Chapter X

Enzymatic Amino Acid Deprivation Therapies Targeting Cancer

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X.1 INTRODUCTION

Proteins are large and complex molecules that play critical roles in the cells. They are involved in virtually all cell functions, including their regulation and structure. Any alteration on the concentration or composition of the proteins will disturb the function of the cells and can compromise cellular viability. In severe cases, this can ultimately lead to the death of the organism.

Proteins are made up of hundreds or thousands of smaller units called amino acids, which are attached to one another through peptide bonds in long chains. There are 20 different types of natural amino acids that can be combined and used by the cells to synthetize proteins. It is, therefore, important that these molecules are available in sufficient amount in the cells to maintain their normal function.

Most of the mammals, and particularly humans, have long ago ceased the capability to produce efficiently ten of the twenty natural amino acids. This means that for the healthy development, growth, metabolic, and physiological functions of their cells, these compounds must be obtained from the diet. Otherwise, they would starve to death. These amino acids are termed essential and include isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. This class of amino acids can also include histidine and arginine. Both histidine and arginine are non-essential in healthy adults, but

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become essential when their requirement increases and they need to be obtained from the diet. This usually occurs during the periods of growth, illness, or metabolic stress. For this reason, they are called semi-essential amino acids. All the other amino acids are termed non-essential and are efficiently synthesized by the enzymes present in the cells (alanine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, proline, serine, and tyrosine).

The metabolism of the amino acids has often been disregarded for clinical purposes. However, recent studies have shown that the amino acid can represent

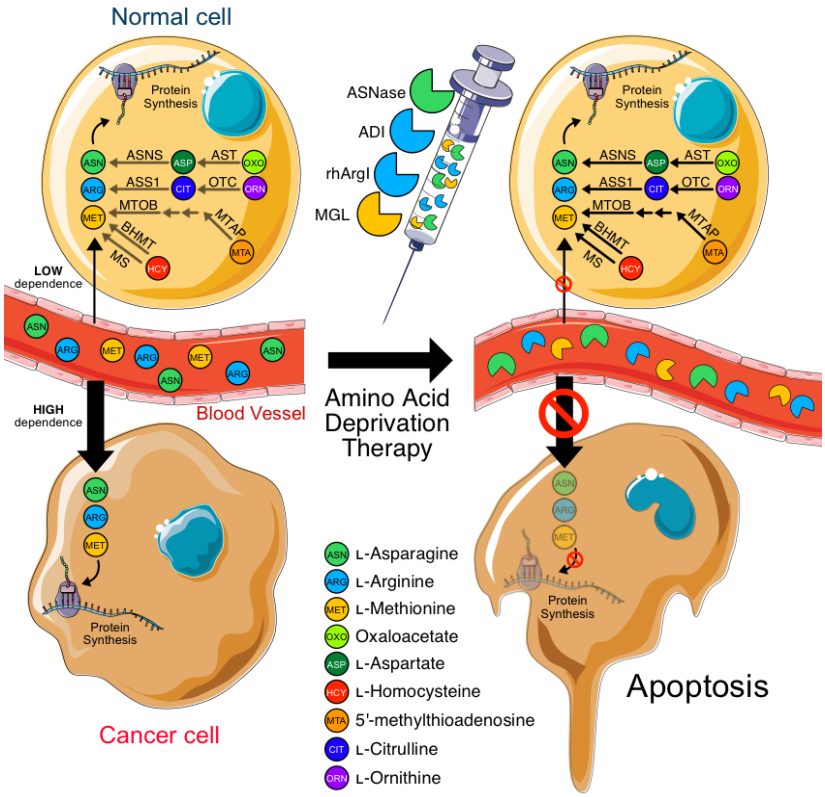

Figure X.1 Schematic representation of the metabolic mechanisms involving the amino acid depletion therapies, using methionine-γ-lyase (METase), asparaginase (ASNase), arginase (rhARGI), and L-arginine deiminase (ADI).

a great clinical opportunity, still poorly explored, for the treatment of several types of cancer that require high concentrations of one or more amino acid residues that are essential in their development, survival, and spread. In order to overcome these problems, tumours rely on the extracellular pool of these amino acids to satisfy protein biosynthesis demands and continue to grow without limitations. This means that if the concentration of those amino acids decreases, the development of tumour cells can be impaired or even annihilated. At the same time, healthy cells remain almost unchanged since they are less demanding

(in the case on the essential amino acids) or can synthesize these compounds (non-essential or semi-essential amino acids) in sufficient amounts by alternative pathways.

The amino acid deprivation cancer therapy relies on these principles, and its main objective is to starve tumours to death while keeping normal cells unharmed (Fung and Chan G. 2017). The optimal conditions for this therapy thus occur when the tumours are sensitive to one of the non-essential or semi-essential amino acid residues. However, a simple diet is not enough to achieve a therapeutically relevant amino acid deprivation for cancer therapy, and therefore, other strategies are used for this purpose.

During the last 50 years, enzymatic therapies have been found to be quite efficient for this purpose. These treatments involve the use of heterologous enzymes (from other organisms, such as bacteria), recombinant, or engineered human enzymes to decrease the concentration of a specific amino acid in the bloodstream that is essential for the development of a tumour (Pokrovsky et al., 2017).

There are successful examples where the enzymatic amino acid deprivation therapies are used to treat cancer. One example involves the use of arginine deiminase (ADI) in the hepatocellular carcinoma (HCC), melanoma, and other urea cycle-deficient cancer cells that have a high demand for L-arginine (semi-essential amino acid). Another example, involves the use of the enzyme methionine-γ-lyase (MGL) in the treatment of several types of tumours, such as, colon, breast, prostate, ovary, lung, brain, kidney, stomach, and bladder cancers, as well as larynx melanoma, sarcoma, leukaemia and lymphomas that require a high concentration of L-methionine (essential amino acid) (Fernandes et al., 2017). The non-essential amino acids can also be used in enzymatic amino acid depletion therapies. This only happens in tumours where the normal metabolism of one non-essential amino acid in the cells is disrupted or when there is some sort of defect in the ability to use a certain amino acid (DeBerardinis and Chandel, 2016). This is the case of childhood acute lymphoblastic leukaemia (ALL), and to a lesser extent, of non-Hodgkin's lymphoma, whose tumours require a high concentration of L-asparagine. In these cases, the use of L-asparaginase (ASNase) has been found to be remarkably successful in the treatment of these tumours and this method has already been approved by FDA.

Taking into account the therapeutic potential of these enzymes in cancer therapies, they have been extensively studied in the last two decades. These therapies have been showing very promising results, albeit some challenges have been reported in clinical trials. In the following sections, the structure, function, and catalytic mechanism of therapeutic heterologous enzymes involved in L-ARG, L-MET, and L-ASN degradation will be revised. Particular attention will be given to the heterologous enzymes L-arginase (rhARGI), L-arginine deiminase (ADI) (Ni et al., 2008), L-methionine-γ-lyase (MGL) (Tan et al., 1996), and L-asparaginase (ASNase) (Rytting, 2010) taking into account their current therapeutic potential.

X.2 AMINO ACID DEPRIVATION ENZYMES

Eukaryotic cells control the concentration of the amino acids in the cell through a tight regulation of their concentration. From this control results a delicate state of dynamic equilibrium that is known as proteome homeostasis. A crucial step in the maintenance of the proteome homeostasis is the synthesis of proteins that are required for cell growth and proliferation. Therefore any perturbation in this equilibrium may affect permanently cell metabolism and even its survival. The amino acid deprivation therapies have, therefore, a very narrow window to work with, since the decrease in the concentrations of any amino acid in the bloodstream needs to be carefully addressed in order to prevent any catastrophic effect on the normal cells and in the full organism. For this reason the amino acid deprivation therapy cannot be used in all types of cancers that are autotrophic for a certain amino acid, but only in the ones where a small decrease in that amino acid can lead to a therapeutic effect while keeping the other cells safe. The following sections describe cases where these assumptions have been fully addressed.

X.2.1 *Arginine*

Arginine (L-ARG) is in different ways, an important amino acid for all living organisms. Besides its common role as a building block for protein synthesis, in the case of mammals, it is also a key precursor/mediator of a series of metabolic, immunological, neurological and signalling pathways in the cells (Barbul et al., 1977, 1983; Billar, 1995; Rogers and Visek, 1985).

For adult mammals, L-ARG is considered a semi or a conditionally essential amino acid. This means that although for healthy individuals arginine is not an essential dietary amino acid, the emergence of a catabolic stress, such as an inflammation or infection, raises the metabolic demands to levels that cannot be supplied by the endogenous synthesis, turning it into an essential amino acid (Daly et al., 1988; Morris Jr., 2007; Reeds, 2000).

A healthy individual obtains the metabolic required L-ARG from the diet, endogenous de novo synthesis and cellular breakdown of proteins (Morris Jr., 2006). When deprived of L-ARG, healthy cells undergo a cell cycle arrest at Go/G1 phase and become quiescent. This effect is reversible for the majority of the cells who recover to their normal proliferation status if reinstated with L-ARG [16].

In the case of tumour cells, its metabolism is so faulty that when deprived of arginine they continue the cell cycle, undergoing unbalanced growth, which eventually leads to the activation of apoptotic pathways (Patil et al., 2016). This particularity, common to all tumour cells, has been widely explored in the development of new anticancer therapies against some cancers with auxotrophy toward L-ARG (Dhankhar et al., 2018).

The susceptibility of some cancers to L-ARG deprivation was observed for more than 60 years (Bach and Swaine, 1965) although, by the time, the

mechanism behind it was unknown. To date, there is evidence of two enzymes with proven ability to efficiently deprive L-ARG auxotrophic tumour cells from its essential amino acid: a Mycoplasma arginine deiminase (ADI) and a human recombinant arginase (rhArg). They have different catalytic mechanisms and are effective in cancer cells with different characteristics.

A. Arginine deiminase

In adult mammals, 5 to 15% of the plasma arginine is obtained by de novo biosynthetic pathway, which involves the conversion of citrulline to L-ARG catalysed by argininosuccinate synthase (ASS1) and argininosuccinate lyase (ASL). ASS1 catalyzes the condensation of citrulline and aspartate to form argininosuccinate that is split into fumarate and L-ARG by ASL. ASS1 is a ubiquitous enzyme whose expression, localization, and regulation differ significantly depending on the tissue-specific needs for L-ARG (Haines et al., 2011).

Some cancers such as metastatic melanoma (MM), prostate carcinomas, hepatocellular carcinoma (HCC) (Dillon et al., 2004) non-Hodgkin's lymphoma, Hodgkin's lymphoma, pancreatic carcinoma, osteosarcoma, and malignant pleural mesothelioma and some breast tumours (Qiu et al., 2015) have an elevated requirement for L-ARG due to the deficient expression of ASS1 that results in L-ARG auxotrophy (Feun et al., 2008; 2012; 2015). Arginine deiminase (ADI, EC 3.5.3.6) is an enzyme with high affinity to L-ARG that catalyses the conversion of L-ARG into citrulline and ammonia. The cancer cells who do not express ASS1 are unable to re-synthesize L-ARG from citrulline, which culminates in the full deprivation of an amino acid that is imperative for their survival (Shen et al., 2003).

ADI, together with ornithine transcarbamylase (OTC, EC 2.1.3.3) and carbamate kinase (CK, EC 2.7.2.2) belong to the arginine deiminase pathway (ADI pathway), which is used by many L-ARG-dependent microorganisms to catabolize the amino acid generating ATP. It has been identified, purified, and characterized in bacteria, archaea and some anaerobic eukaryotes (Horn, 1933; Knodler et al., 1994, 1995; Leopoldini et al., 2009), but there is no evidence of its existence in higher eukaryotes. It was demonstrated that ADI only possesses anti-tumour activity in ASS1 deficient tumours, being ineffective when ASS1 is restored (Ensor et al., 2002; Qiu et al., 2014). Further evidence suggests that ADI mediates the inhibition of tumour growth not only by exhausting the supplies of L-ARG, but also by its antiangiogenic activity via suppression of NO generation (Beloussow et al., 2002; Park et al., 2003; Yoon et al., 2007).

The first evidence of the in vitro anti-tumour activity of ADI was reported by Jones in 1981 who applied ADI isolated from *Pseudomonas putida* on murine leukaemia lymphoblast (Ni et al., 2008). A decade later, in 1990, Miyazaki and colleagues demonstrated that the addition of a small dose (5 ng/ml) of Mycoplasma ADI to the culture media depleted all the available L-ARG , which in turn lead to the inhibition of various human cancer cell lines (Miyazaki et al.,

1990). From there onwards several studies confirmed the potential use of Mycoplasma ADI as an anti-tumour agent in vitro and in vivo (Ashikaga et al., 1994; Sugimura et al., 1992; Takaku et al., 1992).

Although there are studies with other ADI sources (Zhang et al., 2015), the most commonly used ADI protein is derived from *M. arginini*. It is composed of 2 identical subunits with a molecular weight of 45 kDa each and a isoelectric point of 4.7. It is stable at neutral pH and exerted maximal enzyme activity at pH 6.0–7.5 and at 50°C (Takaku et al., 1992).

Since ADI from *M. arginini* has high immunogenicity and its half-life time is too short (aprox. 5 hours) to be directly used in vivo, it was conjugated with polyethylene glycol (PEG) via a succinimidyl succinate amide bond (Takaku et al., 1993). A formulated ADI-PEG20 (molecular weight: 20 kDa) showed significantly reduced antigenicity and an increased half-life time from 5 hours to 7 days in mice and rabbit. In addition, it was also demonstrated that it efficiently degraded plasma arginine to undetectable levels (<5-7 μmol/L) (Ensor et al., 2002; Holtsberg et al., 2002) leading to the L-ARG deprivation in humans (Izzo et al., 2004).

Several studies showed that ADI is a potentially better therapeutic agent for the treatment of leukaemia than ASNase, which has been used for the treatment of acute leukaemia for over 20 years (Ensor et al., 2002; Durden and Distasio, 1980). ADI inhibited the growth of cultured leukaemia cells at concentrations about 20–100 times lower and does not have the serious side-effects associated with ASNase (Gong et al., 2000; Muller and Boos, 1998).

The anti-tumour activity of ADI-PEG20 has been further demonstrated through laboratory studies in HCC (Dillon et al., 2004), pancreatic(Bowles et al., 2008), prostate (Kim et al., 2009), small cell lung (Kelly et al., 2012), head and neck (Huang et al., 2012), and breast cancers (Qiu et al., 2014), lymphoma (Delage et al., 2012), myxofibrosarcomas (Huang et al., 2013), melanoma (Dillon et al., 2004; Feun et al., 2012), and glioblastoma (Syed et al., 2013).

Therapeutic uses

By 2018, there are records of more than twenty clinical trials in different phases (phase I,II, and III) and different recruiting status whose general objective is to evaluate the safety and therapeutic efficacy of ADI-PEG20 in patients suffering from different cancers (ClinicalTrials.gov. Available from: https://clinicaltrials.gov).

The first clinical trials were completed 15 years ago and showed that the plasma L-ARG levels decrease from approximately 130 μmol/L to below the level of detection (< 2 μmol/L) and remain low for more than 7 days after the first intramuscular injection of 160 IU/m^2 of ADI-PEG20 in both HCC (NCT00056992) and MM patients (NCT00029900). The response rates of these studies are in the range 25-47%, and they demonstrated good safety and tolerability (Izzo et al., 2004; Ascierto et al., 2005).

All the posterior ADI-PEG20 clinical trials completed confirmed that monotherapy with ADI-PEG 20 in patients with both MM and HCC is generally well tolerated.

In a HCC trial with patients with metastatic or inoperable histologically confirmed HCC, it was observed that the L-ARG levels remained below baseline for 50 days and had an overall mean survival of 15.8 months. The authors also observed a low rate of significant immunogenic events (2.5%) suggesting good tolerability of ADI (Glazer et al., 2010).

Another phase II study in Asian patients with HCC confirmed that weekly treatment with 160 IU/m^2 was sufficient to deplete circulating L-ARG for 7 days. Additionally, patients with depletion of circulating L-ARG for ⩾4 weeks had a trend towards better survival than those with depletion of arginine for <4 weeks (Yang et al., 2010).

Based on these encouraging results, a phase III trial (NCT01287585) was conducted with HCC patients who had failed prior chemotherapy versus placebo. The results were not as encouraging as expected. ADI-PEG20 monotherapy at the dose of 18 mg/m^2 proved to be ineffective in prolonging overall survival in the selected HCC patients. However, it was also observed that the patients submitted to prolonged L-ARG depletion from ADI-PEG20 showed a relative superior overall survival. Based on these results, the authors are now focused on the development of new strategies to enhance prolonged L-ARG depletion and synergize the effect of ADI-PEG20 (Abou-Alfa et al., 2018).

The results from recent clinical trials concerning MM are not so encouraging as the ones from HCC. Feun and colleagues performed a clinical trial with ocular and cutaneous melanoma patients (NCT00450372) whose results demonstrated that the response to L-ARG-depleting therapy was indeed correlated with lack of ASS expression. However, contrary to previous results, they also observed that it was necessary to raise the dose of ADI-PEG20 from 160 to 320 IU/m^2 per week to observe any patient response (Feun et al., 2012). In another contemporary clinical trial with unresectable stage III or stage IV melanoma patients (NCT00520299), it was also observed that ADI-PEG20 has limited clinical activity as a single agent at doses of 160 IU/m^2 or less. This study also revealed an inverse correlation between mean levels of ADI-PEG20 and ADI-PEG20 antibody levels which is suggestive of some immunogenicity associated with ADI-PEG20. Unexpectedly, their results suggested that uveal melanoma might be more sensitive to L-ARG deprivation than cutaneous melanoma (Ott et al., 2013). A recently completed Phase II clinical trial with malignant pleural mesothelioma patients (NCT01279967) showed that ADI-PEG20 significantly improves progression-free survival, and possibly the overall survival (Szlosarek et al., 2017).

There are also several on-going phase I and II clinical trials using ADI-PEG20 monotherapy in patients with different tumours, such as relapsed sensitive or refractory small cell lung cancer (SCLC) (NCT01266018), prostate cancer and non-small cell lung cancer (NCT01497925), acute myeloid leukaemia (NCT01910012), advanced non-Hodgkin's lymphoma (NCT01910025).

Other phase-I and -II clinical trials testing ADI-PEG20 in combination with existing chemotherapy are completed or ongoing for advanced gastrointestinal cancer (NCT02102022), HER2 negative metastatic breast cancer (NCT01948843), advanced Pancreatic Cancer (NCT02101580), malignant pleural mesothelioma (NCT02709512), soft tissue sarcoma (NCT03449901), HCC (NCT02101593), and MM (NCT01665183), among others.

Structure and Reaction Mechanism

As already discussed, most studies performed to date used ADI isolated from *Mycoplasma arginini*. Based on the Protein Data Bank existing crystallographic structures and on their crystallographic structures, Das et al. (2004) proposed a

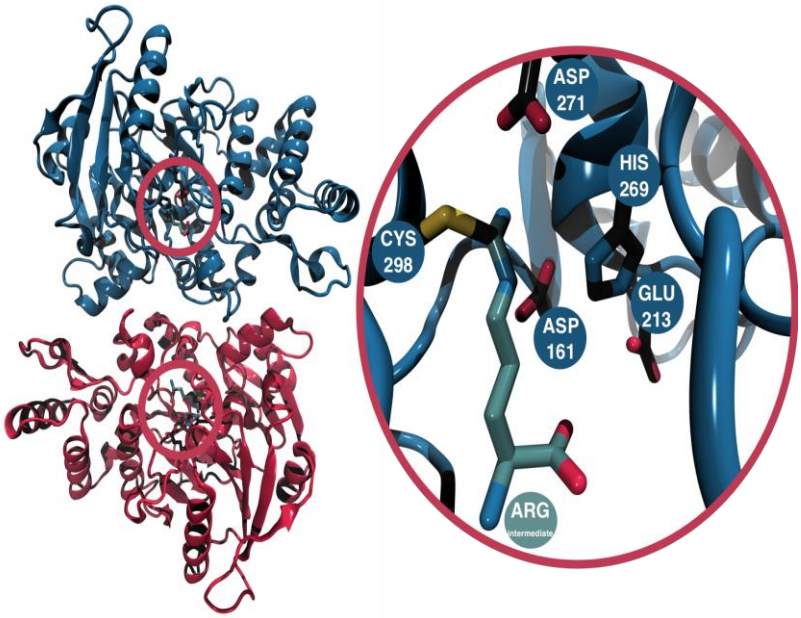

Figure X.2 – (left) Quaternary structure of ADI homodimer (PDB code: 1S9R) coloured by subunit; (right) Close-up view of the active site of ADI.

catalytic mechanism for ADI. Some of its the most important structural features (Das et al., 2004; Galkin et al., 2005; Lu et al., 2004; Lu et al., 2006; Smith et al., 1978) are represented in Fig. X.2 (PDB code 1S9R) (Das et al., 2004).

The analysis of existing data suggests that L-ARG enters into the enzyme's active site and is oriented to its more favourable catalytic position by a network of polar interactions between its guanidino moiety and the side chain carboxylates of ASP161 and ASP271 residues (**Fig.** X.2).

The proposed mechanism for the enzymes is represented in **Fig.** X.3 and comprises six sequential steps.

The ADI mechanism starts with a nucleophilic attack from CYS398 to Cζ carbon of the substrate and a series of simultaneous proton transfers, from

CYS398 to Nη2 nitrogen of the substrate, from Nη2 to HIS2696 and from HIS269 to GLU213. At the end of the first step, a tetrahedral intermediate is generated with the Cζ carbon of the substrate covalently bonded to CYS398 side chain (Step1).

In the second step, HIS269 gives a proton to nitrogen Nη2, generating an ammonia molecule. Simultaneously, HIS269 receives a proton from GLU213. At the end of this step, the ammonia molecule is released from the active site, and the reaction intermediate remains covalently bonded to CYS398 (Step 2).

The next step of the catalytic process requires the intervention of a water molecule that is in close proximity to the ASP271 and HIS269 residues. This water molecule is involved in a nucleophilic attack to carbon Cζ of the reaction intermediate and simultaneously donate a proton to HIS269 that, by its turn, donates a proton to GLU21. The structural data suggests that this reaction may be endorsed by the close proximity of ASP161 that establishes two hydrogen bonds with the -NH groups of the intermediate that turns Cζ carbon more electrophilic. At the end of this reaction, another tetrahedral intermediate is generated covalently bonded to CYS398 . Contrary to the first step (Step 1), this one has a hydroxyl group attached to Cζ carbon instead of an amino group (Step

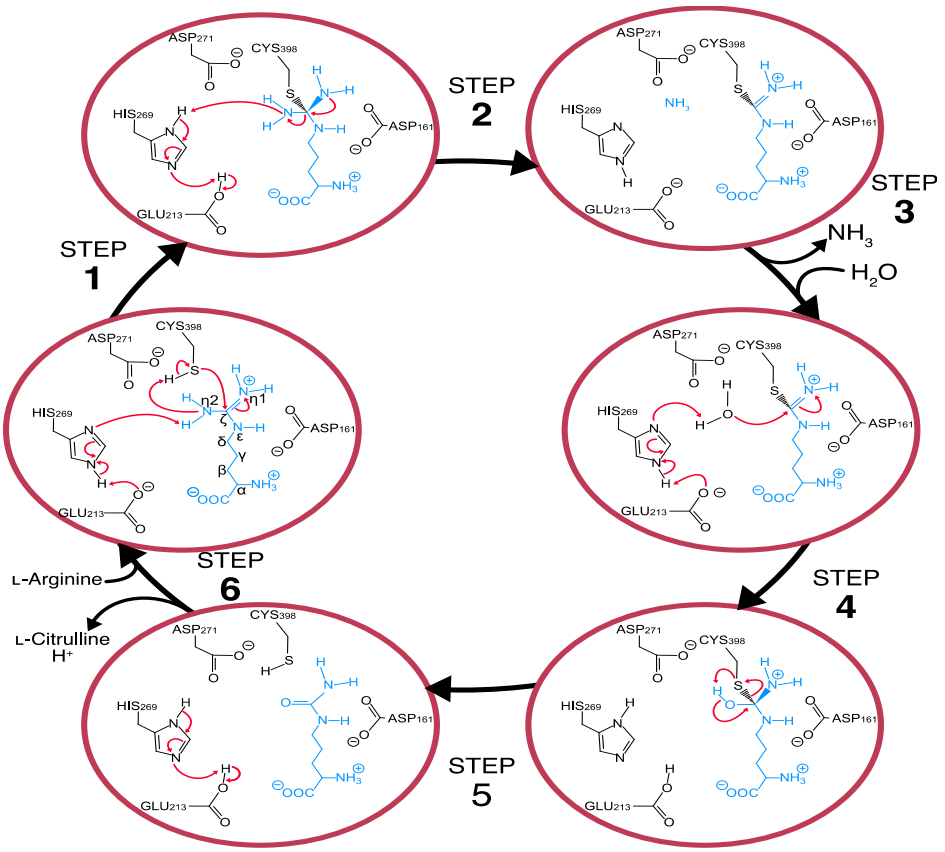

Figure X.3 Schematic representation of the current proposal of the catalytic mechanism of ADI.

4).

In the following step, a proton is transferred from the hydroxyl group of the reaction intermediate to CYS398, with the consequent cleavage of the covalent bond between CYS398 and the substrate. Simultaneously, there is a proton transfer from GLU213 to HIS269. This reaction generates a molecule of L-citrulline that is ready to leave the active site (Step 5).

The last step is the enzymatic turnover in which HIS398 loses a proton to the solvent or an unknown neighbouring active site residue and the enzyme becomes ready for a new catalytic cycle (Step 6).

B. Arginase

Arginase (EC 3.5.3.1) was the first enzyme used to deprive cancer cells of L-ARG. It is found in organisms from the five kingdoms and is one of the very few enzymes that specifically requires a spin-coupled Mn^{2+} - Mn^{2+} cluster for catalytic activity *in vitro* and *in vivo* (Maharem et al., 2018; Reczkowski and Ash, 1992).

There are two genetically distinct isozymes named types I and II. They share approximately 60% amino acid sequence homology and differ in their expression, regulation, tissue distribution, subcellular locations in mammals, 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).

Arginase I (ArgI) is the only of the two isozyme types with demonstrated anti-cancer therapeutic potential. It is a cytosolic enzyme found predominantly in liver hepatocytes, where it catalyses the hydrolysis of L-ARG to L-ornithine and urea. This is the final cytosolic step of the urea cycle, which detoxifies ammonia in mammals (Krebs, 1973). L-ornithine can be further metabolized to polyamines via ornithine decarboxylase (ODC). Then, it can generate L-proline via ornithine aminotransferase (OAT), or it can be converted to citrulline by ornithine transcarbamylase (OTC) (Morris Jr., 2002).

Recent studies show that tumour inhibition mediated by ArgI is only effective in OTC deficient cells and is independent of ASS expression (Cheng et al., 2007). OTC is an enzyme highly expressed in liver and small intestine, but its expression in cancer and other normal tissues is mostly down-regulated (Delers et al., 1984).

A metabolic functional cell possessing OTC will avoid the intracellular depletion of arginine by converting the ArgI product ornithine into citrulline, which can be converted back to L-ARG via ASS. An OCT deficient cell when deprived of L-ARG is not capable to generate the intermediate metabolite citrulline, which is the substrate of ASS, culminating in the complete depletion of intracellular L-ARG. In the absence of its substrate, the ASS presence in the OTC-deficient cells is irrelevant. A recent study showed that this simple explanation might not be fully correct and applicable to all cancer cells. In particular, the results showed that some OTC and, partially ASS, deficient cancer cells exhibit

high resistance to L-ARG deprivation *in vitro* (Bobak et al., 2010). This means that the inhibitory effects of L-ARG depleting agents on tumour growth require further investigation in order to be fully understood.

The individual evaluation of the expression of both OTC and ASS in each cancer seems to be a useful request before deciding which of the two enzymes should be used as in the L-ARG depriving therapy.

The administration of ArgI for the treatment of tumours has been reported since the 50's (Simon-Reuss, 1953; Wiswell, 1951). Although the *in vitro* experiments were very promising at the time, the scale-up to *in vivo* tests was discouraging due to ArgI low affinity for L-ARG (K_M: 6 mmol/L at physiologic pH), its alkaline optimum pH (pH 9.6), and its very short circulatory half-life time (aprox. 30 minutes) (Savoca et al., 1979).

The construction of a recombinant human arginase I (rhArgI), which was only possible after the advent of recombinant DNA technology, brought a renewed interest in the use of arginase as an anti-tumour agent.

In the last years a pegylated cobalt-modified enzyme was developed. The substitution of the Mn^{2+} metal centre in human ArgI with Co^{2+} (Co-rhArgI) results in an enzyme that displays 10-fold higher catalytic efficiency for L-ARG hydrolysis, 12–15 fold reduction in the IC_{50} towards a variety of malignant cell lines and a half-life time of 22 hours in serum. The enhanced pharmacological properties of Co-hArgI translate into a 12–15 fold improvement in the killing of melanomas and hepatocellular carcinomas *in vitro* (Stone et al., 2010). Later, the same group reported that the modification of Co-hArgI with PEG-5K NHS esters increased the retention of the enzyme in circulation by about 2 orders of magnitude (Glazer et al., 2011).

This modified version of bioengineered Co-rhArgI–PEG was proven to be cytotoxic in HCC (Cheng et al., 2007), melanoma (Lam et al., 2011), pancreatic carcinoma tumour xenografts (Glazer et al., 2011), acute myeloid leukaemia (Tanios et al., 2023), and glioblastoma cells (Khoury et al., 2015).

Beyond the already discussed particularities between rhArgI and ADI sensitive cancers, there are other differences that must be mentioned. One is the fact that contrary to ADI, rhArgI is a human enzyme and consequently non-immunogenic. The other is the greater toxicity of the rhArgI relative to ADI, which according to some authors may be due the lack OTC in normal tissues and the consequent impossibility to rescue L-ARG from ornithine. The authors proposed that providing citrulline supplementation to patients undergoing rhArgI mediated L-ARG deprivation could lower the treatment toxicity and eventually improve the therapeutic index, particularly in patients with ASS-negative metastatic cancers (Mauldin et al., 2012).

Therapeutic uses

By 2018, the number of clinical trials using rhArgI is quite lower than the number of clinical trials with ADI. Also, to our knowledge, to date there is no phase III clinical trial completed. There are at least four clinical trials completed, involving

phase I and II. One of them aimed to determine whether rhArgI-PEG was safe and effective in the treatment of advanced HCC (NCT01092091). It was concluded that rhArgI-PEG has a manageable safety profile and is potentially a superior L-ARG depleting agent than ADI in the treatment of HCC due to its low toxicity profile, apparently inexistence of immunogenicity and sustainable L-ARG depletion. The optimal therapeutic dose of rhArgI-PEG was defined as 1600 IU/kg in advanced HCC patients. In contrast to ADI-PEG20, rhArgI-PEG was administrated intravenously (Yau et al., 2013). This clinical study followed a first one performed by the same research group, where pegylated recombinant human ArgI was administrated in 8 escalation doses alone and in combination with standard doses of doxorubicin (NCT00988195).

Two other completed studies evaluated the safety and efficacy of rhArgI-PEG in combination therapy in patients with advanced HCC (NCT02089633) and the safety of increasing dose levels of Co-rhArgI-PEG in patients with Relapsed or Refractory Acute Myeloid Leukaemia or Myelodysplastic Syndrome (NCT02732184).

There are at least two clinical trials recruiting patients: one of them intents to evaluate the safety of rhArgI-PEG to treat patients with L-ARG auxotrophic advanced solid tumours (melanoma, renal cell carcinoma, prostate cancer, and hepatocellular carcinoma), and that have progressed after receiving approved or established therapies (NTC02285101). The other one aims to study the safety of increasing dose levels of Co-rhArgI-PEG in patients with advancer cancers (NCT02561234). One phase II clinical trial is active but not recruiting. Its objective is to evaluate the efficacy of rhArgI-PEG in patients with relapsed or refractory acute myeloid leukaemia in terms of remission rate (NCT02899286). Finally, there is an international phase I/II trial that is not yet recruiting and whose objective is to evaluate the safety and activity of rhArgI-PEG in children and young people with relapsed/refractory leukaemia, neuroblastoma, sarcoma, and high grade gliomas (NCT03455140).

Structure and Reaction mechanism

hArgI is an oligomer with 107 kDa with optimum pH at 9.3 and a K_M for L-ARG of about 10.5 mM (Beruter et al., 1978). The enzyme has some particular structural features that includes spin-coupled Mn^{2+} – Mn^{2+} cluster and an S-shaped tail, 19 amino acids long, located at the subunit-subunit interface. This S-shaped tail mediated 54% of all the intersubunit interactions (Lavulo et al., 2001).

There are several proposals for the catalytic mechanism of hArgI, (Leopoldini et al., 2009; Christianson and Cox, 1999; Cox et al., 2001; Khangulov et al., 1998), although many aspects remain elusive. The analysis of the crystallographic structure of the human ARGase in complex with L-ornithine (PDB code: 3GMZ) (Fig. X.4) (Ilies et al., 2011) reveals some clues about the important catalytic residues, and their possible role in the catalytic mechanism. GLU277 is one of the residues that stands out. It is an active site residue that is apparently implicated in the substrate binding through the formation of a strong electrostatic

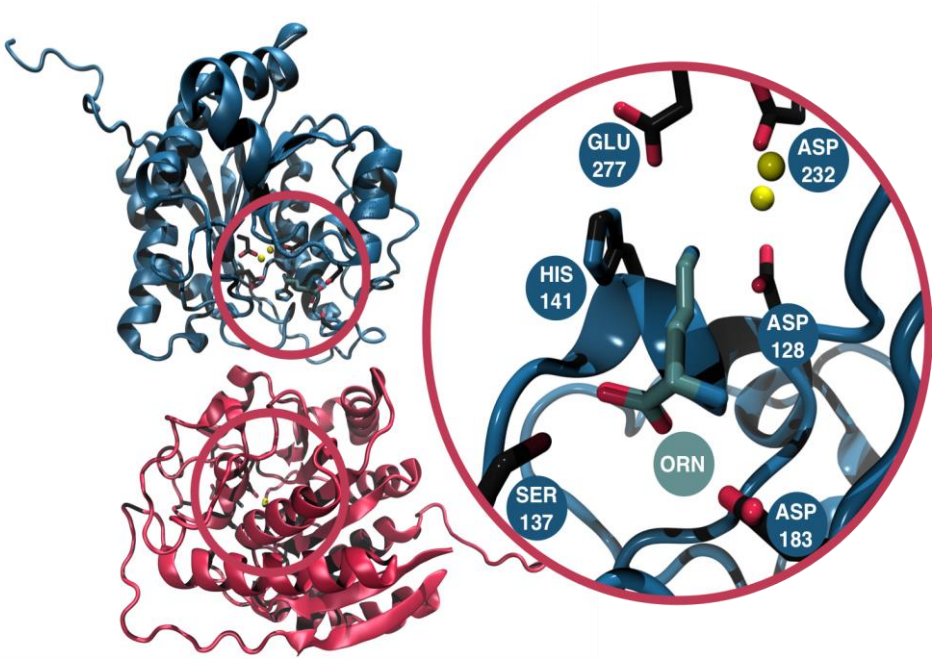

Figure X.4 – (left) Quaternary structure of hArgl homodimer (PDB code: 3GMZ) coloured by subunit; (right) Close-up view of the active site of the enzyme.

interaction with the guanidium group. This interaction places the substrate in a favourable 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). Additionally, GLU277, along with a hydroxide anion and ASP232 and ASP128 are coordinated with the Mn^{2+} ions.

The proposed mechanism for this reaction is represented in **Fig.** X.5 and comprises six sequential steps.

The reaction starts with the binding of the L-ARG's guanidine group to Mn^{2+} ions. After its correct positioning, L-ARG undergoes a nucleophilic attack from the bridging hydroxy anion to its carbon Cζ (Step 1) originating a tetrahedral intermediate. In the second step, ASP128, which is thought to be deprotonated (Leopoldini et al., 2009), acts as an acid/base catalyst, abstracting a proton from the hydroxyl group and shuttling it to nitrogen Nε of the substrate (Step 2). In the third step, the bond between nitrogen Nε and Carbon Cζ from the substrate breaks, generating the reaction products: L-ornithine and urea (Step 3). The next steps will promote product dissociation from the enzyme. It requires the entry of a water molecule that will bridge the binuclear Mn^{2+} cluster, causing the urea product to move to a terminal coordination site on Mn^{2+} (Step 4). Then, HIS141 residue acts as a proton shuttle from the bulk solvent to nitrogen Nε of ornithine. This proton transfer facilitates product release and regenerates the nucleophilic metal-bridging hydroxide anion (Step 5). Finally, ornithine dissociates and the enzymatic turnover takes place preparing the enzyme for a new catalytic cycle (Step 6).

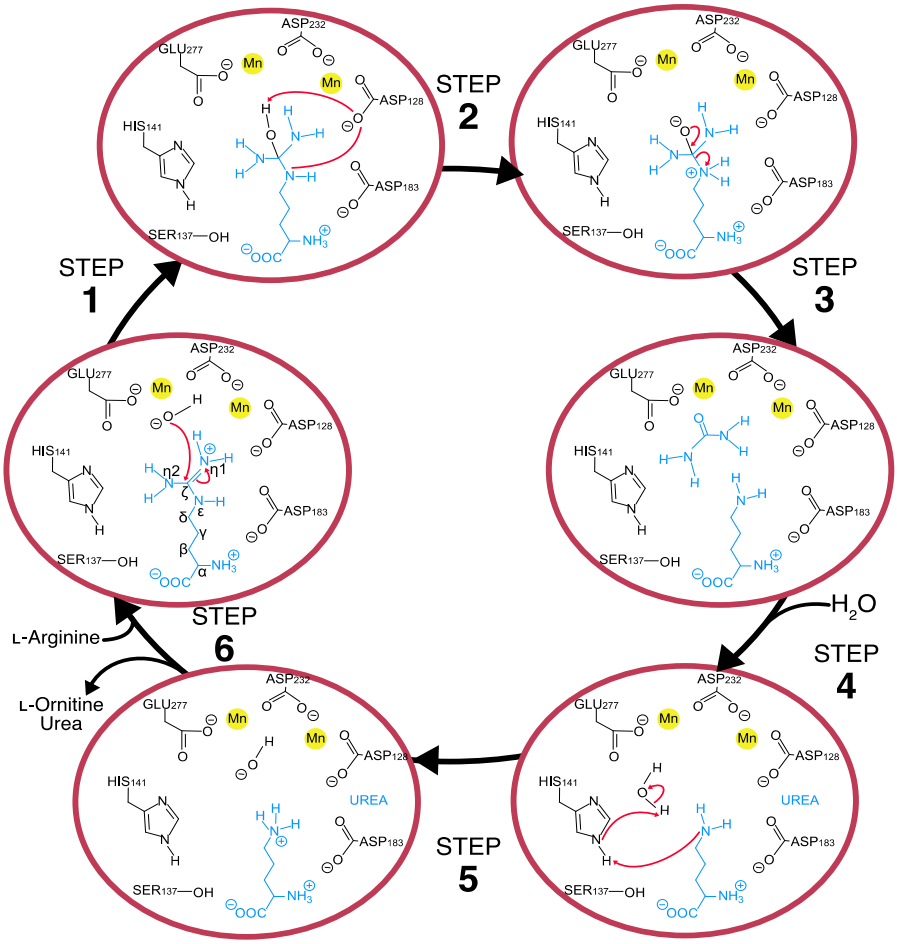

Figure X.5 Schematic representation of the Current proposal of the catalytic mechanism of hArgI.

X.2.2 *Asparagine*

L-Asparagine (L-ASN) is a non-essential amino acid that is synthesized in humans by the asparagine synthetase enzyme (Lomelino et al., 2017). However, L-ASN is indispensable for the synthesis of several proteins, in particular, the ones that are glycosylated through N-glycosylation (Cherepanova and Gilmore, 2016) Moreover, this amino acid is involved in the control of some cell functions in the nervous system, and also in the metabolism of ammonia to enable the elimination of this toxic metabolic by-product (Erecinska et al., 1991; Chen and Chen, 1992).

In humans, the asparagine synthetase catalyses the production of L-ASN through an ATP-dependent reaction, where L-aspartate and L-glutamine are converted to L-ASN and L-glutamate (Lomelino et al., 2017; Richards and Kilberg, 2006).

Although cells can produce their own supply of L-ASN, some types of cancer, such as leukaemia (Shahriari et al., 2017; Wetzler et al., 2007), breast (Knott et al., 2018; Yang et al., 2014), gastric (Yu et al., 2016), and lung (Xu et al., 2016) cancers, are impaired in the pathways related to the synthesis of L-ASN. Consequently, these tumours become dependent on an external supply of L-ASN, which offers a specific opportunity window to target cancer using L-ASN deprivation therapies.

L-Asparaginase (ASNase) is an enzyme available in bacteria, plants, and fungi that catalyses the hydrolysis of L-ASN to L-aspartate and ammonia (Gesto et al., 2013). The adequate administration of ASNase in the bloodstream can reduce the plasma levels of L-ASN and promote tumour growth slowdown by starving the cancer cells.

Kidd observed, for the first time, the therapeutic potential of ASNase as an anti-tumour approach in two different types of lymphoma. (Kidd, 1953; 1953a) However, at the time, the role of ASNase was unknown because Kidd only reported that the gynae pig serum was able to regress the lymphomas in murine. Later on, Broome and Dolowy were able to identify and isolate ASNase from the gynae pig serum, and then, they understood that the antitumor effect was due to the ASNase (Broome, 1961; 1963; Dolowy, 1966). These studies were pioneer to point out ASNase as an enzyme with great therapeutic potential against cancer.

ASNase (EC 3.5.1.1) is an enzyme available in several bacteria genus, namely *Bacillus*, *Pseudomonas* (Saeed et al., 2018), *Serratia*, *Aerobacter*, *Photobacterium, Xanthomonas*, (Peterson and Ciegler, 1969) *Proteus* (Tosa et al., 1971), *Streptomyces* (DeJong, 1972), *Vibrio* (Kafkewitz and Goodman, 1974), and *Aspergillus* (Sarquis et al., 2004). Besides the broad spectrum of microorganism that has ASNase in their proteome, ASNases derived from *Escherichia coli*, and *Erwinia chrysanthemi* showed the most promising results in anticancer therapy. The *E.coli* form is the main source of ASNase because of its capability to produce the enzyme in great quantities. The *E. chrysanthemi* form is an alternative when hypersensitivity reactions occur in patients treated with the *E.coli* ASNase. In fact, the immunogenicity is the biggest drawback of the use of these non-human enzymes in anticancer therapy.

ASNase from *E.coli* presents two different forms, ASNase-I and ASNase-II, which are different regarding its cellular location and affinity for the substrate. ASNase-I is present in the cytosol, and it has a lower affinity for the substrate (L-ASN) when compared with ASNase-II that is located in the periplasmic space (region between the inner cytoplasmic membrane and the bacterial outer membrane) (Yao et al., 2005). Due to the higher affinity of ASNase-II for the substrate, this enzyme has been extensively studied for application in anticancer therapy.

Therapeutic uses

ASNase-II has been used as a general therapy agent for the treatment of acute lymphoblastic leukaemia, and other related blood cancers (Shrivastava et al., 2016). The administration of ASNase causes a decrease in the circulating pool of

L-ASN, starving the susceptible cancer cells whose cell cycle becomes arrested in the G1-phase, which causes apoptosis (Lee et al., 1989; Keating et al., 1993).

Besides the excellent therapeutic efficiency, ASNase has a short half-life time in the bloodstream, causing a higher demand for injections to keep the therapeutic level. Consequently, this procedure leads to some side effects, namely allergies, exacerbated immune responses, and even anaphylactic shock (Mashburn and Wriston, 1964; Moola et al., 1964Soares et al., 2002; Henriksen et al., 2015).

Similarly to what happens with other enzymes used in therapy, pegylation is one of the most appropriate manners to improve de plasma half-life and reduce the immunogenicity of the ASNase. Pegylation is a process in which the enzyme is modified with 5 kDa units of monomethoxypolyethylene glycol (PEG) to reduce de exposition of immunogenic patterns in the enzyme's surface (Rytting, 2010). The pegylated ASNase keeps its enzymatic activity, but the half-life time in the bloodstream increases about 5 times, and the immunogenicity is also lower (Zeidan et al., 2008).

Currently, pegylated ASNase is being used as first-line therapy in the USA, UK, Australia, and New Zealand, and as second-line therapy in Europe, where it is used when the patients are known to have an allergy to the free ASNase (Fu and Sakamoto, 2007). The application of ASNase was approved by FDA in 2006 and it is commercialized under the name Oncaspar®. Ten years later, in 2016, EMA approved Oncaspar® to be used in European Union.

Although all these formulations make possible the use of ASNase as an anticancer therapeutic target, the formulations currently available are far from perfect. The major side effect continues to be the immunogenicity derived from the bacterial source of the ASNase. There are well-known side effects caused by the administration of ASNase in patients with cancer, namely hypersensitivity reactions (Avramis and Tiwari, 2006), coagulation disorders (Hernandez-Espinosa et al., 2006), pancreatitis, hyperglycaemia (Raja et al., 2012), and hepatotoxicity (Bodmer et al., 2006). These side-effects rise an intense search for alternatives in order to identify other isoforms, and enzymes from other organisms, that show, at least, the same efficacy, and attenuated side-effects (Thomas et al., 2010; Shrivastava et al., 2012; Mendes et al., 2012).

Structure and Reaction Mechanism

ASNase-II from *E.coli* is a homotetramer whose subunits have 326 amino acids each (Fig. X.**6**). The pocket where the enzyme binds the substrate and actively catalyses the reaction is composed of amino acid residues from two adjacent subunits: THR12, TYR25, SER58, GLN59, THR89, ASP90, and LYS162 from one subunit, and ASN248 and GLU283 from the adjacent subunit. These residues establish a strong set of hydrogen bond interactions that includes a conserved water molecule (Fig. X.6). There were several plausible proposals for the catalytic mechanism of ASNase-II, but the symmetry of the location of THR12 and THR89

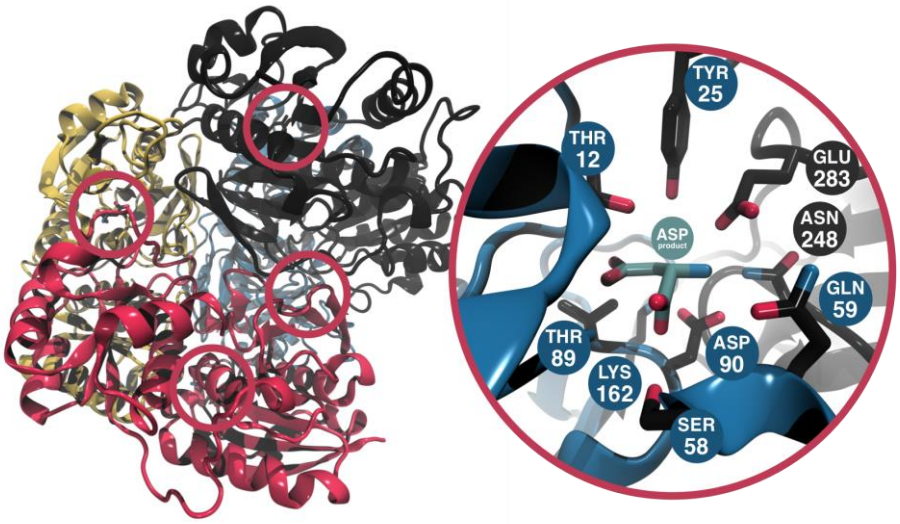

Figure 6 - (left) Quaternary structure of the ASNase (PDB code: 3ECA) colored by subunit. (right) Representation of the important amino acid residue, and the product in the active site of the ASNase. The molecules are tagged by residue name and residue index.

residues, which enclose the C2 carbon of the substrate, suggests a direct involvement in the catalytic mechanism (Swain et al., 1993).

Currently, the catalytic mechanism of ASNase-II is known and supported by several computational, and experimental studies (Gesto et al., 2013; Borek et al., 2014; Schalk et al., 2016).

The catalytic process starts with the formation of a tetrahedral intermediate

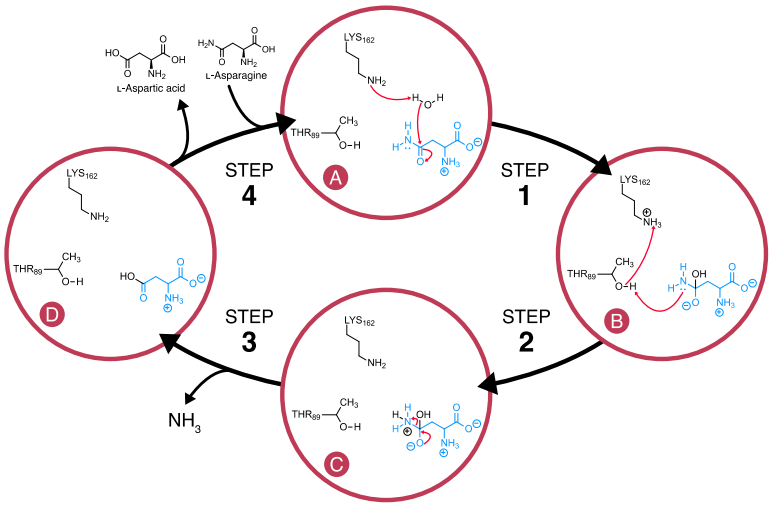

Figure X.7 - A schematic representation showing a proposal for the catalytic mechanism of the ASNase. A-D are reaction intermediates.

after the nucleophilic attack of the water molecule to carbon Cα of the L-ASN (substrate) (Fig. X.7 – Step 1). Since the water molecule establishes a new covalent bond with the substrate, one of its protons is passed to the amino group of LYS162 that becomes positively charged.

In the second step, LYS162 residue protonates the amino group of the substrate in a proton transfer mediated by the THR89 residue (Fig. X.7 – Step 2). Afterwards, the amino group is eliminated as ammonia, and the aspartic acid is formed (**Fig.** X.7 – Step 3). Finally, both products are released, and the enzyme becomes available to bind a new substrate molecule to initiate another catalytic cycle (**Fig.** X.7 – Step 4) (Gesto et al., 2013).

X.2.3 *Methionine*

L-Methionine (L-MET) is one of two sulphur-containing proteinogenic amino acids. This essential amino acid for humans (Young, 1994) acts as an essential precursor for the synthesis of cysteine (through the production of S-adenosylmethionine), taurine, glutathione (a vital tripeptide that reduces reactive oxygen species (ROS), protecting cells from oxidative stress), and polyamines spermidine and spermine (crucial for nuclear and cell division (Cellarier et al., 2003; Davis and Uthus, 2004; Ho et al., 2011; Thivat et al., 2007). Although all these roles are essential for the cell wellbeing, the most important role of L-MET is as a source of methyl units for the DNA methylation through its intermediate S-adenosylmethionine.

It is well known that the DNA methylation is one of the most significant epigenetic signalling markers, controlling the gene expression in several processes such as X-chromosome inactivation, genomic imprinting, repression of repetitive elements, and carcinogenesis (Geiman and Muegge, 2010; Ramani et al., 2010). Regarding the carcinogenesis, in some types of cancer, e.g. endometrial (Fiolka et al., 2013), breast (Chimonidou et al., 2011), lung (Powrózek and Małecka-Massalska, 2016), and myeloma (Wong and Chim, 2015) cancers, the tumorigenic process has been associated with the hypermethylation of the promoter regions of tumour-suppressor genes (Kulis and Esteller, 2010). Considering the dependence of cells of L-MET for the DNA methylation event, it was suggested that strategies leading to L-MET deprivation could be used as therapy against cancer. Since, L-MET is a crucial amino acid in the synthesis of almost all proteins of the cell, this means that the enzyme cannot be applied in long treatments and must be inactivated.

Since L-MET is an essential amino acid, the L-MET deprivation could be harmful for the human body, but it was proved that long-term nutritional deprivation of L-MET does not compromise the human life because cells have several pathways to ensure the L-MET recycle. L-MET can be recovered through the re-methylation of homocysteine by the L-Methionine Synthase (MS) or, in the liver, by the Betaine-Homocysteine Methyltransferase (BHMT) (Cellarier et al. 2003; Hoffman, 1984; Guo et al., 1993). Additionally, Methylthioadenosine

Phosphorylase (MTAP) is also able to catalyse the production of L-MET from 5'-methylthioadenosine.

Although all of these salvage pathways can restore the required levels of L-MET, several malignant cell lines, including breast, lung, colon, kidney and bladder cancers, glioblastoma and melanoma, have suppressed these salvage pathways (Cavuoto and Fenech, 2012). Consequently, the cancer cells are dependent on an exogenous supply of L-MET, manifesting a growth inhibition when this supply is suppressed (Jeon et al., 2016; Chaturvedi et al., 2018).

The high demand for L-MET in the tumour cells as a result of their great protein synthesis and the lack of L-MET salvage pathways make L-MET essential for the tumour growth and proliferation. Therefore, the L-MET restriction can be exploited as a cancer therapy, in particular for these L-MET-dependent tumours (Chaturvedi et al., 2018).

In the literature, two different approaches have been documented with therapeutic promising results using : the dietary deprivation of L-MET and the use of L-MET depletion enzymes. The nutritional restriction of L-MET showed promising results in animal studies, but this diet showed a limited efficiency in clinical trials (Chaturvedi et al., 2018; Thivat et al., 2009). For example, a phase I clinical trial reported a poor reduction of L-MET in plasma (58%) after 17.3 weeks of L-MET dietary restriction (Epner et al., 2002).

Due to the lack of effectiveness in reducing the serum levels of L-MET through nutritional restriction, Methioninase or Methionine γ-lyase (MGL) has been studied to achieve the desired level of reduction. Several studies reported a fast and effective depletion of L-MET from serum, showing a vast efficiency against human tumours graphs implanted in mice. A pilot study was conducted in three patients showing a drastic reduction in plasma L-MET from 23.1 μM to 0.1 μM (reduction of aprox. 99.6%) when the enzyme was administrated by a 10-hour intravenous infusion (Tan et al., 1996a). Based on this report, MGL corroborated its efficiency in reducing serum L-MET levels. Moreover, a slowdown in the tumour growth and spread necrosis in tumour regions were observed in either the MHL administered alone or in combination with other drugs (Yoshioka et al., 1998; Tan et al., 1999; Murakami et al., 2017, Kawaguchi et al., 2017, Kokkinakis et al., 2001).

Beyond the depletion effect of MGL, this enzyme can also be administered in combination with the prodrug selenomethionine. The MGL can produce methyl-selenol from the selenomethionine, which causes oxidation of thiol groups generating toxic superoxide molecules that cause cell death. By this way, MGL can be fused with a specific antigen to target the tumour cell and, then, the selenomethionine is administrated, being active only in the area where the MGL is located (Zhao et al., 2006; Guillen et al., 2014).

Therapeutic uses

MGL has a broad range of application in cancer therapies. – Since this enzyme is absent in mammals, MGL was initially isolated from *Clostridium sporogene*

(Sharma et al., 2014). Later on, the enzyme from *Pseudomonas putida* was chosen for clinical purposes, due to its greater stability and affinity for the substrate (low K_M) (Esaki and, Soda, 1987). Therefore, the MGL gene from *P. putida* was isolated and expressed in *Escherichia coli* to produce the recombinant enzyme in great quantities for administration in humans (Hori et al., 1996).

The use of an enzyme from another organism raises complications related to the immunogenicity of the recombinant enzyme and its low stability in the bloodstream. In order to overcome this problem, the recombinant MGL was modified with polyethylene glycol (PEG). The pegylated enzyme presented low immunogenicity, a longer half-life time, and greater resistance against degradation by other enzymes in the bloodstream (Tan et al., 1999; Kudou et al., 2007, Takakura et al., 2006). Pegylation can overcome these issues but causes a decrease in the affinity for the PLP cofactor which decreases the efficiency and specificity of the MGL. Fortunately, this issue was easily solved through the supplementation of the patients with PLP and oleic acid (Takakura et al., 2006).

Although all these promising results, there are only one phase I clinical trials addressing the viability to administered intravenously MGL to deplete plasma L-MET. The pegylated MGL was tested in three healthy people reporting no significant toxicity and a considerable capacity to decrease serum L-MET level. In sum, this pilot phase I clinical trial reported that the administration of MGL is safe and able to effectively reduce L-MET level in the bloodstream (Tan et al., 1996; Tan et al., 1997). However, the clinical trials must continue to corroborate the effectiveness of MGL to treat cancer.

Since the recombinant MGL presents strong applicability for the cancer treatment, the molecular details, in particular, the catalytic mechanism can provide valuable insights to enhance its efficiency.

Structure and Reaction Mechanism

MGL (EC 4.4.1.11) is a pyridoxal-5'-phosphate (PLP)-dependent enzyme that catalyses the reversible conversion of L-MET into methanethiol, ammonia, and 2-oxobutanoate (Kreis and Hession, 1973). The enzyme is a homotetramer with 398 amino acid residues in each subunit, and it has four active sites placed in each interface of two adjacent subunits (Fukumoto et al., 2012). Each one of the four active sites binds a PLP cofactor molecule, which is required for binding the substrate (L-MET) and triggering the reaction (Kudou et al., 2007, Motoshima et al. 2000; Tone, 2005).

Although MGL belongs to the γ-family of PLP-dependent enzymes, there is an important cysteine residue (CYS116) in the active site that is absent in the other enzymes of the same family. Mutagenic studies reported that the CYS116 residue is crucial for the enzymatic activity (Kudou et al., 2008) and substrate specificity (Kudou et al. 2007, Inoue et al., 2000). Beyond the CYS116 residue, there are five more residues with a key role in the mechanism based on structural, enzymatic, and mutagenesis studies: TYR59, ARG61, TYR114, ASP186, LYS211, LYS240, and ASP241 (Fig. X.8).

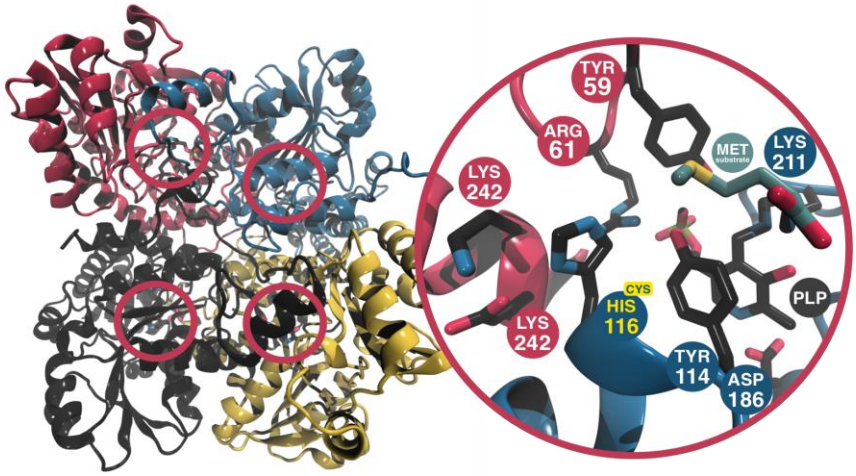

Figure X.8 – (left) Quaternary structure of the MGL (PDB code: 3VK3) colored by subunit. (right) Representation of the important amino acid residues, the PLP cofactor, and the substrate in the active site of the MGL. The molecules are tagged by residue name and residue index. The CYS116 was mutated by a HIS residue to allow the co-crystallization of the MGL with the natural substrate (L-MET).

The proposed catalytic mechanism for MGL is based on these studies and the available knowledge for other PLP-dependent enzymes (Kudou et al. 2007, Cerqueira et al., 2011; Oliveira et al., 2011; Fernandes et al., 2017; 2018).

All PLP-dependent enzymes wait for their substrate in a steady state where the PLP is covalently bonded to a lysine residue (LYS211) forming an internal aldimine (Fig. X.9A). When a molecule of substrate enters inside the active site, its amino group makes a nucleophilic attack (Fig. X.9 – Step 1) to the C8 of the PLP cofactor generating a gem-diamine intermediate (Fig. X.9B). Immediately after, the bond between the LYS211 residue and the PLP cofactor is broken, and the substrate (L-MET) becomes covalently bonded to the PLP cofactor (Fig. X.9 – Step 2), originating an intermediate commonly called external aldimine (Fig. X.9C).

In the next step, the proton bonded to the Cα of the L-MET is abstracted from an unknown base from the active site (Fig. X.9 – Step 3). Based on the crystallographic structure and the catalytic mechanism of other PLP-dependent enzymes, the LYS211 residue could be the base involved in this reaction. The resulting electron pair is stabilized across the PLP conjugated system forming a quinonoid intermediate (Fig. X.9D).

In the fourth step, the proton abstracted in the previous step is transferred to carbon C8 of the PLP cofactor, which increases the acidity of the protons bonded to the Cβ of the L-MET. Consequently, in the fifth step, the base can get one of these protons.

The abstraction of the proton bonded to carbon Cβ atom triggers the elimination of the methanethiol group (γ-elimination) (Fig. X.9 – Step 6), which is rapidly protonated by the base in the active site. In the meantime, the obtained

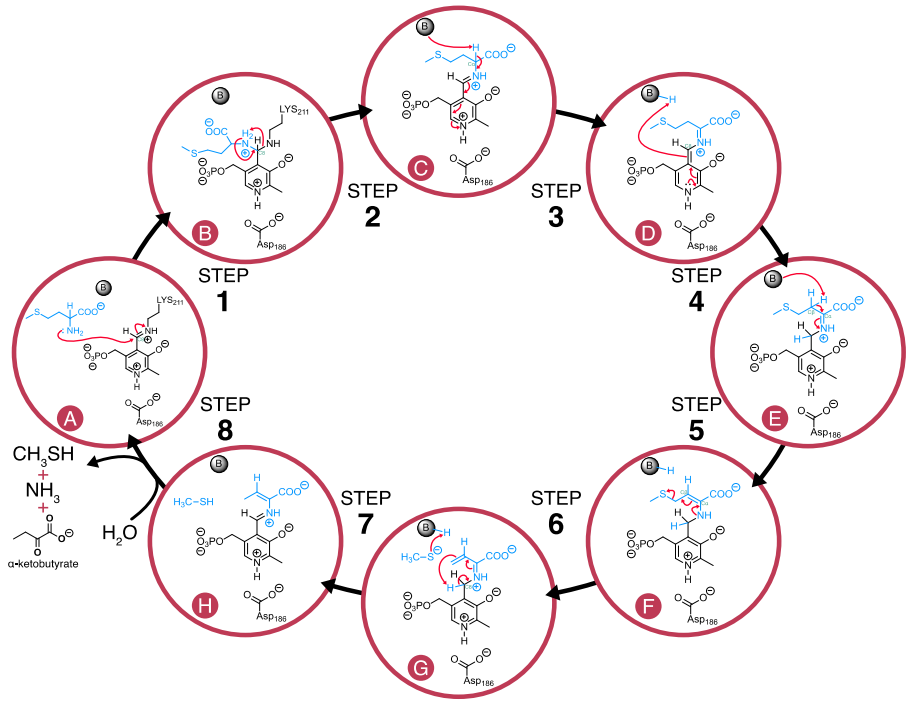

Figure X.9 – A schematic representation showing a proposal for the catalytic mechanism of the MGL. A-H are reaction intermediates, and the "B" sphere represents a generic molecule in the active that can behave as a base.

amino-crotonate intermediate (Fig. X.9H) undergoes hydrolysis of its amino group. In this eighth step, the amino group is eliminated as an ammonia molecule, and a water molecule replaces that position as a carbonyl group.

In the end, the LYS211 becomes covalently bonded to the PLP cofactor, and the enzyme restores its initial configuration where the internal aldimine (Fig. X.9A) is ready to catalyse a new reaction as soon as the substrate becomes available.

X.3 CONCLUSIONS

Amino acids are no longer regarded solely as building materials but also as sensing markers that regulate important signalling pathways that can be used for therapeutic purposes.

In the last decades, research on amino acid depletion in cancer cells using enzymes has provided valuable insights on the potential impact of metabolic control and regulation in the tumour microenvironment. These studies have shown that decreasing the concentration of specific amino acids in the bloodstream, which are required in high demands by tumour, can be an effectiveness way to treat the tumour.

The arginine dependence of some tumour cells, although known for more than 60 years, has failed to reunite by 2018 all the safety and efficacy conditions to become approved for use in therapy.

Although arginase was initially pointed as a potential new medicine against cancer cells, its inherent limitations, insurmountable at the time, turned the attentions to ADI. In 2018 there are more than twenty clinical trials ongoing or completed whose aim is to evaluate the potential of ADI as a safe and effective anti-cancer treatment target. The results from a recently finished phase III clinical trial (NCT01287585) were not as encouraging as expected but did not withdraw the researcher's attention from ADI.

In the meanwhile, new strategies to improve arginase potential have arouse and at the moment there is a completed clinical trial (NCT01092091) were it was shown that rhArgI-PEG has a manageable safety profile and is potentially a superior arginine depleting agent than ADI in the treatment of HCC. At the moment there are about ten clinical trials in different stages evaluating the effectiveness and safety of arginase as a potential anti-cancer therapeutic targets. The combination of the different arginine depleting enzymes with existing therapy is also being tested. The observation that patients submitted to prolonged arginine depletion showed a relative superior overall survival is a valid reason to keep the exploitation of arginine deprivation as a potential therapy against some cancers.

The L-ASN depletion has already been used in therapy after some modifications to overcome some of the most common side-effects as immunogenicity. Therefore, the pegylated ASNase is used as first-line therapy in the USA, UK, Australia, and New Zealand, and as second-line therapy in Europe against acute lymphoblastic leukaemia and other related blood cancers. Although the evolution of these formulations, the research on improvements that can increase the efficacy and suppress the side-effects goes on, in particular, to overcome some cases of hypersensitivity reactions, pancreatitis, hyperglycemia, and hepatotoxicity. Contrary to the L-ASN, the L-MET depletion therapies against cancer are taking the first steps. The bacterial origin of the MGL enzyme is the source of major complications in the use of this enzyme in therapy. Several cases of immunogenicity reactions have been reported, and although the pegylation strategy suppressed the immunogenicity and low half-life time of the enzyme in the bloodstream, this modifications cases a decrease in the affinity of the enzyme for the substrate. Thus, the amino acid deprivation strategy to treat cancer targeting the serum L-MET supply is currently in an early stage of development comparatively to L-ASN, for example.

As any new therapy targeting cancer, amino acid deprivation therapies must be explored with caution in order to eliminate potential negative impacts that can be caused by the side effects observed when the metabolism of normal cells is also affected. It is thus important to understand at the atomic-level the underlying mechanisms of metabolic function of the enzymes that are used in these therapies. At the same time this knowledge can be used to guide the development of new and more specific therapies alone or in combination with

other drugs that can decrease the number of side effects, and turn these treatments more effective.

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