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Review

Circumventing the side effects of L-asparaginase

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ABSTRACT

L-asparaginase is an enzyme that catalyzes the degradation of asparagine and successfully used in the treatment of acute lymphoblastic leukemia. L-asparaginase toxicity is either related to hypersensitivity to the foreign protein or to a secondary L-glutaminase activity that causes inhibition of protein synthesis. PEGylated versions have been incorporated into the treatment protocols to reduce immunogenicity and an alternative L-asparaginase derived from *Dickeya chrysanthemi* is used in patients with anaphylactic reactions to the *E. coli* L-asparaginase. Alternative approaches commonly explore new sources of the enzyme as well as the use of protein engineering techniques to create less immunogenic, more stable variants with lower L-glutaminase activity. This article reviews the main strategies used to overcome L-asparaginase shortcomings and introduces recent tools that can be used to create therapeutic enzymes with improved features.

1. Introduction

Asparagine is a non-essential amino acid that is important for cell growth and development. L-asparaginase (EC number 3.5.1.1) is an enzyme employed in the treatment of childhood acute lymphoblastic leukemia (ALL) that has successfully contributed to increasing the survival rates to 90% [1,2]. L-asparaginase works as an antileukemic agent because the amount of asparagine synthesized endogenously by leukemic blast cells is insufficient to produce *de novo* asparagine, forcing them to rely upon an external supply for growth. If the treatment with L-asparaginase reduces the circulating concentration of asparagine to 3μM or less, it disrupts the cell cycle progression of leukemic cells, ultimately killing them by starvation [3]. The treatment does not affect healthy cells which manage to endogenously synthesize asparagine using asparagine synthetase (ASNS), an enzyme that catalyzes the conversion of aspartate to asparagine using glutamine as source [4].

1.1. History of L-asparaginase

The hydrolysis of asparagine was first observed in bovine muscles [5] and later confirmed in horse and pig organs [6]. In a tour de force screening, Clementi [7] detailed the enzymatic properties of a hydrophilic "ferment" found in the tissues of several animals across all classes of vertebrates capable of hydrolyzing asparagine and called it L-asparaginase. Upon enumerating the animals whose tissues contained L-asparaginase, the author astutely observed that only Guinea pigs also had it in the blood serum. Years later, Guinea pig serum was reported to have beneficial effects in the regression of lymphomas induced in mice and rats, whereas the lymphomas treated with horse, pig, and rabbit serum enlarged and killed their hosts [8]. When Neuman [9] demonstrated that exogenous asparagine was needed for leukemia tumor cell growth, Guinea pig blood serum became a conceivable therapeutic agent against transplanted lymphomas due to the presence of L-asparaginase [10,11].

Despite its therapeutic potential, L-asparaginase from Guinea pig serum was disregarded for clinical use because the production was not adequately abundant, so researchers turned to bacterial sources [12]. Ohnuma [13] identified two L-asparaginase isozymes in *Escrerichia coli*. Type I includes cytosolic enzymes, expressed constitutively and with low affinity for L-asparaginase, resulting in non-therapeutic applications. Type II enzymes are localized in the periplasmic space, with induced expression during anaerobiosis and high affinity for L-asparaginase. *E. coli* L-asparaginase Type II demonstrated antineoplastic potential comparable to that found in Guinea pig serum [14] with the advantage

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of being suitable for large-scale production [15].

These efforts culminated in the approval of the first *E. coli* L-asparaginase Type II (EcA) for therapeutic use by the Food and Drug Administration in 1978 [16]. Current available formulations of L-asparaginase include native formulations of *E. coli* and *Dickeya chrysanthemi*, PEGylated formulations of *E. coli* and *D. chrysanthemi* (DcA) [17,18] and one recombinant *E. coli* variant [19].

2. Immunogenicity

All therapeutic proteins have the potential to induce the production of anti-drug antibodies (ADA), which can be classified as neutralizing or non-neutralizing. Neutralizing antibodies can bind to the active site of the therapeutic protein, inhibit activity, and reduce drug efficacy, causing the need for larger or more frequent doses to achieve the desired clinical effect and are common when therapeutic products that share significant similarities with host proteins [20,21]. Although non-neutralizing antibodies do not bind to the active site, they are still able to accelerate drug clearance by forming immune complexes with the biotherapeutics and remove them from circulation via the reticuloendothelial system [21,22].

L-asparaginase is one of the most notable examples of a well-established therapeutic protein with the potential to elicit an immune response in patients, partly due to its large size and bacterial origin [23,24]. When L-asparaginase is administered to the human body, normal and leukemic lymphoblasts may cleave it through the lysosomal proteases cathepsin B and asparagine endopeptidase [25], potentiating antigen processing and promoting an immune response [26–28].

The development of hypersensitivity is the main reason for treatment interruption with L-asparaginase [29], as it causes anaphylaxis, edema, serum sickness, bronchospasm, urticaria and rash, itching and swelling of extremities, and erythema [27]. The first anaphylactic reactions were reported as early as 1971, with 6 patients out of 40 developing anti-L-asparaginase antibodies [28]. Currently, 30–75% of patients show some form of hypersensitivity, with up to 70% of patients developing ADA [30]. Anti-L-asparaginase antibody production results in a series of side effects that range from allergies to more generalized reactions with potentially life-threatening symptoms [31]. A study performed in mice traces L-asparaginase hypersensitivity to IgG and IgE interacting with the immunoglobulin receptors FcγRIII and FcϵRI, respectively [32].

Besides, it is estimated that 46% of patients produce ADA that alters drug pharmacokinetics and clearance, which may be related to many of the reported cases of drug potency reduction [54–58]. This leads to a condition termed subclinical hypersensitivity or silent inactivation, where clinical signs are absent but drug efficacy is reduced or eliminated, resulting in poor therapeutic outcomes [33,34].

2.1. Managing immunogenicity

A number of works report that immunogenicity can be partially addressed by PEGylation, a process by which the enzyme is covalently coupled with polyethylene-glycol [[35], PEG]. When L-asparaginase is conjugated to PEG, surface-exposed positions not involved in the binding site are covered and the molecular size is increased, diminishing the probability of generating ADA and preventing the L-asparaginase uptake by the reticuloendothelial system [36].

Due to its smaller immunogenicity, PEGylated L-asparaginases are now employed in the frontline therapy in some countries and is quickly becoming the preferred preparation [37–39], but there are also accounts of side effects and silent inactivation [40]. Patients often exhibit significant cross-resistance between these formulations [41] and there are reports of non-fatal, toxic adverse effects, mostly hepatotoxicity, prominently for individuals over 40 [42,43]. As of recently, clinical trial studies remain necessary to confirm the potential of PEGylated L-asparaginases as a first-line treatment to ALL [44].

In case the patient develops hypersensitivity to the treatment with EcA, it is a common procedure to switch to DcA. Due to the differences between the amino acid compositions of the two enzymes, anti-EcA and anti-DcA antibodies have been shown not to cross-react immunologically [45–47]. DcA largely addresses the problem of hypersensitivity by reducing the probability of immunogenic response to 12–20%. However, it has a shorter half-life and studies report a significant number of patients that did not achieve complete remission [31,37].

Several other approaches have been suggested to reduce the risk of antibody formation. Concurrent administration of L-asparaginase with immunosuppressive steroids decreases the immunogenic risk [48] but may mask the presence of subclinical hypersensitivity that tampers with L-asparaginase activity [41]. Finally, it is possible to resort to enzyme engineering to create less immunogenic variants of L-asparaginase. This will be detailed in the next session.

2.2. Deimmunization

A major driver of immunogenicity is the presence of T-cell epitopes within the protein sequence, resulting in the sustained production of antibodies that neutralize the therapeutic effect.

The first challenge of L-asparaginase deimmunization is to map the epitopes. Using alanine scanning, Jianhua revealed that the placement of 3 consecutive alanines 195-AAA-197 substantially diminished the immunogenicity in EcA [49]. Moola used mice and rabbits anti-DcA antibodies produced against 10 continuous hexapeptides to map epitope positions in DcA and found the majority of the immune responses was raised against the peptide 282–292, while residues 285–289 were common to all epitopes. The individual contribution of each residue in the two overlapping hexapeptides 283-IVPPDE-288 and 287-DELPG-292 was evaluated and each residue was replaced by the other 19 naturally occurring amino acids. Overall, most mutations were found to reduce immunogenicity, especially those which caused a change in charge, aromaticity, or size, with Pro291 reported to be of particular importance [50].

Once the epitopes are mapped, the next step in deimmunization requires the modification of epitope content via site-directed mutagenesis. The use of a saturated-mutagenesis technique on positions P1, P4, P6, and P9 of the core of three selected epitopes (115-MRPSTSMSA, 216-IVYNYANAS, and 304-VLLQLALTQ) allowed the creation of a variant of EcA with 8 mutations significantly less immunogenic than wild-type EcA, proving it is possible to obtain a deimmunized variant with many mutations [51]. As a general rule, a higher number of mutations makes it more difficult for the variant to retain the same stability and behavior as the wild type. Ramya and Pulicheria [52] narrowed the search space to the mutations less likely to compromise the structure using a phylogenetic approach to map the most conserved residues in the similar epitopes across L-asparaginases of several microorganisms. The authors found tyrosine, histidine, threonine, and serine to contribute the most to L-asparaginase immunogenicity.

Currently, two Human Leukocyte Antigens (HLA) are linked to ALL. At first, a strong association was reported between HLA-DRB1*04:01 and childhood ALL in males [53] and has proven useful to eliminate immunogenic sequences in EcA [51]. In a different work, data from ALL patients of European ancestry revealed a significantly higher rate of anti-L-asparaginase antibodies among patients with the HLA-DRB1*07:01 allele [54]. L-asparaginase hypersensitivity was linked to 10 EcA peptides, where the peptide between positions 212–231 presented a high-score for epitope prediction. To confirm the link with ALL, the authors built homology models for the DRB1*07:01 receptor and 3 for other low binding affinity receptors as control and estimated the binding free energy via docking and molecular dynamics. The binding free energy between the epitope and the DRB1*07:01 protein was found to be significantly lower than with the other 3 receptors, which indicates the interaction between this peptide and the MHC is highly stable and plays a central role in EcA immunogenicity [54].

The use of a human L-asparaginase is regarded as a viable option for treatment as an alternative to a deimmunized, bacterially-derived enzyme [55]. Human-like enzymes have been used in lieu of the bacterially-derived for a number of other diseases because they are typically immunotolerant [56]. As such, the replacement of currently approved L-asparaginases by human homologs could overcome the problem of immunogenicity associated with the administration of bacterial L-asparaginases. However, the native form of the human L-asparaginase lacks therapeutic potency [24,57,58] because its KM is in the millimolar range [55] and the concentration of asparagine in the blood is approximately 50 μM [59].

3. Improving stability, activity, and half-life

An alternative approach to overcome the short half-life of L-asparaginases is to improve the structural stability of the enzyme. However, reengineering L-asparaginases is burdened by the complexity of the structure. The most functional form of the enzyme is a quaternary structure in which two monomers form a closely paired "intimated dimer", and the two intimate dimers combine into a catalytic active tetramer [[60], Fig. 1-left]. Other functional states such as monomer, octamer, and dodecamer, albeit not as effective as the tetramer, may be found in smaller percentages of commercially available formulations [37]. The binding cavity is partially formed when a monomer folds and is complete when two monomers join, resulting in a binding site that partially rests at the dimer interface (Fig. 1-center).

The top of the binding site is covered by a loop that works as a "lid" (Fig. 1-right). One of the lid loop residues of EcA, N24, participates in a hydrogen bond network close to the catalytic site [26] and was subject to investigation via a series of mutations. Mutations that increase the flexibility of the loop were found to reduce the catalytic reaction, whereas mutations that stabilize the loop lead to an increase in the catalytic effect, possibly because the substrate is more firmly fastened to the binding site [26].

Some works suggest the most stabilizing mutations cause charge neutralization and charge reversal since they remove adverse electrostatic interactions [61]. Kotzia [62] verified the importance of the residue N133 for thermostability by improving the melting temperature of DcA by almost 10° C with a single point mutation. More recently, a single-point mutation N24S lead to the generation of an EcA variant with higher thermal stability that also kept levels of catalytic activity equivalent to native, had reduced long-term storage degradation, and showed greater resistance to tumor cell proteases [63]. Molecular dynamics and structural analysis of the mutant revealed an overall rigidity of the molecule caused by modifications in the hydrogen bond network of the mutated residue.

Enhancing L-asparaginase activity is also important to improve the therapeutic potency of promising molecules like the human L-asparaginase that lacks therapeutic efficacy [64,65]. Therefore, human L-asparaginase must be re-engineered to enhance its catalytic properties to make it suitable for clinical use. Recently, the crystal structure of a human L-asparaginase-like protein was solved and 3 double mutants and 2 quadruple mutants that allowed up to a 6-fold increase in the catalytic activity were produced through rational engineering [66]. Another achievement was the identification of a new human-like L-asparaginase in Guinea pig [57] with micromolar KM, which was used to produce a variant with a 140-fold increase in catalytic efficiency and killing potency [38].

Stability and activity are generally thought to be antagonistic. A number of examples ratify this hypothesis, showing that more stable enzymes tend to display poorer levels of catalysis than their orthologs [67–69], but this negative correlation between activity and stability is based on factual observation, and not necessarily enforced by some natural constraint. Hence, there are also several counterexamples to this rule [70]. Evidence indicates that L-asparaginase is one of these exceptions, as variants with increased thermostability either have improved or uncompromised catalytic efficiency, even if the modifications are applied to the subunit interfaces and its flanking residues [26, 64,65]. As part of the binding site lies on the monomer-monomer interface, on possible explanation is that mutations that enhance the interaction of subunits tightly trap the substrate as well as improves the compactness of the tetramer.

Knowledge of the three-dimensional structure is essential to shedding light on the delicate equilibrium of forces between atoms that allows the protein to be stable and flexible and is a critical step towards mutants more stable and therapeutically potent mutants.

4. L-glutaminase activity

Another problem displayed by commercial L-asparaginases is a secondary L-glutaminase activity. In addition to hydrolyzing asparagine, both EcA and DcA are able to hydrolyze L-glutamine to L-glutamic acid and ammonia with different degrees: the rate of L-glutaminase/L-asparaginase activity in EcA is approximately 2%, whereas for DcA it can be as high as 10% [24,39,71]. The role of this L-glutaminase co-activity is not fully understood and the literature contains conflicting evidence of both beneficial and harmful effects.

There are reports that indicate the L-glutaminase cofunction is necessary to potentiate the anti-tumor effect in cancer cells [65,72], with some even suggesting it is indispensable for the antitumoral property of the enzyme [26,73]. The antitumoral role of L-glutaminase activity has been associated with the ASNS expression levels. The L-glutamine depletion is believed to be required for the cytotoxic effect in leukemia clones expressing ASNS [26,74,75] but not necessary in ASNS-negative cancer cells [28,74]. The anti-tumor effect of the L-glutaminase cofunction can be explained by the fact that the concentration of L-glutamine is essential for cell cycle progression from G1 to S phase in various cells and that abundant L-glutamine may augment the L-asparagine biosynthesis by ASNS [76].

Contrastingly, since L-glutamine is the most abundant amino acid in the blood, a donor of the amino group for many biosynthetic reactions, and the major form of transport of nitrogen in the blood, its deprivation is attributed to several deleterious effects [39,77,78]. Some of the side effects linked to the L-glutaminase coactivity are reduction of the

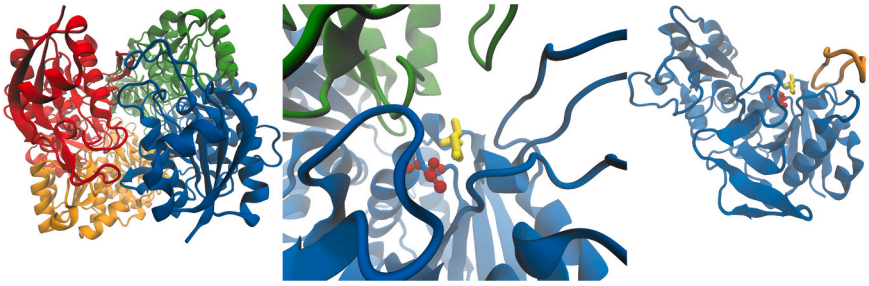

Fig. 1. Left: Quaternary structure of the tetramer of L-asparaginase. Two monomers closely bind together and form an intimate-dimer (blue-green, red-orange). Two intimate dimers form the tetramer. Center: Binding site of the monomer (blue). The binding site lies in part at the monomer-monomer interface because the other subunit of the close pair (green) stabilizes the substrate (yellow). Right: Lid-loop (orange). The loop lays at the top of the binding site and works as a lid, modulating the affinity for the substrate (yellow) shown here interacting with residue Thr-89 (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

synthesis of several important proteins such as albumin, insulin, fibrinogen, and protein-C, resulting in hepatotoxicity [79], pancreatitis, hyperammonemia [80,81], neurotoxicity, hyperglycemia [27,82], leukopenia and coagulation abnormalities such as thrombosis and hemorrhage in patients receiving L-asparaginase treatment [38,46]. Additionally, there is evidence that L-glutaminase activity is not required for *in vivo* tumor cytotoxicity, and that the reduction of L-glutaminase activity leads to less adverse effects [83].

Reengineering L-asparaginases to reduce L-glutaminase activity consists in producing an enzyme with low L-glutamine turnover while keeping the L-asparaginase and anticancer activities unaffected. This is challenging because, due to the structural similarities between L-asparagine and L-glutamine, an undesirable rise in the secondary L-glutaminase activity is a common byproduct of the attempt to engineer a variant with higher catalysis. Experiments with L-asparaginase from *Helicobacter* sp. resulted in mutants with undetectable L-glutaminase activity, but that were also negatively affected with regards to cytotoxicity [83,84].

Other examples, however, demonstrate that L-glutaminase activity can be modulated regardless of the L-asparaginase activity via site-directed mutagenesis on residues N248 [85,86] and Q59 [74] in EcA and Q63E in *H. pilorum* L-asparaginase. Knowledge of the three-dimensional structure allows the identification of residues that preferentially interact with glutamine or asparagine and has been used to infer the rate of L-glutaminase/L-asparaginase activity of recombinant enzymes. Molecular dynamics simulations and experimental studies that assessed the interaction of EcA mutants with glutamine confirmed glutaminase activity was particularly low for variants with a reduced binding cavity size and allowed researchers to precise which residues strongly favor the interaction with L-glutamine over L-asparagine [87].

5. Alternative sources and formulations

L-asparaginases are found in bacteria, plant, fungi, and animal cells, and resorting to different sources allows the characterization of enzymes with different properties, such as less adverse reactions [88] and varying levels of L-glutaminase activity [84], even though studies with non-bacterial L-asparaginases indicate the vast majority cannot be used for human applications.

Most examples of reengineered L-asparaginases attempt to build on those commercially available, but it is also beneficial to resort to alternative sources or reengineer the other natural variants [89]. Recently, anti-proliferative effects of L-asparaginase obtained from *Sarocladium strictum* have been shown [90], and a strong *in vitro* efficacy against five human tumor cell lines using a L-asparaginase from *Streptomyces rochei* was reported [91]. Sannikova and collaborators worked with Gram-negative bacteria Wolinella succinogenes and were able to produce a mutant that exhibited higher therapeutic efficacy in mice with a ratio of L-glutaminase/L-asparaginase catalytic activity close to half as that presented by EcA [92]. Similarly, a work with *Bacillus licheniformis* L-asparaginase resulted in a mutant that displayed higher than native specificity, thermal stability, and half-life [93]. Despite the promising initial results, the L-asp-KM only improved from 0.671 to 0.42 mM, indicating opportunities for continued advances.

Several alternative L-asparaginases with reduced glutaminase activity were also isolated from *Wolinella succinogenes* [84] *Aspergillus terreus* [94], *Erwinia carotovora* [85], *B. licheniformis* [95], *H. pylori* [75], *Enterobacter cloacae* [139], and *Mesoflavibacter zeaxanthinifaciens* [96]. Among these organisms, we highlight the interest in *E. carotovora* L-Asparaginase as this enzyme has promising catalytic and therapeutic efficiencies when tested against different cancer strains and their use inhibited the growth of human and animal tumor cells [97], and is likely less immunogenic according to computational analysis [98]. Another L-glutaminase-free human L-asparaginase showed levels of cytotoxic activity towards leukemia cells similar to that of Guinea pig and EcA type I [24,55]. More recent examples include *B. subtilis* bacterium from marine sponges [99], *Leucosporidium scottii*, an extremophile yeast living in low-temperature, high salinity environments [100], *Bacillus antitudinis* [101], B. licheniformis [102], *Anoxybacillus flavithermus* [103].

Several attempts to mitigate the shortcomings of L-asparaginase involve alternative formulations. In a series of works lead by Burhan Ateş, thorough research was conducted using various magnetic nano vehicles to covalently immobilize L-asparaginase resulting in formulations with higher stability, a higher affinity for the substrate, and better shelf life. This indicates enzyme immobilization techniques can be satisfactorily applied to L-asparaginase and should be further explored [104–111].

6. Conclusion

L-asparaginase has become a widely used enzyme in ALL treatment because it hydrolyses circulating L-asparagine tumor cells depend upon for growth. Despite its benefits, it is clear from clinical studies that bacteria-derived enzymes are capable of stimulating undesirable immune responses in patients. In general, the beneficial effects of the therapeutic applications of L-asparaginase surpass the probability of evoking an immune response, but the control of anti-drug antibodies is needed for the long-term success of the treatment and allows greater expansion in the approval and use of biopharmaceuticals.

Immunogenicity and short *in vivo* stability are two disadvantages commonly observed in native formulations partially addressed by PEGylation. Additionally, all formulations display different degrees of secondary L-glutaminase coactivity linked to several dangerous side effects.

The immunogenicity of protein therapeutics has proven to be difficult to predict in patients so far. Methods to evaluate immune reactions have greatly advance since biopharmaceuticals were first used and allow a better understanding of the relation between products, patients, and diseases. The prediction of immunogenicity combined with deimmunization, a technique in which key amino acids are substituted to remove epitopes, has led to the creation of a number of partially deimmunized variants of L-asparaginase. In addition, protein engineering has been successfully used to obtain more stable variants with better catalytic activity and reduced L-glutamine affinity.

The desire of the time is to explore the knowledge to design enzymes with less immunogenic effects, novel functionalities, longer half-life, and smaller L-glutaminase/L-asparaginase ratio. Considering the challenges pointed in this review, enzyme engineering is an invaluable tool to improve the therapeutic importance and circumvent the shortcomings of currently available L-asparaginase for improved, patient-tailored therapy.

Conflict of interest statement

There is no conflict of interest.

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