Abstract. L-asparaginase enzymes have been a vital component of acute lymphoblastic leukemia therapy for >40 years. L-asparaginase acts by depleting plasma L-asparagine, which is essential to the survival of leukemia cells. In contrast to normal cells, tumor cells cannot synthesize L-asparagine and thus depend on its external uptake for growth. Currently, three bacterial L-asparaginases are used in therapy; however, they are associated with severe side-effects related to high toxicity and immunogenicity. The introduction of human L-asparaginase-like protein 1 in acute lymphoblastic leukemia treatment would avoid the problems caused by the bacterial enzymes; however, a major difficulty in the therapeutic use of the human enzyme comes from the fact that human L-asparaginase must be activated through an autoprocessing step, which is a low-efficiency process in vitro that results in reduced enzymatic activity. The present review article aimed to contribute to the understanding of the enzyme self-activation process and focuses on the efforts made for the development of a therapeutic variant of human L-asparaginase.

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1. Historical overview
L-asparaginases are enzymes that catalyze the hydrolysis of the amino acid L-asparagine in L-aspartate and ammonia. The research on enzymes with L-asparaginase activity reached the first milestone in 1904 when Lang first observed the hydrolysis of L-asparagine in bovine muscle tissues (1). In 1910, Fürth and Friedmann found the same hydrolytic activity in horse and pig organs, confirming Lang's discovery (2). A number of applications have been suggested for L-asparaginase activity in tissues of different species; however, it was the discovery of L-asparaginase activity in the blood of Cavia porcellus (guinea pig) in 1922 (3) that gave direction to the research that emerged 31 years later to change the panorama of leukemia treatment. In 1953, Kidd found that the serum of guinea pigs inhibited the proliferation of lymphoma in mice (4); however, the element of the serum responsible for the antitumoral activity remained unknown. After 10 years, Broome demonstrated that serum-susceptible lymphoma cells were not able to proliferate in culture media deficient in L-asparagine (5,6), and thus, the antitumor action was attributed to L-asparaginase. This set of discoveries (Table I) rapidly led to the search for new sources of the enzyme, which resulted in the discovery of tumor inhibition capacity by the L-asparaginase of Escherichia coli (7,8). In 1966, the first clinical tests in children with acute lymphoblastic leukemia revealed successful treatments with the L-asparaginase from C. porcellus (9) and, later, with the L-asparaginase from E. coli (10). Impressively, the first complete remission of a patient with ALL was reached in 1967 (11). Since 1978, L-asparaginase has been used in the treatment of ALL (12).

2. L-asparaginase in the treatment of acute lymphoblastic leukemia
ALL is known as the most common type of childhood cancer and corresponds to approximately 30% of all malignant neoplasms in children from 0 to 14 years of age (13). In addition, children under the age of 5 years constitute the group that presents the highest risk of ALL development (13). Although 80% of cases affect children, ALL also manifests in adults, in whom it tends to be more severe (14). While the 5-year survival rate for children is 91%, in adults >20 years of age, this rate
L-asparagine is an important non-essential amino acid for the growth and development of both healthy and neoplastic cells. As it acts on protein biosynthesis, the depletion of L-asparagine hinders cell proliferation (38). Reducing the concentration of L-asparagine in circulating blood from 50 to ≤3 µM during treatment with L-asparaginase (38) prevents leukemia cells from continuing the cell cycle, thereby activating apoptotic signaling (39). This mechanism comports with the evidence that depletion of an amino acid can lead to the induction of apoptosis or autophagy (36). In other types of cancer [ovarian cancer (40), chronic myeloid leukemia (41) and pulmonary adenocarcinoma (42)], L-asparaginase has been reported to be able of inducing not only apoptosis but also autophagy, since L-asparaginase functions as a negative modulator of this process (36,43).

Despite its primordial use in the treatment of ALL, L-asparaginase has therapeutic potential for use in other types of cancer, such as acute myeloid leukemia (41), ovarian cancer (40,43,44), brain cancer (45), prostate cancer (46), pulmonary adenocarcinoma (42), non-Hodgkin lymphoma (47), chronic lymphoid leukemia and sarcomas such as lymphosarcoma, reticulosarcoma and melanosarcoma (48).

Currently, three L-asparaginases are employed in ALL therapy: Native E. coli L-asparaginase II, a pegylated form of this enzyme [conjugated with the polymer polyethylene glycol (PEG)] and L-asparaginase isolated from Erwinia chrysanthemi (49). The selection of the type of L-asparaginase for the chemotherapeutic regimen depends on the country in which the treatment is performed. The choice of the enzyme isoform reflects the level of priority given to the reduction of side-effects and the maintenance of treatment efficacy (Table II). The cost of the intensification phase of ALL treatment with L-asparaginase can fluctuate between $47,610 and $133,554 per patient, depending on the selection of L-asparaginase and immunological reactions (50).

L-asparaginase II from E. coli induces the highest levels of toxicity and immunogenicity among the three products available. Following administration, circulating L-asparaginase II from E. coli is soon recognized by the cells of the immune system. Once the immune response is activated, the effect of the enzyme is neutralized (27,37,38). Even with a reduction of 50% in the dose of this enzyme from the BFM 95 to the BFM 2000 protocol (51,52), in 60% of patients, a hypersensitivity reaction associated with drug inactivation is induced. However, antibodies produced against L-asparaginase II from E. coli are not always accompanied by symptoms characteristic of a hypersensitivity reaction (anaphylaxis, edema, serum sickness, bronchospasm, urticaria and rash, pruritus, swelling of the extremities, or erythema); in approximately 30% of the patients, the enzyme inactivation is silent (27,37,38).

The pegylated E. coli ASNase and the unpegylated E. chrysanthemi enzymes are indicated as substitutes in these cases of patient hypersensitivity and/or inactivation. PEG-L-asparaginase is less immunogenic. However, its administration following treatment with the native enzyme may result in the silent inactivation due to a cross-reaction with anti-L-asparaginase antibodies already present in the patient (27,37,38). In addition, some patients present a genetic predisposition to E. coli L-asparaginase formulations, as variants in CNTO3 (53), NFATC2 (54), GRIA1 (55) and human leucocyte antigen (HLA) genes (53,54).
E. chrysanthemi L-asparaginase largely solves the problem of hypersensitivity since the chances of developing antibodies against this enzyme are 12-20% (38). In addition, the enzyme from E. chrysanthemi has fewer toxic effects, as hypertriglyceridemia and hepatic toxicity, compared to E. coli preparations (56). However, it has a shorter half-life time, and studies have reported a significantly higher number of patients who do not achieve complete remission of leukemia cells (37, 38, 57).

Although possible, the exchange of L-asparaginase during treatment due to hypersensitivity reactions is quite costly. Substituting pegylated L-asparaginase with E. chrysanthemi L-asparaginase can increase the treatment cost by 3-fold. Treatment using the native enzyme from E. coli is the option with the lowest cost (18% lower than the pegylated version); however, its subsequent substitution by other L-asparaginases may result in a cost increase by 5-fold (58). In addition to the effects resulting from the activation of the immunological response, the administration of multiple doses of L-asparaginase may generate toxic effects. The high toxicity induced by bacterial L-asparaginases (particularly L-asparaginase from E. coli) is related to its lack of hydrolytic specificity, also leading to the depletion of glutamine, which is converted into glutamate and ammonia by these enzymes.

Table I. Timetable of important discoveries related to L-asparaginases.

<table>
<thead>
<tr>
<th>Year</th>
<th>Discovery</th>
<th>(Refs.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1904</td>
<td>Identification of L-asparaginase activity in several muscular tissues</td>
<td>(1)</td>
</tr>
<tr>
<td>1910</td>
<td>Identification of L-asparaginase activity in horse and pig organs</td>
<td>(2)</td>
</tr>
<tr>
<td>1922</td>
<td>Identification of L-asparaginase activity in the blood of Cavia porcellus</td>
<td>(3)</td>
</tr>
<tr>
<td>1953</td>
<td>Identification of antitumor properties of C. porcellus serum</td>
<td>(4)</td>
</tr>
<tr>
<td>1961-1963</td>
<td>Antitumor activity of serum of C. porcellus attributed to L-asparaginase</td>
<td>(5,6)</td>
</tr>
<tr>
<td>1964-1965</td>
<td>Identification of Escherichia coli L-asparaginase in the inhibition of tumor growth</td>
<td>(7,8)</td>
</tr>
<tr>
<td>1966</td>
<td>Clinical trials with L-asparaginase of C. porcellus and E. coli for the treatment of children with acute lymphoblastic leukemia (ALL)</td>
<td>(9,10)</td>
</tr>
<tr>
<td>1967</td>
<td>First complete remission of a patient with ALL after treatment with E. coli L-asparaginase</td>
<td>(11)</td>
</tr>
<tr>
<td>1978</td>
<td>Approval by the Food and Drug Administration (FDA) regulatory agency of United States for the use of E. coli L-asparaginase (Elspar®) for ALL treatment</td>
<td>(12)</td>
</tr>
<tr>
<td>1994</td>
<td>Approval by FDA for the use of pegylated E. coli L-asparaginase (Oncaspar®) for ALL treatment</td>
<td>(106)</td>
</tr>
<tr>
<td>2006</td>
<td>Pegylated E. coli L-asparaginase approved as first-line use by FDA</td>
<td>(106)</td>
</tr>
<tr>
<td>2011</td>
<td>Approval by FDA for the use of Erwinia chrysanthemi L-asparaginase (Erwinaze®) for ALL treatment</td>
<td>(12)</td>
</tr>
</tbody>
</table>

ALL, acute lymphoblastic leukemia.
This non-specific hydrolysis is related to a large part of the side-effects, such as liver diseases, acute pancreatitis, hyperglycemia, glycosuria, ketoacidosis, central nervous system disorders, hypoalbuminemia, hypofibrinogenemia, and hypercoagulation, among other dysfunctions (38,59).

Although the use of L-asparaginase benefits a considerable subset of patients with ALL, some relapse due to the development of resistance occurs, leading to a refractory disease (60). The suggested mechanisms of resistance are enzymatic resistance by antibodies (45), the upregulation of asparaginase synthetase activity in resistant (61-63), opioid receptor µ1-mediated mechanism for Erwinase resistance (64) and the loss of huntingtin associated protein 1 (HAP1) (60). L-asparaginase-induced apoptosis is essentially mediated by Ca$^{2+}$ and the loss of HAP1 acts by preventing the formation of the HAP1-huntingtin (Htt)-inositol 1,4,5-triphosphate receptor (InsP3R) ternary complex, resulting in reduced [Ca$^{2+}$]i and the downregulation of the Ca2+-mediated calpain-1-Bid-caspase-3/12 apoptotic pathway (60).

Despite the need to solve the issues associated with resistance, immunogenicity and toxicity, the enzyme L-asparaginase is a potent tool in the fight against ALL as it causes the selective death of leukemic cells (65). Therefore, researchers using L-asparaginase have sought to produce an enzyme with a high affinity for L-asparagine and a long half-life (66). These characteristics can be found in human L-asparaginase enzymes, which can replace bacterial-based L-asparaginases. However, the in vitro activity of human L-asparaginases still hampers their path for clinical use in ALL therapy.

There are a number of reviews on the use of bacterial L-asparaginases in the treatment of ALL (37,67-71), particularly about the application of Erwinia asparaginase (37) and the description of optimal clinical regimens of bacterial asparaginase (71). The present review article aims to bring together all the state-of-the-art structural and functional studies concerning human L-asparaginase activation providing knowledge on enzymatic autoprocessing, an essential in vitro process for clinical application.

### 3. Human L-asparaginase

In 1968, Lee and Bridges demonstrated for the first time that human serum exhibited L-asparaginase activity (72). It has since been well-established that the human genome codes for three enzymes that hydrolyze L-asparagine: Aspartylglucosaminidase (AGA), 60-kDa lysophospholipase and L-asparaginase-like 1. AGA is a lysosomal enzyme that participates in the final stage of glycoprotein degradation by breaking the amide link between an L-asparagine and carbohydrate clusters (73). Therefore, this enzyme is not an alternative in ALL treatment as it does not hydrolyze L-asparagine to aspartate. The 60-kDa lysophospholipase is a poorly characterized protein and is homologous to the L-asparaginase I from *E. coli*, which also acts on the hydrolysis of lysophospholipids; however, despite its poor characterization, the 60-kDa lysophospholipase has strong positive allosteric regulation by the substrate L-asparagine (74). The majority of investigations have focused on human L-asparaginase-like 1 (ASRGL1), which is also known as ALP (75), CRASH (76) or hASNase 3 (77) and is the primary topic of the present review and is discussed in detail.

In addition to the ability for L-asparagine hydrolysis, ASRGL1 also hydrolyzes isoaspartyl peptides. The formation of isoaspartyl peptides in proteins is a signaling mechanism for proteolysis; thus, mistaken formation is related to several pathophysiological situations that may lead to an increase in susceptibility to proteolysis or loss of function (78). ASRGL1 can act in the removal of this signaling through the hydrolysis of isoaspartyl peptides and is important for the correct cell functioning (79).

In human cells, ASRGL1 is located in the cytosol, and its expression occurs mainly in the brain, testicles, uterine
endometrium and liver. In these and other tissues, ASRGL1 functions through L-asparaginase activity and isoaspartyl peptidase activity. In the brain, it is regarded as a long-life protein and is involved in the formation of L-aspartate, a neuroactivator in the general metabolism of the nerve cell (76,80,81).

ASRGL1 belongs to the superfamily of N-terminal nucleophile hydrolases (Ntn-hydrolases) (81). Ntn-hydrolases are enzymes produced in an inactive form, and their activation is dependent on an autoprocessing step that exposes a catalytic nucleophile (threonine, serine or cysteine) in the N-terminal region of the newly formed β subunit (81,82). This superfamily includes aspartylglucosaminidases (73), penicillin acylases (83), Taspase1 (84), proteasomes (85) and plant-type L-asparaginases (79,81,86); the latter is the group to which ASRGL1 belongs. The amino acid sequences of proteins belonging to the Ntn-hydrolase superfamily are not conserved, but they have a common three-dimensional structure comprising an αββα sandwich consisting of antiparallel β-strands (79,81).

As an Ntn-hydrolase, ASRGL1 presents the core of αββα sandwich, as shown in Fig. 3A. By comparison with other Ntn-Hydrolases, it was strongly suggested that the autoprocessing of ASRGL1 involves residues near the active site (80).

The recognition of three peptides by specific antibodies in different tissues of rats was the first evidence of the need for ASRGL1 cleavage for enzymatic activation (75,80). Bush et al (75) were the first to hypothesize that a full-length precursor is cleaved into two subunits of about 25 (α subunit) and 15 kDa (β subunit). In 2003, Dieterich et al (80) demonstrated that peptides derived from the 25 kDa band belonged to the N-terminal region of the precursor and that the 15 kDa band would be in the C-terminal region. Interestingly, Bush et al (75) observed the presence of a human homolog of rat L-asparaginase due to cross-reactivity of anti-rat L-asparaginase antibodies with human sperm extract on western blots (SDS-PAGE bands at 25 and 17 kDa).

Currently, it is known that this autoprocessing is autocatalytic and indeed results in two subunits: The α subunit in the N-terminal region, and the β subunit, which is smaller, in the C-terminal region (81). The nucleophilic N-terminal residue responsible for autocatalysis and the hydrolysis of the substrate is threonine 168 (T168). The inactive form of the enzyme is cleaved between glycine 167 (G167, which becomes the end of the α subunit) and T168 (which becomes the beginning of the β subunit) to enable its hydrolytic activity on L-asparagine (Fig. 3B) (81).

Studies on human L-asparaginase have intensified in recent years due to the interest in its potential therapeutic use in some types of cancer, particularly ALL.

### 4. Human L-asparaginase: A challenging solution

Due to the benefit of L-asparaginase in the treatment of ALL, the use of a human enzyme could clinically be very advantageous. ASRGL1 could overcome several obstacles that have challenged the treatment and well-being of patients with ALL.

As a human protein, ASRGL1 can markedly reduce the immunogenicity of treatment (77), fulfills the requirement for the high thermal stability essential for use in medicines (87), has a high affinity for L-asparagine and does not have glutaminase activity (81). The great challenge regarding the use of ASRGL1 in the therapeutic protocol of ALL relies on its enzymatic activity *in vitro*. The clinical prerequisite for the $K_M$ of an L-asparaginase protein should be on the order of micromolar, and although bacterial L-asparaginases fulfill this requirement, the $K_M$ of ASRGL1 *in vitro* is inadequate for therapeutic use, on the order of millimolar (81,88). The comparative kinetic data of L-asparaginases are presented in Table III (76-78). ASRGL1, as a member of the subfamily of plant-type L-asparaginases, needs to undergo autoprocessing to become active; while bacterial L-asparaginases used in ALL treatment belongs to another subfamily of L-asparaginases: Bacterial-type L-asparaginases, which do not need autoprocessing (81).

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**Table II. Treatment of acute lymphoblastic leukemia with L-asparaginase.**

<table>
<thead>
<tr>
<th>Patient type</th>
<th>Type of case</th>
<th>North America, United Kingdom, Australia and New Zealand</th>
<th>Western Europe</th>
<th>Other countries</th>
</tr>
</thead>
<tbody>
<tr>
<td>Children</td>
<td>First diagnosis</td>
<td>1st) PEG-EcA 2nd) EwA</td>
<td>1st) PEG-EcA 2nd) EwA</td>
<td>1st) EcA 2nd) EwA or PEG-EcA</td>
</tr>
<tr>
<td></td>
<td>Relapsed</td>
<td>1st) PEG-EcA 2nd) EwA</td>
<td>1st) PEG-EcA 2nd) EwA</td>
<td>1st) EcA 2nd) EwA or PEG-EcA</td>
</tr>
<tr>
<td>Adults</td>
<td>First diagnosis</td>
<td>1st) EcA or PEG-EcA 2nd) EwA or PEG-EcA</td>
<td>1st) EcA or PEG-EcA 2nd) EwA</td>
<td>1st) EcA 2nd) EwA or PEG-EcA</td>
</tr>
</tbody>
</table>

EcA, L-asparaginase II native from *Escherichia coli*; PEG-EcA, pegylated *E. coli* L-asparaginase; EwA, L-asparaginase isolated from *Erwinia chrysanthemi*. The selection of the L-asparaginase variant used in treatments varies according to the treatment region and depends on the age of patient (child, age 0 to 18 years; adult, >18 years of age), and the type of diagnosis (first diagnosis or recurrence); these factors indicating the first (1st) and second (2nd) choice of the treatment, depending on the need for substitution. The information presented in this table is derived from previous studies (12,37).
As autoprocessing is a crucial event for enzyme activation and as it has been demonstrated that asparaginase activity is proportional to the degree of autoprocessing (89), it is assumed that the low enzymatic activity of ASRGL1 is due to incomplete autocleavage. Advancements in genetic engineering have enabled the realization of several modifications to increase the efficiency of autoprocessing in vitro; however, to date, efforts to generate an alternative to the therapeutic enzyme of bacterial origin have been unsuccessful.

The majority of enzymes that are synthesized as inactive precursors need to undergo a cleavage process, such that the active protein domain is separated from the remainder of the protein (89). However, following autoprocessing, the ASRGL1 subunits continue to interact strongly such that they remain a single functional unit. Although they are no longer connected, the interaction between them is fundamental for their ability to hydrolyze the substrate. Therefore, the expression of the β subunit alone does not result in enzymatic activity (89). Attempts to express the two subunits individually failed as they must be complexed in vitro, which was difficult to achieve (90). Even the co-expression of the two subunits under the pretext of producing a completely cleaved protein did not generate the expected results. Only one of the constructs resulted in a functional protein, although its specific activity on L-asparagine was similar to that of the wild-type protein (91). The co-expression attempts of the α and β subunits have presented a major disadvantage due to the inadequate formation of the complete enzyme and the consequent low L-asparaginase activity (87).

Karamitros and Konrad (92) developed a strategy for the identification of catalytically improved ASRGL1 variants for clinical application. They already found a variant with 6-fold better activity than wild ASRGL1. The potential therapeutic use of ASRGL1 in ALL is also supported by antileukemic efficacy from other human Ntn-hydrolases such as AGA, which led to apoptosis of leukemia cells (93). Moreover, other human hydrolases are being used extensively and satisfactorily in therapy (94), as hyaluronidase as an anesthetic in ophthalmology (95), β-glucocerebrosidase in Gaucher's disease (96), α-galactosidase A in Fabry disease (97) and α-glucosidase in Pompe disease (98). Notably, the use of a human enzyme brings safety and fewer complications to patients in all these examples.

5. Structural characterization of autoprocessing

Despite the difficulties in recreating the complete autoprocessing of ASRGL1, studies have enabled the elucidation, at least in part, of the mechanisms that result in the activation of the enzyme.

The autoprocessing mechanism begins with an acceptor base of the proton from the hydroxyl group of T168. Following deprotonation, T168 (with the increased nucleophilic character) attacks the carbonyl group of G167, forming a covalent bond that is subsequently hydrolyzed. The complete cleavage

Figure 3. Three-dimensional structure of ASRGL1 monomer indicating the (A) αββα sandwich folding motif and (B) evidencing the α and β subunits formed following autoprocessing. The yellow antiparallel β-sheets are flanked by red α-helices. PDB 4O0C.
Table III. Comparative kinetic data of L-asparaginases.

<table>
<thead>
<tr>
<th>L-asparaginase</th>
<th>Origin</th>
<th>$K_M$</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>(Refs.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASRGL1</td>
<td>Human</td>
<td>2.9±0.16 mM</td>
<td>3.19±0.07 (Ref.)</td>
<td></td>
</tr>
<tr>
<td>L-asparaginase I</td>
<td>Guinea pig</td>
<td>57.7±6.4 µM</td>
<td>38.6±1.4 (Ref.)</td>
<td></td>
</tr>
<tr>
<td>EcA</td>
<td><em>Escherichia coli</em></td>
<td>15.0±0.5 µM</td>
<td>44.4±0.3 (Ref.)</td>
<td></td>
</tr>
<tr>
<td>EwA</td>
<td><em>Erwinia chrysanthemi</em></td>
<td>47.5±3.5 µM</td>
<td>207.5±3.6 (Ref.)</td>
<td></td>
</tr>
</tbody>
</table>

ASRGL1, human L-asparaginase-like 1; EcA, L-asparaginase II from *Escherichia coli*; EwA, L-asparaginase isolated from *Erwinia chrysanthemi*. $K_M$ and $k_{cat}$ were determined using a spectroscopy NADH-dependent enzyme-couple assay.

between the two residues leaves the amino group of T168 free to catalyze the hydrolysis of L-asparagine (89). It is noteworthy that the essential residue T168 of ASRGL1 plays a double role: First, its lateral chain is necessary for autoprocessing. Second, with the breaking of the peptide bond between G167 and T168, the free T168 amino group participates in the catalysis of L-asparagine hydrolysis.

A full understanding of the exact mechanism of ASRGL1 autoprocessing is essential for achieving a solution to the low activity on the substrate in vitro. However, biochemical and structural studies of the human enzyme have proven to be a challenge, since the recombinant proteins generated for the study constitute a mixture of the unprocessed (inactive) and processed (active) states. Thus, the low activation rate (only 50% of autoprocessing with the wild-type enzyme was achieved) makes both structural and enzymatic characterization difficult (81,90).

The resolution of the structures of wild-type ASRGL1 in the processed and unprocessed states (77) and the ASRGL1 structure constructed by the circular permutation technique (CP-ASRGL1), in which the N- and C-terminal regions are joined by a linker (90), have revealed new knowledge of ASRGL1 autoprocessing and have paved the way for further studies.

In the inactive precursor protein, the distance between T168 hydroxyl and carbonyl carbon of G167 is 4.0 Å, which does not favor the chemical events necessary for autoprocessing and indicates the need for a conformational change to instigate cleavage (77). The inactive state of autoprocessing created by the T168A mutation caused a large increase in the thermal stability of the mutant protein ($\Delta T_m=10^\circ$C), providing evidence for a mechanism of activation by steric tension. This tension is due to the orientation of T168 in the inactive protein. The electrons in the methyl group are very close to the hydroxyl of this residue, creating repulsive forces unfavorable to interaction. Autoprocessing causes a relaxation in the lateral chain of T168, which permits the hydroxyl of T168 to come closer to the active site since the distance between this hydroxyl and the amino group of T168 is reasonable at 2.7 Å (90).

The conformational alteration in the autoprocessing region can be facilitated by glycine 9 (G9) (77). The comparison between the structures of ASRGL1 before and after processing indicates a 180° rotation of the G9 carboxylic group. This change in G9 was also observed in the enzyme L-asparaginase type III in the guinea pig (99) and can promote the repositioning of G167 (77), which approximates the T168, thus favoring autoprocessing (77,90). G9 is part of a glycine-rich loop called the HGG loop (histidine 8-glycine 9-glycine 10). This loop is highly conserved (~100%) throughout the phylogeny of the plant-type L-asparaginase (90). Individual substitutions of G9A, G10A or G11A have resulted in reduced autoprocessing and kinetic activity (90). These observations indicate that the HGG loop is critical for both autoprocessing and hydrolytic activity.

In the structure of the inactive protein (PDB ID 3TKJ, (90)), the position of the G10 carbonyl favors the hydrogen bond between G11 and T219, forcing the HGG loop to remain in the closed conformation (shown in green in Fig. 4). In addition, the proximity (1.6 Å) between G9 and L166 contributes to this closed conformation. By contrast, in the active enzyme [PDB ID 4ET0, (90)], the rotation of G9 modifies the position of the G10 carbonyl, which results in the changed position of the HGG loop (shown in blue in Fig. 4).

In addition to the importance of the T168 and G9 residues in the autoprocessing of ASRGL1, asparagine 62 (N62) and threonine 186 (T186) residues participate in autoproteolysis (87). Their role is not essential as that of T168, but they are considered relevant. This discovery was made through the structural resolutions of ASRGL1 variants with mutations in the residues N62, T168 and T186, as shown in Table IV.

The T186 hydroxyl interacts with the hydrogens of the G187 and G188 residues, which directs the T186 hydroxyl to function as a hydrogen donor for T168 hydroxyl. Furthermore, in an even more significant manner, hydrogen from N62 also forms a hydrogen bond with the hydroxyl of T168. Autoprocessing requires the hydrogen bond network provided by N62 and T186 to assist in deprotonation of the T186 $\gamma$-hydroxyl group. The nucleophilic character of T168 increases and being now further activated by a base, its oxygen can target the carbonyl of G167 to initiate autoprocessing (87,88).

Through another set of mutations, it was possible to also determine the details of the catalytic mechanism of L-asparaginase hydrolysis by ASRGL1. The hydrolysis of L-asparagine begins with the activation of the lateral chain of T168, which then initiates the nucleophilic attack on the carbonyl of the lateral chain of the substrate. In the cleavage state, T168 is a free amino group that acts as a base to activate the hydroxyl of T186 (a mechanism similar to the proton donation in the autoprocessing reaction). The nucleophilic attack proceeds through the generation of a covalent bond between the amide group of L-asparagine and T168 of the enzyme. The reaction generates an excess of negative charges in L-asparagine that is stabilized by the ammonia that is released through the interaction with the hydroxyl of T219. Finally, the covalent bond between T168 and the substrate is cleaved by...
the action of a water molecule, thereby releasing the aspartate (87,88).

The presence of another threonine near the catalytic site may favor the binding of the substrate L-asparagine. By being more polar and shorter than isoleucine 189, the mutant I189T gives more space for the active site, which favored the catalytic activity of the mutant enzyme. This mutant was generated from libraries of ‘site saturation mutagenesis’ by randomization of specific residues. However, it is worth noting that, in some cases, the increased activity was observed only by the activation of the prior enzyme. The authors also emphasized that their mutations resulted in an increased rate of conformational stability rather than increased catalytic efficiency. Even if random mutagenesis could prioritize the ASRGL1 peptidase function despite the L-asparaginase activity and also generate inactive enzymes due to nonfunctional nucleotide substitutions; the substitution of a single residue may be effective in achieving improved L-asparaginase activity (92).

To date, all mutations generated in different residues have aided in the understanding of the mechanistic bases of ASRGL1 functioning; however, to date, they failed to increase satisfactory its autoprocessing rate or its hydrolysis of L-asparagine.

6. Free glycine contributes to autoprocessing

In 2013, Su et al (89) described an important finding on the autoprocessing of ASRGL1. Glycine in solution exerts a critical influence on the autoprocessing of ASRGL1. The rate of autoprocessing is dependent on the glycine concentration, and a concentration of only 5 mM is able to promote even 40% of the processing, and complete cleavage was achieved with the addition of 250 mM glycine. It is noteworthy that all the processed structures described thus far had been obtained only by incubation with glycine.

The impact of solutions with alanine, serine, asparagine and small metabolites in the autoprocessing of ASRGL1 were also tested, although the results were not similar to the incubation with glycine (89). Among 13 other molecules tested by Li et al (87), glycine was the main activator of autoprocessing. Only three other molecules (ammonium carbamate, ammonium phosphate and sarcosine) were able to increase the level of processing; however, the levels were significantly lower to that induced by glycine. Glycine accelerates autoprocessing by 200-fold.

High concentrations of glycine in medium were also shown to be effective in promoting the processed state of ASRGL1 in human cell lines. Complete autoprocessing was achieved from treatment with 5 mM glycine (89). The glycine concentration found in human tissues varies from 3 to 8 mM. The activation of ASRGL1 may be linked to the pathophysiological conditions associated with these high glycine concentrations. Some of these conditions represent the environments of cancerous cells, where high concentrations of glycine can promote increased cell proliferation or where high concentrations of L-aspartate are required. However, high glycine concentrations can also be found in normal physiological situations, such as in sperm extract, a finding that may indicate that this high glycine concentration is related to the highly expressed ASRGL1 found in testicular tissues (89). In agreement, Bush et al (75) observed only processed ASRGL1 in human sperm extract (bands at 25 and 17 kDa on western blots).

The mechanism of action of free glycine was proposed by Su et al in 2013 (89). It is now known that glycine is anchored to the active site of ASRGL1 through several interactions: Its amino group has electrostatic interactions with aspartate 199...
Critical step for ASRGL1 autoprocessing and other members

Previous studies have demonstrated that dimerization is a necessary to evaluate the therapeutic use of ASRGL1 activated by a pre-clinical evaluation of the efficacy and toxicity in animals lead to an increase in autoprocessing (87). Therefore, the studies of active sites demonstrate that glycine does not always need to remove it by dialysis (91). In addition, mutational steps must be taken following incubation with glycine, which can inhibit the reactions to the substrate such that there is a need to dialysis (91). In addition, mutational studies of active sites demonstrate that glycine does not always lead to an increase in autoprocessing (87). Therefore, the pre-clinical evaluation of the efficacy and toxicity in animals and, in case of success, clinical trials in humans, is still necessary to evaluate the therapeutic use of ASRGL1 activated by a glycine-rich solution.

7. Oligomerization of human L-asparaginase

Previous studies have demonstrated that dimerization is a critical step for ASRGL1 autoprocessing and other members of the Ntn-hydrolase family, such as human aspartylglucosaminidase and E. coli L-asparaginase type III (79,87,100).

The structure of ASRGL1 deposited in the PDB under the code 4ZM9 has provided relevant information as to the mechanisms through which the dimerization of enzyme activates autocalysis in one of the monomers (87). Researchers have highlighted the role played by the dimeric interface during the autoprocessing of ASRGL1 (87). To determine this role, they studied the variants C202A and C220S. Cysteine 202 (C202) is one of the three cysteines present in the α subunit of the enzyme and is specifically located in the dimer interface. The crystallographic structure (PDB ID 4ZM9) indicates that the C202 of each subunit is positioned adjacent to each other. Even if they are not S-S linked in crystallographic structure, the dimerization interface of the variants C202A and C220S was weakened, and the authors observed that both modifications caused a reduction in the melting temperature ($T_m$) of the mutant proteins in relation to the wild type. Moreover, neither of the two variants were able to achieve any level of autoprocessing (87). Based on this finding, the importance of the dimer interface and the C202 residue in both ASRGL1 stability and autoprocessing is notable.

<table>
<thead>
<tr>
<th>Residue of mutation</th>
<th>Mutation</th>
<th>Autoprocessing</th>
<th>Catalytic activity</th>
<th>(Refs.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asparagine 62</td>
<td>N62D</td>
<td>Reduction</td>
<td>Reduction</td>
<td>(87)</td>
</tr>
<tr>
<td></td>
<td>N62A</td>
<td>S. D.</td>
<td>Reduction</td>
<td>(87)</td>
</tr>
<tr>
<td>Arginine 143 and Arginine 147</td>
<td>R143E, R147K</td>
<td>S. A.</td>
<td>S. A.</td>
<td>(92)</td>
</tr>
<tr>
<td>Threonine 168</td>
<td>T168S</td>
<td>S. D.</td>
<td>N. M.</td>
<td>(88)</td>
</tr>
<tr>
<td>Threonine 186</td>
<td>T186V</td>
<td>S. D.</td>
<td>S. D.</td>
<td>(88)</td>
</tr>
<tr>
<td></td>
<td>T186A</td>
<td>S. D.</td>
<td>Increase</td>
<td>(87)</td>
</tr>
<tr>
<td></td>
<td>T186S</td>
<td>S. A.</td>
<td>Reduction</td>
<td>(87)</td>
</tr>
<tr>
<td>Isoleucine 189 and Valine 190</td>
<td>I189T, V190I</td>
<td>S. A.</td>
<td>Increase</td>
<td>(92)</td>
</tr>
<tr>
<td></td>
<td>I186V, V190I</td>
<td>S. A.</td>
<td>S. A.</td>
<td>(92)</td>
</tr>
<tr>
<td>Threonine 219</td>
<td>T219V</td>
<td>Reduction</td>
<td>S. D.</td>
<td>(88)</td>
</tr>
<tr>
<td></td>
<td>T219A</td>
<td>S. A.</td>
<td>Increase</td>
<td>(88)</td>
</tr>
<tr>
<td></td>
<td>T219A</td>
<td>S. A.</td>
<td>Reduction</td>
<td>(87)</td>
</tr>
<tr>
<td></td>
<td>T219S</td>
<td>S. A.</td>
<td>Reduction</td>
<td>(87)</td>
</tr>
</tbody>
</table>

S. A., without alteration; S. D., without detection; N. M., not measured. The state of autoprocessing is shown in the absence of glycine and compared to wild-type ASRGL1. Reduction or increase: Relative to the wild-type ASRGL1 subject to the same conditions.
by measuring the $T_{m}$ of each step by fluorometry. The initial, unprocessed state had a $T_{m}$ of 61°C, which was converted over time into an intermediate state of $T_{m}$ equal to 65°C. The intermediate state was obtained after the autoprocessing of one of the subunits of the dimer due to the torsional strain of the scissile peptide bond (G167-T168). In the intermediate state, a 50% autoprocessing rate was achieved as the autoprocessing of one of the subunits leads to a relaxed conformation with a reduction of torsional strain. The completely cleaved state, which had a $T_{m}$ of 71°C, was achieved only following incubation for 48 h in the presence of 1 M glycine (87).

A previous study on ASRGL1 revealed a new trimeric conformation (102). The trimeric structure was obtained by the combination of biophysical techniques (small-angle X-ray scattering and hydrogen/deuterium exchange) and molecular dynamics simulation. Trimerization reinforces the important role the oligomerization plays, ASRGL1 trimers show increased thermal stability against monomers ($T_{m}$ deviation of 4.33°C), improved enzymatic activity (3.4 times higher than monomer activity) and full autoprocessing (without previous incubation with glycine). The authors of that study proposed that the inclusion of a third monomer may lead back to a torsional strain that allows complete autoprocessing of the ASRGL1 oligomer (102).

8. Conclusions

Proteins have become a potent tool in the treatment of several chronic diseases, such as diabetes, cancer and neurodegenerative diseases. Among their advantages, they can be produced on a large scale and have high specificity, low cross-reactivity, relatively few side effects and new modes of action (103). In addition, protein engineering extends the benefits of therapeutic proteins because it enables the adoption of various strategies to adapt the protein properties such as efficacy, stability, solubility, specificity, immunogenicity and pharmacokinetics (104).

Unfortunately, the commonly used strategies in protein engineering, such as fusion proteins, pegylation, glycosylation, the alteration of the oligomeric state and mutagenesis (104,105), were not able to produce a satisfactory active ASRGL1. As noted herein, some important residues for autoprocessing are currently known: The influence of oligomerization for ASRGL1 activation and the influence of a glycine-rich-medium for protein cleavage. However, there remain some important questions which require answers. Among these, identifying the acceptor base in the autoprocessing is one of the promising topics for studies for enabling autoprocessing in vitro. This has remained elusive.

Nevertheless, the potential clinical application of ASRGL1 is supported by the safety and efficacy of other hydrolases used in the treatment of human diseases and the antileukemic activity of another human Ntn-hydrolase, such as AGA (84). The present review article contributes to the knowledge of ASRGL1 activation and the information presented herein may guide the development of therapeutic variants of ASRGL1.

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Authors' contributions

SBdM and TdACbDs conceived and designed the study. SBM prepared and processed the figures. TdACbDs revised the manuscript critically for important intellectual content. Both authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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