedback mechanism makes normal cells and many tumors resistant to ARGase inhibition. Fortunately, there are many tumors that present deficient expressions of OCT 0and/or ASS enzymes and in these cases ARGase can be efficiently used to decrease ARG intracellular levels and therefore tumor growth (Cheng et al., 2007).

ARGase is found in organisms from the five kingdoms (Reczkowski and Ash, 1992). In most mammals, two isoforms of this enzyme exist and are denoted as types I and II. They share approximately 60% amino acid sequence homology and differ in their expression regulation, tissue distribution, subcellular locations, immunologic reactivity, physiologic function, and certain enzymatic properties (Dizikes et al., 1986; Herzfeld and Raper, 1976; Jenkinson et al., 1996; Spector et al., 1983; Vockley et al., 1996). ARGase-I is a cytosolic enzyme located predominantly in the liver (Krebs, 1973). ARGase-II is a mitochondrial enzyme that does not appear to function in the urea cycle and is more widely distributed in numerous tissues (Morris et al., 1997).

In mammals, ARGase-I is present in the liver and it is one of the most important enzymes involved in nitrogen metabolism. It is involved in the principal route for disposal of excess nitrogen resulting from amino acid and nucleotide metabolism. The flux of nitrogen through this pathway is considerable, given that the average individual excretes about 10 kg of urea per year (Ash et al., 1998).

In humans, h-ARGase-I is an oligomer of 107 kDa and shows optimum pH at 9.3 and has a Km for ARG of about 10.5 mM (Beruter et al., 1978). The key structural features of mammalian ARGase-I include a binuclear manganese cluster (Mn), and an S-shaped tail composed by 19 amino acids at the C terminus of each monomer. The S-shaped tails are located at the subunit-subunit interface, and 54% of the intersubunit interactions are mediated by this region of the protein (Lavulo et al., 2001).

The catalytic mechanism of this enzyme is still not fully understood but several suggestions have already been proposed, some of them more consensually accepted (Christianson and Cox, 1999; Cox et al., 2001; Leopoldini et al., 2009) than others (Khangulov et al., 1998). Among the active site residues, GLU277 is apparently implicated in the substrate binding through the establishment of electrostatic interactions with the guanidium group of the substrate. This interaction appears to orientate the subtract in a favorable position with respect to the metal-bound hydroxide anion with whom it must react (Cavalli et al., 1994; Kanyo et al., 1996; Scolnick et al., 1997). Both manganese ions are coordinated to a hydroxide anion and with two aspartates (ASP232 and ASP128) and one glutamate (GLU277). These structural features can be observed in the crystallographic structure with the PDB code 3GMZ, where the human ARGase is in complex with L-ornithine. (Fig. 24.4) (Ilies et al., 2011).

According to (Leopoldini et al., 2009) the hydrolysis of ARG mediated by ARGase-I is kinetically favored when the ASP128 residue is deprotonated. The hydrolysis starts with the binding of ARG to the Mn(II) ions through the gaunidium groups. These interactions place the substrate in a good position

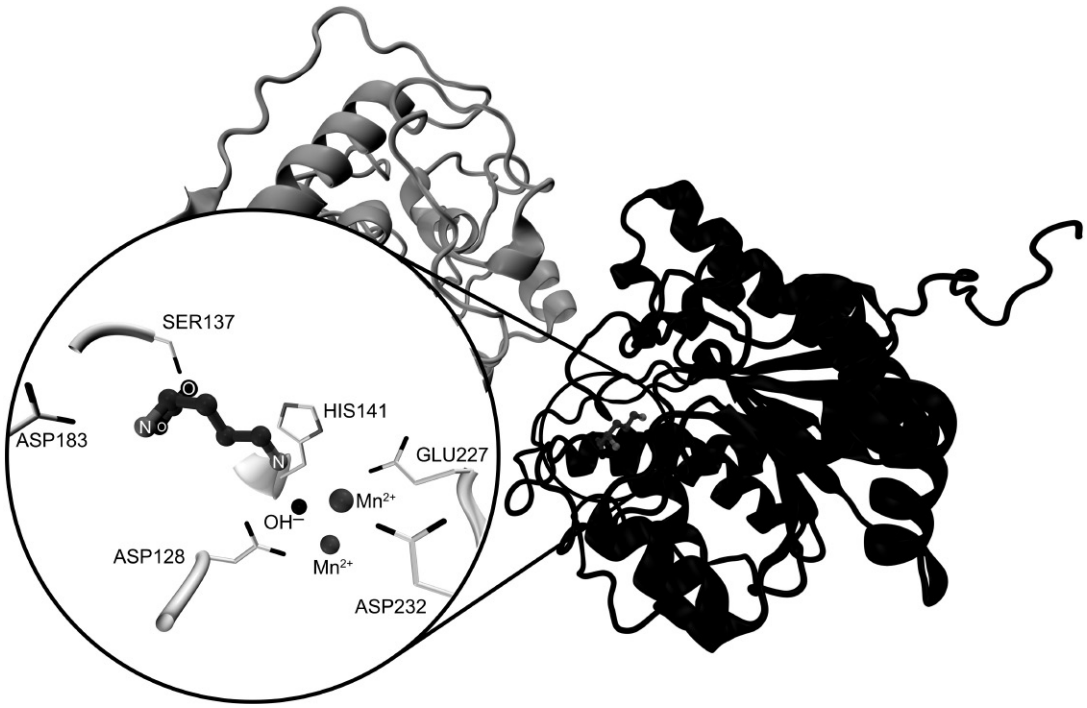

FIGURE 24.4 New Cartoon Representation of the Cocrystalized Structure of h-ARGase With L-Ornithine (PDB Code: 3GMZ)

Each subunit is represented with a different color *(gray and black)* and the substrate in ball and sticks and colored by CPK. In the close-up of the active site are represented: the residues of the active site that are important for the catalytic process in licorice, and the oxygen atom from the hydroxide ion and the two magnesium ions in VDW.

to undergo nucleophilic attack by the bridging hydroxy anion to carbon Cζ (Step 1) from which results a tetrahedral intermediate (Fig. 24.5a). In the second step of the mechanism, ASP128 acts as an acid/base catalyst, abstracting a proton from the hydroxyl group and shuttling it to the nitrogen Nε of the substrate (Step 2). This step is then followed by the bond break between nitrogen Nε and carbon Cζ of the substrate from which results L-ornithine and urea (Step 3). In the following step (Step 4), a water molecule enters to bridge the binuclear Mn(II) cluster, causing the urea product to move to a terminal coordination site on Mn(II). HIS141 appears to facilitate product release by serving as a proton shuttle from bulk solvent to nitrogen Nε of ornithine and regenerating the nucleophilic metal-bridging hydroxide anion (Step 5). Once this step is complete, ornithine dissociates, the enzymatic turnover takes place, and the enzyme is ready for a new catalytic cycle (Step 6).

h-ARGase-I has been tested with success in experimental animals since 1950, and showed promising activity in in vitro conditions (Simon-Reuss, 1953; Wiswell, 1951). However, the therapy failed to produce major responses when tested in in vivo conditions. The reason for this failure was related with the low affinity of this enzyme for ARG with the consequent requirement of a large quantity of enzyme in order to obtain the expected therapeutic results. In addition, this enzyme has a short circulatory

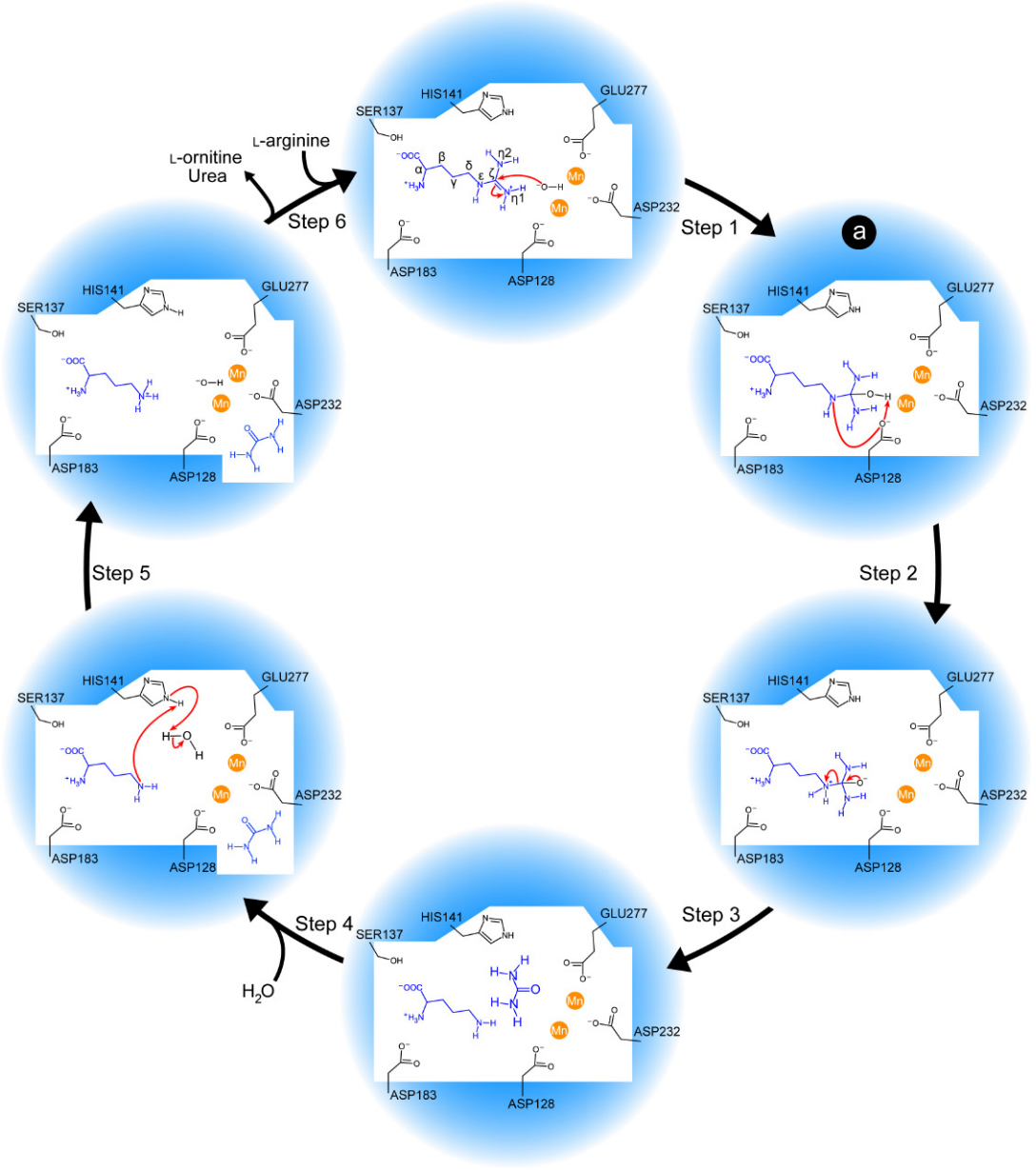

FIGURE 24.5 Schematic Representation of the ARGase Catalytic Mechanism

Each circle represents a different reaction intermediate. The important reaction intermediate is indicated with label (a).

half-life (only a few minutes) (Savoca et al., 1979) and its optimal pH is very high (9.5), which cannot be achieved under physiological conditions. All of these drawbacks limited the use of h-ARGase-I as an antitumor agent in the last decades (Currie et al., 1979). In spite of these drawbacks it was shown in 2010 that a pegylated recombinant human arginase I (PEG-r-h-ARGase-I) impairs the proliferation of malignant T cells in acute lymphoblastic T cell leukemia in in vivo and in vitro studies (Hernandez et al., 2010).

Later on, a pegylated cobalt-modified enzyme was developed and showed that the substitution of the Mn(II) metal center in h-ARGase-I by Co(II) (Co-r-h-ARGase-I) makes the enzyme more efficient for ARG hydrolysis and lowers the IC50 in a variety of malignant cell lines, including melanomas and hepatocellular carcinomas in in vitro assays (Stone et al., 2010). The only-same group also reported a modification of Co-r-h-ARGase-I with PEG-5K esters that showed an increase retention of the enzyme in circulation by about 2 orders of magnitude. The use of PEG(5K)-*co*-h-ARGase-I was later found to be effective in the treatment of hepatocellular and pancreatic carcinomas in xenograft models (Glazer et al., 2011).

2.2.2 L-arginine deiminase

L-Arginine deiminase (ADI, EC 3.5.3.6) is widely distributed among prokaryotic organisms and some anaerobic eukaryotes, but has never been identified in humans or other higher eukaryotes (Fig. 24.3E). The organisms that employ this pathway use ADI to convert ARG to L-citrulline and ammonia (Horn, 1933). L-citrulline is then degraded further, forming ATP (Adenosine Tri-Phosphate), carbon dioxide, and L-ornithine (Arena et al., 1999). The ADI pathway is thus used by these organisms to get energy, carbon, and nitrogen. In addition, it has also been suggested that this pathway can protect some bacteria from acidic conditions, by the production of ammonia (Casianocolon and Marquis, 1988) (Fig. 24.6).

The interest in ADI for therapeutic purposes began in the 1990s when Takaku and coworkers successfully used this enzyme to inhibit the growth of several murine and human tumor cell lines in vitro and in vivo, by exhausting the supplies of ARG (Takaku et al., 1995; Takaku et al., 1992). From that time, several studies confirmed the potential of ADI, purified from Mycoplasma, as an antitumor agent in different types of tumors (Ashikaga et al., 1994; Sugimura et al., 1992; Takaku et al., 1992). Later on, it was also found that beyond the antitumor activity, ADI has an antiangiogenic activity via suppression of nitric oxide generation (Beloussow et al., 2002; Park et al., 2003; Yoon et al., 2007). The cooperative antiproliferative and antiangiogenic activities of ADI are believed to be the key assets that turn ADI very effective in the treatment of several tumors and other diseases.

ADI has been structurally and pharmacologically characterized from various bacteria, but the most studied enzyme is ADI from *Mycoplasma arginini*, taking into account its therapeutic potential (Zhang et al., 2015). ADI from *M. arginini* has 410 amino acids and is composed of two identical subunits with a molecular weight of 45 kDa each and has an isoelectric point of 4.7. Its V_{max} value and K_m value for ARG is estimated to be 50 units/mg protein and 0.2 mM, respectively. It exerted maximal enzyme activity at pH 6.0–7.5 and at 50°C (Takaku et al., 1992).

A proposal for the catalytic mechanism of ADI from M. arginini was published by Das et al. (2004). The crystallographic structures available on the protein databank show that the active site is deeply buried on the protein surface and the substrate binds with the guanidium group pointing toward two aspartates (ASP161 and ASP271). These interactions endorse a specific orientation of the substrate that becomes aligned with HIS296 and CYS398, two residues that play an important role in the catalytic

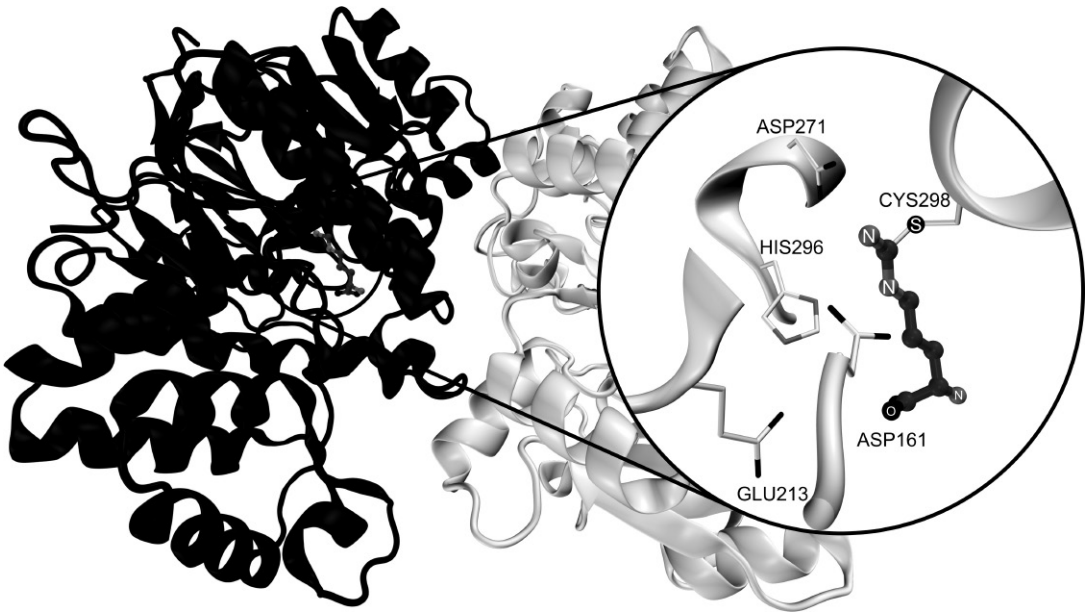

FIGURE 24.6 New Cartoon Representation of the Cocrystalized Structure of ADI Homodimer Structure With a Tetrahedal Reaction Intermediate (PDB Code: 1S9R)

Each subunit was represented with a different color *(gray and black)* and the reaction intermediate in ball and sticks and colored by CPK. In the close-up of the active site the residues of the active site that are important for the catalytic process are represented in licorice, as well as the reaction intermediate that should be formed after the first step of the mechanism.

process. These structural features are represented in Fig. 24.7 (PDB code 1S9R) (Das et al., 2004). The importance of the CYS398 in the catalytic reaction is well established, since it has already been demonstrated through structural studies, mutagenesis, and thiol modifying agents (Das et al., 2004; Galkin et al., 2005; Lu et al., 2004, 2006; Smith et al., 1978).

In the first step of the mechanism there is a nucleophilic attack of CYS398 to carbon Cζ of the substrate. Simultaneously, the proton from CYS398 migrates to nitrogen Nη2 of the substrate. At the same time, one proton from the same nitrogen migrates to HIS296 and the proton from HIS296 is transferred to GLU213. In the end of the first step, a tetrahedral intermediate (Fig. 24.7a) is generated with carbon Cζ of the substrate covalent bonded to CYS398.

In the second step of the catalytic mechanism occurs the formation of ammonia through the migration of one proton from HIS296 to Nη2 of the substrate. Simultaneously, one proton is transferred from GLU213 to HIS296. In the end of this reaction, the reaction intermediate (Fig. 24.7b) remains covalently bonded to CYS398 and one molecule of ammonia is released from the active site.

The next steps of the mechanism (Fig. 24.7, Steps 4–6) involve the release of the product of the reaction (L-Citrulline) and the enzymatic turnover. This requires three sequential steps and involves the reaction of one water molecule that is in close proximity to ASP271 and HIS296. In the fourth step of the catalytic mechanism occurs the nucleophilic attachment of the water molecule to carbon Cζ of

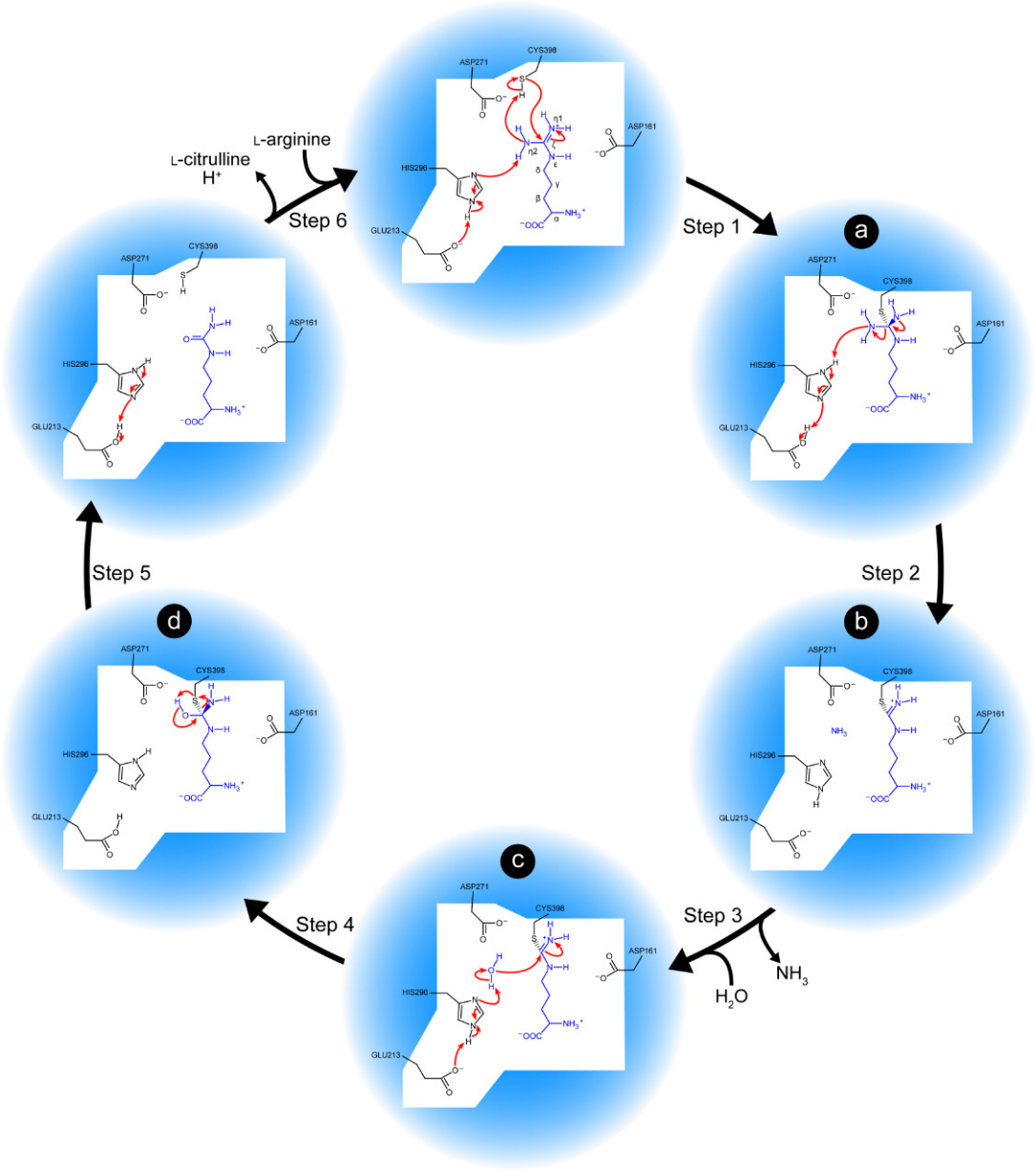

FIGURE 24.7 Schematic Representation of the ADI Catalysis Mechanism

Each circle represents a different reaction intermediate. The important reaction intermediates are labelled (a–d).

the reaction intermediate (Fig. 24.7c). At the same time one proton from the water molecule is transferred to HIS296 and one proton migrates from HIS296 to GLU213. The reaction is endorsed by the close proximity of ASP161 that establishes two hydrogen bonds with the NH groups of the intermediate that turns carbon Cζ more electrophilic. In the end of this reaction, another tetrahedral intermediate is generated (Fig. 24.7d), still bonded to CYS398 but now with a hydroxyl group attached to carbon Cζ instead of an amino group that was found after step 1. Step 5 involves the cleavage of the covalent bond between CYS398 and the substrate. This requires the proton transfer from hydroxyl group of the reaction intermediate to CYS398. Simultaneously, occurs the proton transfer from GLU213 to HIS296. In the end of this reaction, L-citrulline is generated and is ready to leave the active site. The final step of the reaction (Step 6) involves the enzymatic turnover that requires HIS398 to lose a proton to the solvent or any neighbor active site residue.

In recent years there has been an increased interest in ADI as a potential antineoplastic therapy since various studies have demonstrated disturbances in both ARG synthesis and metabolism in malignant processes. To date several types of cancer have already been shown to be sensitive to arginine deprivation via ADI, such as melanoma, lung cancer, renal cell carcinomas, and HCC (Liu et al., 2014). These tumors do not express ASS and are therefore highly sensitive to ARG deprivation and optimal targets for ADI chemotherapies. In spite of the promising results, the application of these treatments presents some limitations. The therapeutic efficiency of ADI is limited to tumors that do not express ASS and/or have an inactivated L-citrulline to ARG recycling pathway (Ensor et al., 2002; Qiu et al., 2014). In addition, ADI has a short serum half-life and when used in prolonged treatments is highly immunogenic. In order to overcome some of these limitations a pegylated form of ADI was formulated, PEG(20)-ADI (molecular weight: 20 kDa), that serves to reduce the immunogenicity of the enzyme while greatly improving its pharmacokinetic half-life in serum (Feun and Savaraj, 2006; Feun et al., 2008; Ni et al., 2008). This enzyme revealed a similar efficiency in in vitro assays with melanomas and HCC, comparing with native ADI, however, it shows a much more effectiveness in in vivo assays (Ensor et al., 2002). Currently, the antitumor activity of PEG(20)-ADI has been also observed in pancreatic (Bowles et al., 2008), prostate (Kim et al., 2009), small cell lung (Kelly et al., 2012), head, neck (Huang et al., 2012), and breast cancers (Qiu et al., 2014), lymphoma (Delage et al., 2012; Huang et al., 2012), myxofibrosarcomas (Huang et al., 2013), melanoma (Feun et al., 2012), and glioblastoma (Qiu et al., 2014; Syed et al., 2013).

The therapeutic efficacy of PEG(20)-ADI has been validated through phase I/II clinical trials in advanced HCC (Glazer et al., 2010; Izzo et al., 2004; Yang et al., 2010) and melanoma patients (Ascierto et al., 2005; Feun et al., 2012; Ott et al., 2013). Additionally, there are several ongoing phase I and/or II clinical trials using PEG20-ADI in patients with advanced non-Hodgkin's lymphoma, acute myeloid leukemia, malignant pleural mesothelioma, prostate and nonsmall cell lung, advanced gastrointestinal, HER2 (Human Epidermal growth factor Receptor 2) negative metastatic breast, advanced pancreatic, and small cell lung cancers.

PEG(20)-ADI has also been reported as a potentially better therapeutic agent for the treatment of leukemia than ASNase, which has been used for the treatment of acute leukemia for over 20 years. ADI can inhibit the growth of cultured leukemia cells at concentrations of 5–10 ng/mL, which are about 20–100 times lower than those of ASNase (Feun et al., 2008). In addition, in vivo studies showed that ADI treatment does not have serious side effects, such as anaphylactic shock, coagulopathies, and liver toxicity, which have been reported for ASNase (Vrooman et al., 2010).

2.3 L-METHIONINE

L-MET is a sulfur containing amino acid that plays many key roles in cells (Guedes et al., 2011; Nozaki et al., 2005). In every cell, MET is essential for protein synthesis and it is the precursor of many compounds, such as Glutathione (a tripeptide that reduces reactive oxygen species, thereby protecting cells from oxidative stress), polyamines spermine and spermidine (which are detrimental on nuclear and cell division) and S-adenosylmethionine (the major source of methyl groups in a myriad of biological and biochemical events) (Cellarier et al., 2003; Davis and Uthus, 2004; Ho et al., 2011; Thivat et al., 2007). One of the most relevant biological role of MET in the cell is DNA methylation, where it serves as precursor for S-adenosylmethionine synthesis. DNA methylation is a common epigenetic signalling tool that cells use to control gene expression and has been associated with a number of key processes including genomic imprinting, X-chromosome inactivation, repression of repetitive elements, and carcinogenesis (Geiman and Muegge, 2010; Ramani et al., 2010).

In spite of its importance in cells, MET is a special essential amino acid as its long-term dietary deprivation is still compatible with life. This happens because unlike other essential amino acids that must be ingested, MET can be recycled by remethylation of homocysteine (de novo synthesis), either by L-methionine synthase (MetS) or in the liver by betaine-homocysteine methyltransferase (BHMT) (Cellarier et al., 2003; Hoffman, 1984; Guo et al., 1993a). In addition, MET can also be obtained by a *salvage* pathway in which MET is generated from 5′-methylthioadenosine toward methylthioadenosine phosphorylase (MTAP). This is true for all normally functioning cells, however, numerous malignant cell lines, such as breast, lung, colon, kidney and bladder cancers, melanoma, and glioblastoma (Breillout et al., 1990; Cellarier et al., 2003; Hoffman, 1985; Kokkinakis et al., 2001; Poirson-Bichat et al., 2000) do not have a functional methionine cycle intact. As a result, they cannot synthesize MET and in the absence of an exogenous supply they experience growth inhibition or eventually die.

The mechanisms responsible for MET dependence in malignant cell lines are still not fully understood. Several studies have shown that MET-dependent tumor cell lines presented relatively low amounts of L-methionine synthase or/and have deficient methionine synthase activity (Christa et al., 1986; Fiskerstrand et al., 1994; Kenyon et al., 2002). Other studies emphasized the importance of the inhibition of the salvage MET pathway in which MTAP is a key enzyme and is also responsible for the production of polyamines, which are critical for cell proliferation. Indeed, the loss of MTAP activity through gene deletion is a common feature in many kinds of human cancers, including non-small cell lung cancer, glioma, T-cell acute leukemia, bladder cancer, osteosarcoma and endometrial cancer (Albers, 2009; Nobori et al., 1993; Subhi et al., 2003; Traweek et al., 1988). In contrast, other studies suggest that MET dependence would result not from an enzymatic deficiency but rather on an increased requirement for this amino acid. In fact, MET requirements of tumor cells are higher than that of normal cells because of increased protein synthesis and DNA trans-methylation reactions. This promotes multiple biochemical reactions necessary for fast growth of tumor cells, and can change DNA expression. Indeed, in several tumors, a variety of growth inhibitory and proaapoptotic genes are transcriptionally silenced as a result of DNA hypermethylation (Baylin et al., 1998; Zingg and Jones, 1997).

The observation that some human tumors are MET-dependent in in vivo presents a therapeutic target for cancer growth control and has been studied in great detail in the past 2 decades (Hoshiya et al., 1995). As MET is sourced mainly from diet, initial studies observed that simple methionine dietary restriction caused regression of a variety of animal tumors and inhibits metastasis in animal models (Breillout et al., 1987, 1990; Goseki et al., 1987; Guo et al., 1993b; Millis et al., 1998). Phase

I clinical trials of dietary methionine restriction for adults with advanced cancer indicate that dietary methionine restriction is safe and provided promising results. However, reduction of methionine levels in humans solely by dietary intervention presents several limitations as MET is practically ubiquitous for all food and the price of such diet is about US$1000 per month.

A more attractive pharmacological approach to lowering MET levels in vitro and in vivo involves the use of MET degrading enzymes. L-methionine-α-amino-γ-mercaptoethane lyase or methioninase (MGL, EC 4.4.1.11) is an enzyme that specifically degrades L-methionine to α-ketobutyrate, methanethiol and ammonia (Kreis and Hession, 1973) very efficiently. In addition, it can also degrade L-homocysteine, a key factor that prevents endogenous MET production (Sato and Nozaki, 2009).

MGL is found in bacterial species and in fungi, but it is absent in mammals (Sharma et al., 2014). This enzyme was originally purified from *Clostridium sporogene*, but later on it was found that MGL from *Pseudomonas putida* (Pp-MGL) yielded a more stable enzyme with a relatively low Km (Esaki and Soda, 1987). Due to problems in purification of Pp-MGL, the MGL gene, isolated from *P. putida*, is now cloned and expressed in great quantities from *Escherichia coli* (r-Pp-MGL) (Hori et al., 1996).

Pp-MGL has 389 to 441 amino acids, and it is found as a homotetramer with a molecular weight of about 149–173 kDa (Nakayama et al., 1984). The active site of this enzyme is located at the interface of the two neighboring subunits, each one containing one Pyridoxal 5'-phosphate (PLP) cofactor that is required for the catalytic process (Kudou et al., 2007a; Mamaeva et al., 2005; Motoshima et al., 2000; Toney, 2005). The cofactor is stabilized by six residues TYR59, ARG61, TYR114, CYS116, LYS240, and ASP241 that have been found to be important for catalysis (Fig. 24.8) (Kudou et al., 2007a).

MGL is classified into the γ-family of PLP-dependent enzymes. However, it possesses a key cysteine (CYS116) in the active site that is not conserved in other PLP γ-family enzymes. Mutagenic studies revealed that this active site CYS is essential for substrate specificity (McKie et al., 1998; Kudou et al., 2007a, 2008; Sato et al., 2008) and also for enzymatic activity (Inoue et al., 2000; Kudou et al., 2008).

The enzymatic mechanism of MGL is proposed based on the knowledge of other PLP γ-family enzymes and the enzymological analyses of *P. putida* wildtype and mutant forms (Cerqueira et al., 2011; Fukumoto et al., 2012; Kudou et al., 2007a; Oliveira et al., 2011). Similar to all PLP-dependent enzymes, in the steady state the PLP cofactor is covalently bonded to an active site LYS, forming what is generally known as an internal aldimine (Fig. 24.9a). When MET is available in the active site of MGL, it binds to PLP (Step 1) generating a geminal diamine (Fig. 24.9b). Afterward, the cleavage of the covalent bond between the PLP cofactor and the active site LYS211 takes place (Step 2) and the substrate becomes covalent bonded to the PLP cofactor through the primary amino group, forming an external aldimine (Fig. 24.9c). The next step (Step 3) involves the abstraction of one proton from carbon Cα of the reaction intermediate by an active site base resulting in the formation of a quinonoide intermediate (Fig. 24.9d). Later on, carbon 7 from PLP is protonated by an active site base (Step 4), from which results a ketamine intermediate (Fig. 24.9e). An active site base then abstracts a second proton from carbon Cβ (Step 5) and an enamine intermediate is generated (Fig. 24.9f) that triggers the γ-elimination (Step 6) and the generation of unprotonanted methanethiol (Fig. 24.9g). Methanethiol is subsequently protonated by the active site base and an aminocrotonate intermediate is obtained (Fig. 24.9h) (Step 7). In the final step of the catalytic process (Step 8), a water molecule mediates the α-ketobutyrate and ammonia generation and the concomitant release of methanethiol. At the same time, the enzymatic

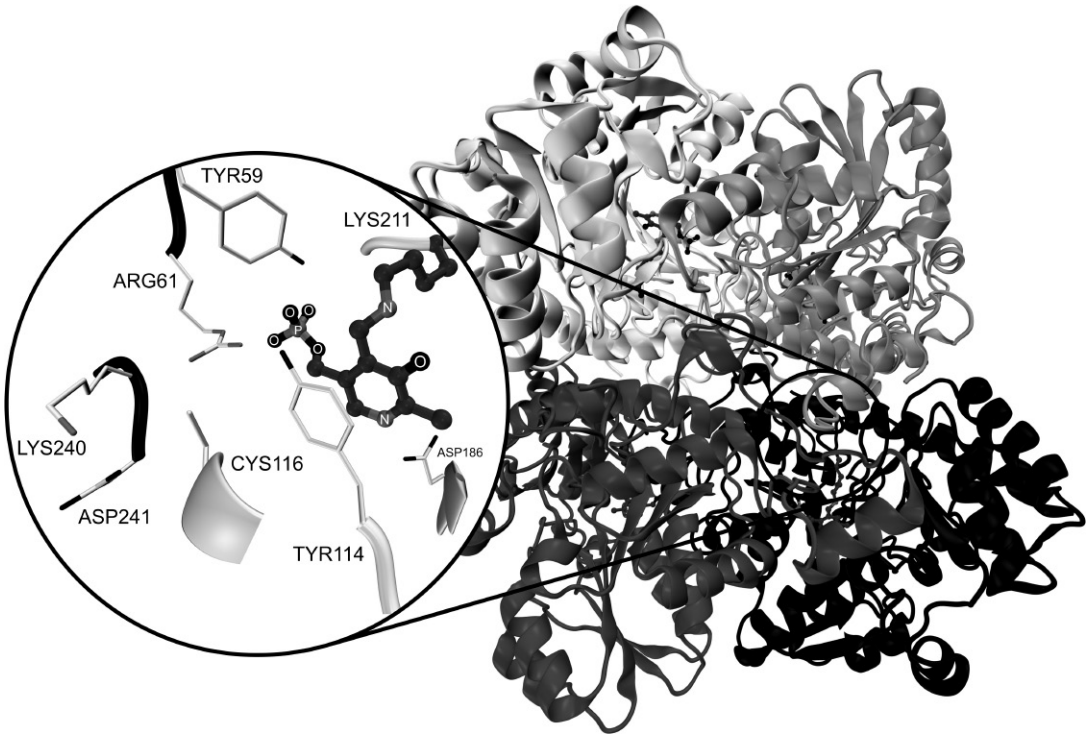

FIGURE 24.8 New Cartoon Representation of the Cocrystalized Structure of the MGL Homotetramer Structure With the PLP Cofactor (PDB Code: 2O7C)

Each subunit was represented with a different black tonality. In the close-up of the active site, the internal aldimine is shown in ball and sticks, where PLP cofactor covalent bonded to an active site LYS. The other active site residues that are important for the catalytic process are represented in licorice.

turnover takes place recovering the steady state of the enzyme, that is, internal aldimine in which the active site LYS is covalent bonded to the PLP cofactor. (Inoue et al., 2000; Kuznetsov et al., 2015; Sato et al., 2008; Toney, 2005).

The therapeutic manipulation of r-Pp-MGL to deplete plasma MET has been widely investigated in the past 2 decades (Jeanblanc et al., 2005) with promising results in a number of L-methionine-dependent cancer cell lines including lung, colon, kidney, brain and prostate cancers, melanoma, and fibrosarcoma (Miki et al., 2000a, 2000b, 2001; Smiraglia et al., 2001; Tan et al., 1996, 1997, 1999; Yamamoto et al., 2003). Preclinical studies in animal models have also demonstrated the potential of r-Pp-MGL to efficiently inhibited the growth of Yoshida sarcomas and a human lung tumor in nude mice xenograft models (Tan et al., 1996).

Several studies have also shown promising results combining the chemotherapeutic effect of r-Pp-MGL with other compounds, such as cisplatin, 5-fluorouracil (5-FU), 1-3-bis(2-chloroethyl)-1-nitrosourea (BCNU), cisplatin, and vincristine (Tan et al., 1999). These studies have shown synergic effects in mouse models of colon, lung, and brain cancers and glioblastoma (Hoshiya et al., 1997;

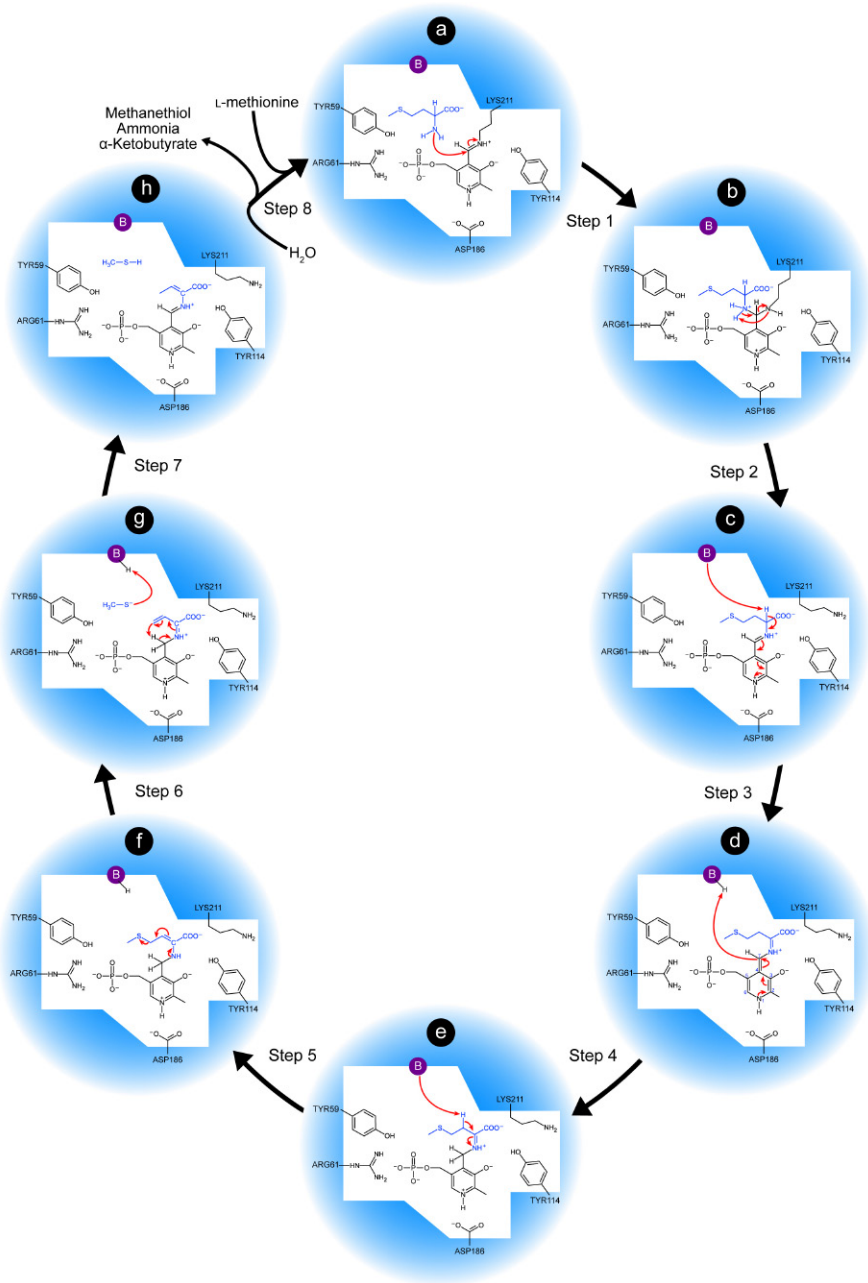

FIGURE 24.9 Schematic Representation of the MGL Catalysis Mechanism

Each circle represents a different reaction intermediate. The important reaction intermediates are labeled (a–h).

Hu and Cheung, 2009; Kokkinakis et al., 2001; Tan et al., 1999; Yoshioka et al., 1998). Other authors have also studied the administration of r-Pp-MGL with selenomethionine (a nontoxic compound). These studies have shown that r-Pp-MGL catalyses the conversion of selenomethionine into methylselenol, and the latter compound is able to oxidate thiol groups and generate toxic superoxide. This compound is very toxic to cells and can cause apoptosis via a mitochondrial pathway (Guo et al., 1993a; Kim et al., 2007). Because in cancer cells the metabolism is faster than in normal cells, this approach has the advantage of being target specific.

In spite of the initial success of r-Pp-MGL for cancer therapy it presents some problems that precludes an easy manipulation, namely, the instability of r-Pp-MGL in plasma caused by immunogenicity, proteolysis, oxidation of the active site residues, release of PLP and the instability of the active dimer (Kudou et al., 2007b). Modification of this enzyme with polyethylene glycol (PEG) has reduced immunogenicity, lengthened the half-life of the recombinant enzyme, and possibly inhibited proteolytic enzymes that could degrade MGL (Tan et al., 1998). The intravenous administration of PEG-r-Pp-MGL was tested in phase I clinical trials. Patients in these trials experienced no significant toxicity, and plasma MET levels fell dramatically. However, the authors did not assess the antitumor activity of this treatment and to date no other clinical trials have been published (Sun et al., 2003; Yang et al., 2004b, 2004c).

3 CONCLUSIONS

A prerequisite for making an effective medication in 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 proved 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 autotrophic 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 acute lymphoblastic leukemia has already been approved by FDA and shows very good results. ARG deprivation by ADI is a novel approach to target tumors that lack argininosuccinate synthetase (ASS) expression. ADI showed promising results during initial clinical trials for the therapy of HCC and melanomas and has currently been granted “orphan status” by the FDA and EMEA for the potential treatment of these diseases (Shen and Shen, 2006). Some studies also suggest that ADI has the potential to become a better therapeutic agent than ASNase in the treatment of leukemia (Gong et al., 2000; Muller and Boos, 1998).

Currently, the major drawback of ADI and ASNase for therapeutic purposes is the heterologous origin. As these enzymes are derived from a microorganism, they are immunogenic in humans and have very short circulating half-life. The chemotherapeutic treatments using these enzymes thus require multiple injections to produce the desired effect and this results in a variety of adverse effects including hypersensitivity reactions, anaphylactic shock, and the inactivation and clearance of the enzyme itself

(Schellekens, 2002). 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 enzyme are much safer and have prompted the use of ADI in many clinical trials and the approval of ASNase by FDA and EMEA for the treatment of some types of tumors.

MGL is another heterologous enzyme discussed in this review. Similar to ASNase and ADI, this enzyme presents several immunogenic effects, but unlike those enzymes the pegylated form of MGL 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 (Cantor et al., 2011; Sun et al., 2003). However, several side effects persist and its utilization in cancer therapies continues to be inappropriate (Miki et al., 2001; Yang et al., 2004a).

Among 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 cancers that are autotrophic for ARG. Indeed, several authors consider ARGase a better chemotherapeutic agent for cancer treatment than ADI because apart from its low immunogenicity, this enzyme also is effective in tumors for which ADI is ineffective (although in these cases OCT cannot be expressed). Its major disadvantage when compared with ADI is its high Km and high optimum pH, which requires large quantities of enzyme in order to accomplish the expected therapeutic results. This problem may be overcome by the utilization of cobalt-substituted ARGase, but more studies are required to evaluate its potential in cancer therapy.

Despite the problems arising from the use of amino acid depleting enzymes in amino acid depriving chemotherapies, the recent progresses made in this field will undoubtedly facilitate the pursuit of new treatments to combat cancers. ASNase was the first enzyme approved by FDA and EMEA for the chemotherapeutic treatment of leukemia with remarkable success. Taking into account the ongoing 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 will not be too distant in the future and will have a profound influence on human health.

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