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REVIEW

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Overview of the structure, side effects, and activity assays of L-asparaginase as a therapy drug of acute lymphoblastic leukemia

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L-Asparaginase (L-ASNase is the abbreviation, L-asparagine aminohydrolase, E.C.3.5.1.1) is an enzyme that is clinically employed as an antitumor agent for the treatment of acute lymphoblastic leukemia (ALL). Although L-ASNase is known to deplete L-asparagine (L-Asn), causing cytotoxicity in leukemia cells, the specific molecular signaling pathways are not well defined. Because of the deficiencies in the production and administration of current formulations, the L-ASNase agent in clinical use is still associated with serious side effects, so controlling its dose and activity monitoring during therapy is crucial for improving the treatment success rate. Accordingly, it is urgent to summarize and develop effective analytical methods to detect L-ASNase activity in treatment. However, current reports on these detection methods are fragmented and also have not been systematically summarized and classified, thereby not only delaying the investigations of specific molecular mechanisms, but also hindering the development of novel detection methods. Herein, in this review, we provided a detailed summary of the L-ASNase structures, antitumor mechanism and side effects, and current detection approaches, such as fluorescence assays, colorimetric assays, spectroscopic assays and some other assays. All of them possess unique advantages and disadvantages, so it has been difficult to establish clear criteria for clinical application. We hope that this review will be of some value in promoting the development of L-ASNase activity detection methods.

1. Introduction

Acute lymphoblastic leukemia (ALL) is an all-age disease that threatens both children and adults. In fact, ALL is the most common malignant tumor among children younger than 14 years of age, accounting for 26% of new cancer diagnoses in this age group.[1] Current feasible treatments for ALL have become increasingly diverse and include chemotherapy, radiotherapy, chimeric antigen receptor T cell (CAR-T) therapy,[2] and hematopoietic stem cell transplantation (HSCT). Chemotherapy still remains the most widely used treatment for ALL while L-asparaginase (L-ASNase) serves as the most common medicine. In-depth studies on L-ASNase were not carried out until it was found that guinea pig serum could inhibit lymphomas.[3] Initially, this inhibition was unknown until it was demonstrated by Broome in 1963.[4] Subsequent studies showed that L-ASNase played an important role in tumor regression.[5] ALL cells abundantly require asparagine because of their malignant proliferation. Owing to the low or lack of asparagine synthetase activity, ALL cells fail to gain sufficient asparagine, which is indispensable for protein synthesis.[6,7] The survival of ALL cells is thus dependent on their capability to absorb sufficient asparagine from the outside. L-ASNase catalyzes the substrate, L-asparagine (L-Asn), and hydrolyzes it into L-aspartic acid (L-Asp) and ammonia. When L-Asn is rapidly depleted in the extracellular fluid, it can result in the death of ALL cells. At present, the World Health Organization (WHO) has added L-ASNase to the List of Essential Medicines. Accordingly, L-ASNase is frequently used as an injectable drug with other chemotherapeutic agents during leukemia and lymphoma treatment, especially in children with ALL.[8] In addition to its use as a therapeutic drug, L-ASNase is also valuable in processed food manufacturing as an additive that can antagonize the formation of acrylamide, which is classified as "probably carcinogenic to humans".[9] Meanwhile, reports on

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the use of L-ASNase as an antimicrobial agent have also been gradually increasing in recent years.[10]

Biopharmaceutical biomolecules are mainly therapeutic recombinant proteins produced through biotechnological processes. As one of these biomolecules, L-ASNase is produced by microorganisms, and clinical applications of L-ASNase enzymes derived from *Escherichia coli* and *Erwinia chrysanthemi* face a number of challenges due to known potential comorbidities, so discovering microorganisms that provide a large number of enzymes with continuously improving biochemical properties is an ongoing challenge.[11] Meanwhile, L-ASNase can be produced both intracellularly (submerged fermentation) and extracellularly (solid state fermentation), so the purification strategies are different for various microorganisms, and it is also quite challenging to match the best purification methods because microbial L-ASNase varies greatly in various biochemical properties (*e.g.*, optimal pH, optimal temperature, *etc.*).[12] Unfortunately, some of these comorbidities may threaten a patient's life.[13] Therefore, the side effects of L-ASNase preparations must be addressed and attenuated. Normally, another L-ASNase that possesses different pharmacological properties can replace the one that was employed as a first-line therapy to reduce the adverse effects observed in patients that experience immunological hypersensitivity or toxicity.[14] Thus, patients with allergic reactions to *Escherichia coli* L-ASNase are often clinically switched to *Erwinia* L-ASNase or PEG-L-ASNase agent.[15–17] The search for an L-ASNase without glutaminase activity and the development of promising approaches, such as nano- and micro-encapsulation, to enhance the *in vivo* performance of L-ASNase are also for alleviating side effects.[18–20] Meanwhile, using effective analytical methods to detect L-ASNase is very important to improve the anti-cancer efficiency and reduce complications.[21]

Different analytical methods have been used to quantify L-ASNase activity, which has enabled timely dosage adjustment during treatment to reduce the accompanying side effects. Assessment of L-ASNase-catalyzed ammonia production using the Nessler reaction is a common and classical method for determining L-ASNase activity.[22] Spectrophotometric methods have also been frequently reported due to their precision. However, the complexity of the operations in this approach cannot be ignored. Chromatography, fluorescent probes, and other methods have also been applied to detect L-ASNase activity. Based on the clinical requirements for the detection of L-ASNase activity, it is believed that fluorescent probes are promising owing to their visualization capabilities, rapid response, low cost, and simple methodology.[23]

In this review, we aim to provide a detailed summary of the structure, anti-tumor mechanism, and side effects of L-ASNase. Further, we give an overview and discuss different detection methods to detect L-ASNase activity, and share our views on the future challenges and developments of L-ASNase in the field of cancer therapy (Fig. 1).

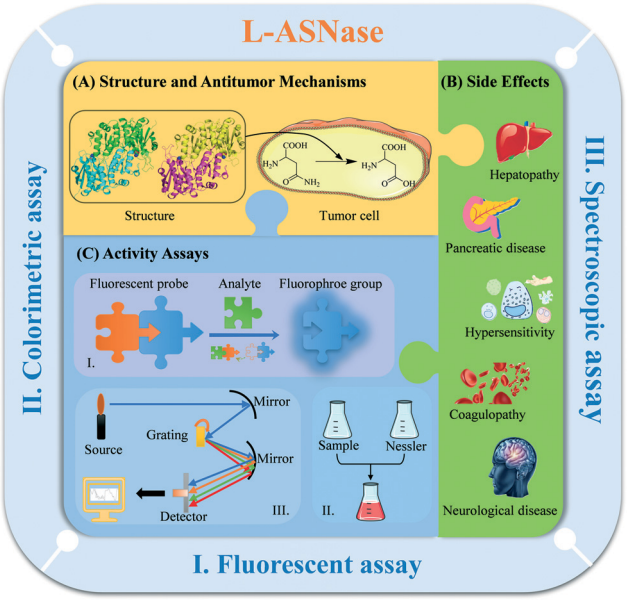

Fig. 1 The schema of this review. It summarized the structure & antitumor mechanism, side effects, and activity assays of L-ASNase. (A) Structure of L-ASNase and its anti-tumor mechanism; (B) the main types of side effects; (C) activity assays: (I) fluorescence assay; (II) colorimetric assay; (III) spectroscopic assay.

2. Structure and antitumor mechanism

Enzymes are proteins or RNAs produced by living cells that are highly specific and catalytically efficient for their substrates. They are also a category of biological catalysts that govern many catalytic processes, such as metabolism, nutrition, and energy conversion. Enzymes can exist intracellularly, extracellularly, and on the surface of cell membranes.[24] Accordingly, in clinical disease diagnosis, it is possible to understand or determine the occurrence and development of certain diseases by measuring enzyme activity in blood, urine, or body fluids. The use of enzyme therapy has become increasingly widespread and several enzyme preparations are employed in clinical practice. L-ASNase is a special enzyme with antitumor activity (Fig. 2A). The active center of L-ASNase catalyzes and hydrolyzes the substrate L-Asn to L-Asp and ammonia, as shown in Fig. 2B.

Currently, L-ASNase can be extracted from bacterial, plant and rhizobial types, while bacterial L-ASNase has the advantage where it can be produced in large quantities by production in large biodigesters, which not only reduces costs but also has a sustainable source of bacteria. Among them, the most successful ones now in commercial production are the *E. coli* type and *Erwinia chrysanthemi* type. In the latest reports, new types of bacteria with production potential such as *Bacillus halotolerans*, *Ganoderma australe*, *etc.* have also been identified.[12,25,26]

According to X-ray crystallographic data, L-ASNase produced by *E. coli* or *Erwinia chrysanthemi* is more of a tetramer, and that isolated from other sources is of

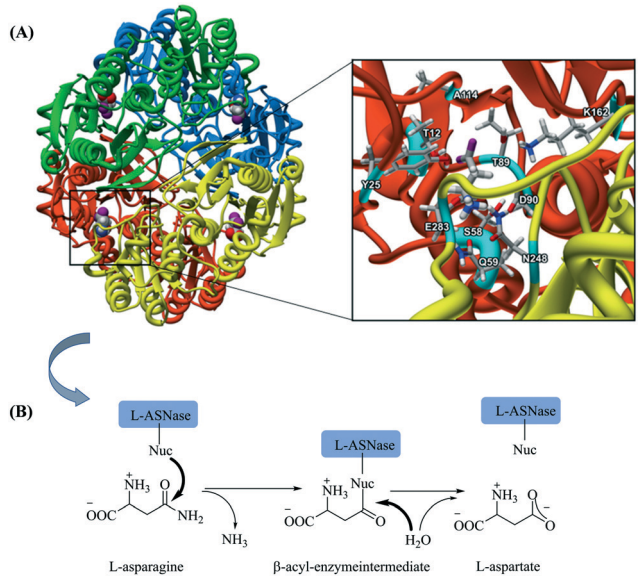

Fig. 2 (A) Crystal structure of the *E. coli* L-asparaginase (L-ASNase) tetramer in complex with the product L-aspartate. The locations of key residues are highlighted in cyan in one of the four homogeneous active sites. Residues including T12, Y25, S58, Q59, T89, D90, A114, and K162 are in the red enzyme subunit, and N248 and E283 are in the neighboring yellow enzyme subunit. Thick tubes represent α-helical regions while ribbons with arrows represent β-sheets. The atoms in these active site amino acids are color coded: hydrogens (white), oxygens (red), carbons (gray), and nitrogens (dark blue). Side-chain oxygens of the Asp ligand are colored magenta for distinction.[30] Reproduced from ref. 30 with permission from Elsevier, copyright 2015. (B) Schematic illustration of the reaction mechanism of L-ASNase catalysis. The covalent intermediate suggested is formed *via* a nucleophilic attack by the enzyme, indicated by bold arrows.

monomeric and dimeric forms.[27] Most bacterial L-ASNases possess quaternary and tertiary structures.[28] Presently, the clinical preparations of L-ASNase are mainly derived from *E. coli* and *Erwinia*; however, the PEG-*E. coli* type is preferred, with a different half-life time (*E. coli* and *Erwinia* types: 8–30 or 8–22 h; PEG type: 5–7 days).[12,29] There are two types of *E. coli* L-ASNases: type I L-ASNase (EC1, K_m = 5 mmol L^{-1}) and type II L-ASNase (EC2, K_m = 12.5 μmol L^{-1}).[30] Type I L-ASNase is expressed in the cytoplasm, where it hydrolyzes L-Asn and L-glutamine (L-Gln) and exhibits a low affinity for L-Asn, while type II L-ASNase is induced to be expressed in hypoxia. Thereafter, it is secreted into the periplasmic cavity and exhibits a high specific hydrolytic activity for L-Asn.[31] As a result, only type II displays anti-cancer activity. Type II L-ASNase is composed of four identical subunits, each containing 326 amino acids that form 14 β chains and 8 α helices aligned into two well-recognized domains: the N-terminal large domain and the C-terminal small domain. These domains are connected by a linker that is composed of about 20 residues (Fig. 2A).[32] According to a prior report, only tetramers with a molecular weight of 141 kDa possess enzymatic activity.[33,34]

The active sites are located between two adjacent monomers and are divided into two motifs.[35] A few residues of the *E. coli*-derived L-ASNase enzyme catalytic site, including T12, Y25, T89, D90 and K162, classified into 4 types by Bartlett *et al.*, were found to critically influence enzyme activity.[36–38] Among these residues, T12 acts as a nucleophilic group during acylation and T89 forms a part of the rigid structure of the protein.[39] Kinetic data have revealed that Y25 is closely related to the stabilization of the catalytic lid loop during its binding to the substrate.[40] However, the specific nucleophile and proton acceptor have not been confirmed.[30]

Since the early 1970s, L-ASNase II has been used as a clinical drug for the treatment of childhood ALL. According to the basic principle, L-ASNase II hydrolyzes L-Asn into L-Asp and ammonia.[32] Both normal and ALL cells require L-Asn for a smooth metabolic process. When injected into blood, L-ASNase rapidly depletes L-Asn in plasma, leading to inadequate uptake of L-Asn for tumor cells that require large amounts of this amino acid. When the exogenous source of L-Asn is impeded, the endogenous L-Asn produced by L-Asn synthetase compensates for the loss while the levels of L-Asn synthetase in tumor cells become limited and are vastly insufficient to meet the demand. As a result, the survival of tumor cells becomes difficult (Fig. 3).[41] Essentially, L-ASNase treatment is based on its catalytic hydrolysis characteristics and the dependence of tumor cells on exogenous L-Asn.

Although the specific molecular signaling pathways of L-ASNase therapy for ALL have not been investigated, numerous functional and structural studies have provided important references and pavements to explore these molecular signaling pathways and response mechanisms. Several advances in the understanding of molecular mechanisms have been reported. A lack of L-Asn was found to inhibit the maturity of ribosomal precursor RNA and the transcription of rRNA. Accordingly, the synthesis of asparaginyl tRNA is depressed, which limits peptide synthesis.[42,43] L-ASNase treatment halts the cycle of tumor cells at the G1 phase prior to DNA degradation.[44] However, the expression level of L-Asn synthase is not upregulated in susceptible tumor cells, which may be due to the inhibition

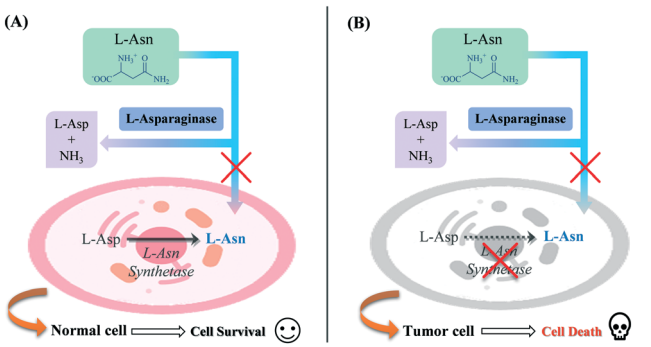

Fig. 3 Anticancer mechanism of L-asparaginase (L-ASNase) treatment. (A) Healthy cells synthesize L-asparagine (L-Asn) intracellularly, *via* L-Asn synthetase, which is prevalent in healthy cells. (B) Tumor cells lacking L-Asn synthetase rely wholly on extracellular L-Asn, which results in their death upon treatment with L-ASNase. L-Asp: L-aspartic acid.

of the phosphorylation of eukaryotic initiation factors (*e.g.* eIF-2a and eIF-4e) by global translation starvation-activated ribosome-associated kinases.[42,45] Ammonium ions produced *via* hydrolysis alters the pH following diffusion into the cytosol. This leads to the activation of signal transduction pathways that are associated with substrate phosphorylation and apoptosis.[44] In addition to this, other signal transduction pathways are yet to be investigated, and it is also of significance to investigate whether the p53 status can be used to forecast susceptibility to asparagine depletion in a more diverse range of tumors, given that p53 often loses potency in cancer.[46]

3. Side effects

L-ASNase alleviates ALL by depleting L-ASN in leukemia cells and exerting a cytotoxic effect in these cells. However, L-ASNase causes numerous side effects and complications when administered as a treatment, including liver function disorders, pancreatitis, diabetes, congestive heart failure, skin rashes, pyrexia, cytopenia, neurological disorders, *etc.*[47,48] The utilization of L-ASNase by leukemia patients may result in normal cell death, despite its potential antileukemic activity.

Since L-Asn is present in human blood at about 50 μM, therapeutic L-ASNase must have a substrate affinity in the low micromolar range. A low Michaelis–Menten constant (K_m) correlates with a high turnover number (K_{cat}), ensuring that therapeutic L-ASNase will adequately reduce endogenous L-Asn at safe doses.[49] The percentage of glutaminase activity associated with L-ASNase should also be low and the enzyme stability and half-life should be high so that it could be the ideal clinical formulation. However, there are no clinical agents that combine these properties, and therefore side effects are still prevalent after dosing.[50]

Hepatic steatosis is one of the complications of L-ASNase therapy. L-ASNase is known to reduce plasma glutamine concentrations through its glutaminase activity. In addition, glutamine deficiency has been reported to be associated with reduced hepatic protein synthesis.[51,52] Glutamine is also a component of glutathione, an important protective agent against oxidative stress.[53] Glutathione deficiency causes hepatocytes to become susceptible to oxidative stress damage, which can lead to mitochondrial damage. Treatment-induced glutamine deficiency is thus a main cause of this complication.[54] Previously, we explained that L-ASNase fights leukemia primarily by depriving L-Asn, on which the synthesis of insulin also depends, resulting in hyperglycemia as one of the most common side effects.[55,56] As L-ASNase in the ALL regimen is a macromolecule of bacterial origin, and it can trigger an immune response in patients.[57] According to Moola, some mild allergic reactions and anaphylactic shock have been ascribed to the uses of L-ASNase-based drugs.[13] In addition to other factors, impaired synthesis of hepatic coagulation factors, including fibrinogen, antithrombin (AT) III, and protein C also leads to coagulation disorders, which may be related to the abnormalities in lipid metabolism during treatment.[53] Adolescents also appear to be more susceptible to L-ASNase-induced neurotoxicity, which causes depression, drowsiness, restlessness, giddiness, *etc.*[58] Side effects of L-ASNase therapy are listed in Table 1.[59]

The *E. coli* expression system is widely employed because of its adaptation to high density fermentation, inexpensive culture process, high expression yield, and short production period.[60] As a result, most of the L-ASNase preparations available for therapy are isolated from *E. coli* and are of the EC2 type. Pegylated L-ASNase preparations are preferred because of their lower immunogenicity and longer half-life; however, they are relatively expensive. Some patients develop a severe immunogenic response, exhibit biotoxicity, and display more side effects when treated with L-ASNase preparations isolated from *E. coli*. These findings may be attributed to the higher glutaminase activity of *E. coli*-derived L-ASNase relative to other strains.[60] A new source of L-ASNase without glutamyltransferase activity is thus urgently required.[61,62]

Presently, if a patient has a vigorous immunogenic response to L-ASNase isolated from *E. coli*, they are switched to an L-ASNase isolated from other sources, such as the plant pathogen, *Erwinia chrysanthemi*. The *Erwinia chrysanthemi* asparaginase (ErA) does not cross-react with *E. coli* asparaginase (EcA) antibodies, thereby serving as an alternative treatment option.[63,64] However, 14% of patients receiving ErA suffer from an allergic reaction to it, which is the main reason for treatment discontinuation.[65] Thus, the employment of longer half-life and more stable formulations avoiding multiple dosing can reduce the chance of activating hypersensitivity reactions.[66] The basic therapeutic strategy presently utilized to avoid severe side effects in patients is the employment of different sources of L-ASNase; therefore, many studies have been focused on identifying different sources of L-ASNase and evading the strong immunogenic

Table 1 Summary of assays that can be used to determine L-ASNase activity

Category	Side effects
Hepatopathy	Hypoalbumin, decreased serum cholesterol concentrations, increased transaminase and bilirubin concentrations, abnormal lipoproteins and alkaline phosphatase
Pancreatic diseases	Diabetic ketosis, acute hemorrhagic pancreatitis, pancreatitis, decreased serum levels of insulin
Hypersensitivity	Mild to severe local reactions, urticaria, bronchospasm, serum sickness, hypotension, anaphylaxis, immunosuppression
Coagulopathic diseases	Increased prothrombin time, decreased plasminogen concentration, hypofibrinogenemia, coma, thromboembolism
Neuropathy	Neurotoxicity, leading to depression, drowsiness, fatigue, restlessness, agitation and giddiness

response induced by the higher glutaminase activity of the EcA drug, such as the screening of L-ASNase from bacteria, fungi, and yeast, for high throughput.[18,67–72]

At the same time, researchers are not only exploring possible sources of production, but also experimenting with different immobilization methods to enhance therapeutic efficacy, such as the novel p(HEMA-GMA) cryogel invented by Noma *et al.* which resulted in a significant increase in enzyme activity and stability, and Ca–ALG/MWCNT–COOH hybrid beads designed by Ulu *et al.* which enhanced the affinity of the enzyme for the substrate.[73–75]

4. Activity assay

L-ASNase alleviates ALL by depleting L-Asn in leukemia cells and exerting a cytotoxic effect on these cells. In order to pursue the best therapeutic effect and reduce the side effects of drugs, it is very important to establish a rapid, sensitive and specific method for detecting L-ASNase activity in serum. Most main detection methods have been summarized in this review, such as fluorescence assays, colorimetric assays, spectroscopic assays and some other assays, which possess both advantages and disadvantages in accuracy, linearity, sensitivity, measurement range and cost.[76] Therefore, these analytical methods are systematically summarized and reviewed to provide reference for the establishment of new analytical methods and the design of standardized analytical schemes in the future.

It is an effective method to analyze protein drugs by absorbance detection. Since proteins are chromophores with the highest absorbance in the UV range, the easiest way to measure L-ASNase concentration is to evaluate absorption at 280 nm. But there is also the possibility of contaminating the compound.[77,78] Additionally, L-Asp or NH_3 produced by enzymatic reactions can be quantitated through numerous established L-ASNase assays as the products of substrate transformation. Meanwhile, alternative substrates are used to react with L-ASNase.[79] Herein, we mainly summarized and discussed the L-ASNase activity detection principles and specific applications of the following three methods: fluorescence method, colorimetry method, and spectrometry method. Other analytical methods are also briefly described, such as chromatography, paper electrophoresis, conductimetry, and kinetic enzymatic methods. A diagram (Fig. 4) was constructed to explain the principles and processes of the main detection methods.

4.1 Fluorescence assay for L-ASNase detection

Recently, fluorescent probes have been used prodigiously as a sensitive means of *in vitro* detection and *in vivo* imaging due to their great selectivity and high specificity.[80,81] The reaction of the fluorescent probe with the enzyme produces changes in the fluorescence signal, which is captured using a fluorescence spectrometer to detect the activity of the enzyme. The configuration of a fluorescent probe mainly

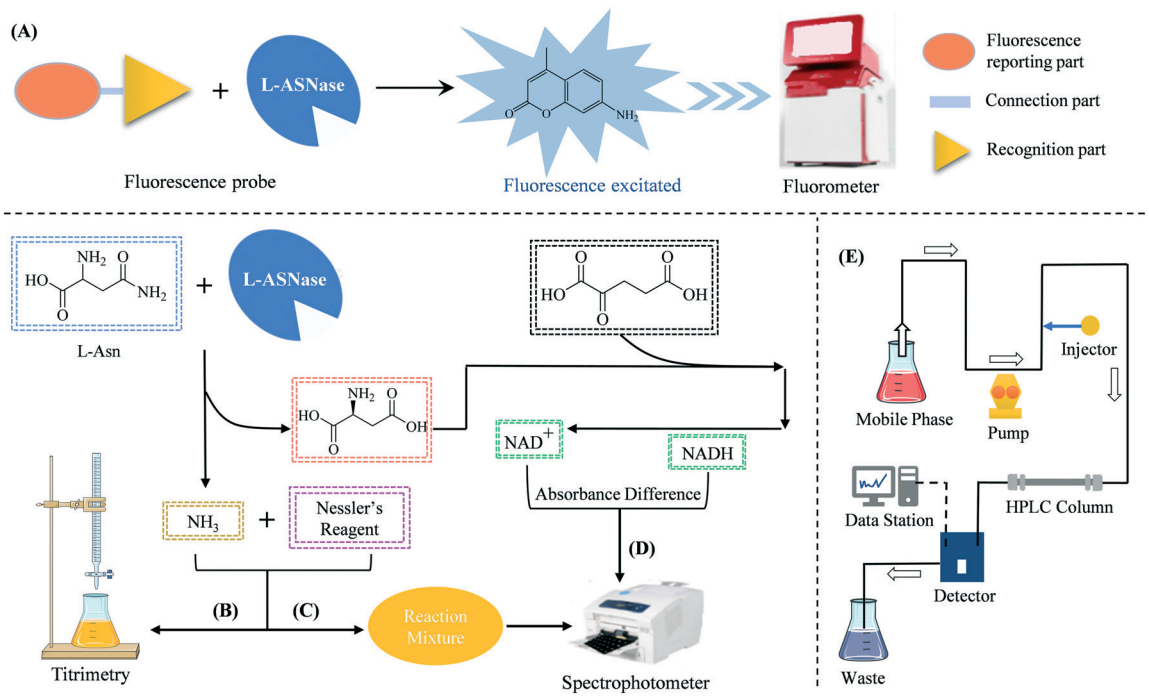

Fig. 4 Process diagram of different activity assays. (A) Fluorescent probe: L-aspartic acid β-(7-amido-4-methylcoumarin) generated markedly enhanced fluorescence and was detected with a fluorometer. (B) Colorimetry: NH_3 produced by enzymatic reaction was titrated using Nessler's reagent. (C) Spectrophotometry based on Nessler's reagent: the reaction mixture was quantified by spectrophotometry. (D) Spectrophotometry based on nicotinamide adenine dinucleotide (NADH) consumption: L-Asp produced by the enzymatic reaction consumed NADH, with a change in absorbance that is directly proportional to the rate of NADH consumption. (E) HPLC flow illustration. L-ASNase: L-asparaginase.

consists of the following parts: fluorescence reporting part, connection part, and recognition part. The role of fluorophores is to convert molecular response into detectable fluorescence signals. The connection part is the linker as it connects the fluorescence reporting part to the recognition part, which affects the signal transmission. The recognition part can specifically bind or react with targets, activating a change in the fluorescence signal of the probe. Accordingly, it plays a role in the specific recognition of the detected object.[82,83]

Previously, Ylikangas *et al.* reported that L-aspartic acid β-(7-amido-4-methylcoumarin) (AspAMC) could be an alternative substrate for the surveillance of L-ASNase activity in the serum of ALL patients during their treatment.[22] The release of the substrate was measured using fluorescence (Fig. 4A). Briefly, the researchers incubated L-ASNase with AspAMC and measured the release of 7-amino-4-methylcoumarin fluorometrically from 30 to 300 min. The hydrolysis rate of the substrate was linear over a 50-fold range of the enzyme concentration. In ALL patients' serum samples, the probe took an average recovery of 106.5%. These results suggest that an enhanced method of assaying L-ASNase activity in bio-samples has been established. This method could thus be used to monitor L-ASNase activity in patients during L-ASNase treatment.

Recently, our group developed a novel fluorogenic probe 6-naphthylamine-2,3-dicarboxylate-asparagine (TPAN-Asn), which was based on the intramolecular charge transfer (ICT) effect and exhibited great selectivity and activity in complex physiological environments.[23] When prolonging the reaction time, the fluorescence intensity of the probe decreased at 425 nm and increased at 500 nm during *in vitro* tests as the reaction time increased. In contrast, when increasing the enzyme concentration from 1000 to 3000 U L^{-1}, the fluorescence intensity ratio increased at both sites (Fig. 5). The calculated detection limit was 264.4 U L^{-1}, showing the good sensitivity of the probe. They also performed tests on ALL patients' samples with a relative standard deviation (RSD) of 2.8%, and the average recovery of TPAN-Asn was 101.1%, indicating excellent accuracy.

Comparing AspAMC with TPAN-Asn, TPAN-Asn had a good selectivity and was not easily disturbed. Meanwhile, its fluorescence intensity had a much wider linear relationship with concentration and it did not respond to 53 common interferents, in which another amidohydrolase glutamyltranspeptidase was included. In addition, in ALL patients' serum samples, TPAN-Asn also had a superior and consistent average recovery value, which means it is more advantageous in real sample testing, whereas AspAMC showed a lower detection limit than TPAN-Asn.

Although current fluorescent probes have some unique advantages, they inevitably also possess some shortcomings, such as the fact that most probes can only detect a single substrate and are susceptible to interference in complex human body environments. Sometimes, alternative fluorescence substrate assays show a relatively high limit of detection and long incubation time required, but the feasibility of fluorescent probes for L-ASNase activity detection was demonstrated. Researchers are currently optimizing the structure of the L-ASNase fluorescent probe to enable improved detection performance and imaging capabilities. The unique advantages of the fluorescent probe could result in this method becoming indispensable for the clinical diagnosis of ALL during L-ASNase therapy.[54,84]

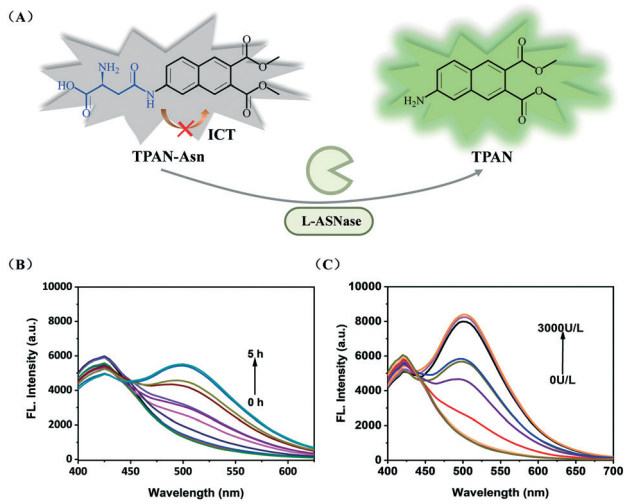

Fig. 5 (A) TPAN-Asn activated the ICT effect upon hydrolysis of the Asn group and showed an onset of fluorescence. (B) Fluorescence emission spectra of 50 μM TPAN-Asn incubated with 1500 U L^{-1} L-ASNase at 50 °C for 0–5 h (λ_{ex} = 360 nm). (C) Fluorescence spectra of 50 μM TPAN-Asn incubated with changing concentration (from 0 to 3000 U L^{-1}) of L-ASNase at 50 °C for 3 h (λ_{ex} = 360 nm).[23] Reproduced from ref. 23 with permission from Elsevier, copyright 2021.

4.2 Colorimetric assays for L-ASNase detection

The traditional analysis method, colorimetry, is used to determine the content of the components to be measured. This is achieved *via* a comparison or measurement of the color depth of a colored substance. The colorimetric Nessler method is generally the simplest and most commonly used method for determination of L-ASNase activity (Fig. 4B).[85] Magri *et al.* reported an approach for the quantitative analysis of reaction mixtures, relying mainly on spectrophotometry. A yellow color was produced in solution by the reaction of Nessler's reagent (dipotassium tetraiodomercurate(II)) with ammonia released during the conversion of L-Asn to L-Asp, and extinction at 450 nm is measured with a colorimeter (Fig. 4C).[86] In the presence of salts, Nessler's reagent usually forms turbidity, resulting in the formation of colorless and insoluble complexes with mercury. Subsequently, it was also experimentally demonstrated that salts of Mg^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+} and Ni^{2+} interfere with ammonium quantification using Nessler's reagent, while salts of Na^{+}, Ca^{2+}, Cu^{2+}, Zn^{2+} and Mo^{2+} do not have an influence.[87,88] An improved Nessler's reagent method has recently been proposed by Simas *et al.* It was

experimentally demonstrated that the combination of tartaric acid salts and PVA can significantly reduce the interference caused by salts in the analyte on the assay results.[87]

Colorimetric methods, such as the Nessler method, exhibit good reproducibility; however, they require meticulous care and require high product concentrations (LOD ≥ 20 U L^{-1}).[89] As these methods are not very selective for ammonia, quantification will be disturbed when a complex medium is employed, ultimately preventing accurate assessment of enzymatic activity. During the quantification, the ammonia in the medium is detected together with the one produced in the enzyme catalysis, which in turn affects the results of the enzyme activity assay, so it is also important to control the original ammonia concentration in the sample *via* commonly used pretreatment methods.[90] In addition, factors including mixing speed and reaction temperature can also affect the rates of color development in solution, resulting in method-associated errors that cannot be ignored.[89] There are few reports on the use of Nessler's reagent to assess L-ASNase activity in serum, mainly due to the complexity of the matrix in serum. The various proteins in the serum will inevitably affect the reaction and measurement of Nessler's reagent, such as the appearance of unobvious discoloration or the formation of precipitates.[87,91]

There are also some colorimetric methods that use substrate substitution for detection. As early as 2002, Lanvers *et al.* showed that in the presence of an oxidizing agent, L-ASNase would catalyze the hydrolysis of L-aspartic acid β-hydroxamate (AHA) to form hydroxylamine, which then reacted with 8-hydroxyquinoline in an alkaline environment. Finally, the produced green indooxine dye could be detected at 710 nm.[92] This method featured a relatively low detection limit (>2 × 10^{-5} U L^{-1}), but the pH range of the test environment was demanding because hydroxylamine was only stable in neutral solution. Additionally, recent research revealed that compared to high performance liquid chromatography (HPLC) methods, the assays using Nessler's reagent overestimated the reported results, while using AHA and indooxine underestimated the results.[86]

4.3 Spectroscopic assays for L-ASNase detection

Spectroscopic methods are often employed to identify substances and determine sample chemical composition and relative content according to their spectra. The most common methods are absorption spectroscopy, circular dichroism (CD) spectroscopy, and Fourier transform infrared (FTIR) spectroscopy.

Once, Peterson and Dunlop *et al.* reported simple methods for L-ASNase detection, and a method using β-nicotinamide adenine dinucleotide (NADH) to probe L-ASNase was developed by Cooney *et al.* as early as 1970.[89,95] Recently, Fernandez *et al.* reported a new type of NADH method that results in high throughput and has a wide linear range (0.025–2.2 IU mL^{-1}).[94] The probing mechanism of the whole chemical process is shown in Fig. 6A. To quantify the release

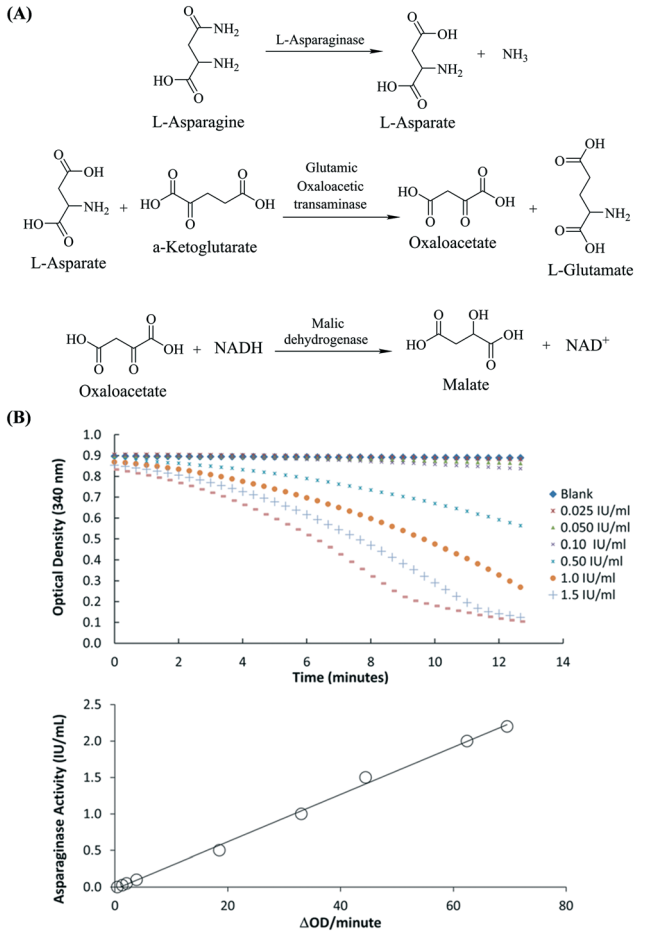

Fig. 6 (A) Synthetic route for NADH-based detection of L-ASNase activity. (B) Spectrophotometric calibration curves for L-ASNase activity measurement.[94] Reproduced from ref. 94 with permission from ICJEM, copyright 2013.

of L-aspartate from L-ASNase, an enzyme reaction mixture, containing L-Asn, glutamic oxaloacetic transaminase, α-ketoglutarate, malic dehydrogenase and reduced NADH, is mixed with the samples. In this method, the variation of absorbance over time is proportional to the rate of NADH dissipation in the sample, also the same as L-ASNase activity (Fig. 4D). Following reaction initiation, absorption values are recorded every 20 s for 15 min to capture the changes in absorbance associated with L-ASNase activity. The linear region of the curve is then used to determine the reaction rate (Fig. 6B). Ultimately, this high-throughput method was found to provide a broader linear range of detection.

The limited sensitivity of absorption-based spectrophotometry is a major limitation of this method. However, better detection is usually achieved by increasing the sample volume. Karamitros *et al.* used Amplex Red to detect L-Asp produced indirectly in a spectrophotometric manner.[96] By using this method, the analytical measurement was found to range from 0.011 to 2.2 IU mL^{-1}, which is more sensitive than the NADH dependent assay. Further, this method required 10-fold less L-ASNase than the NADH

dependent assay. Kudryashova *et al.* developed a new method to determine the catalytic parameters of L-ASNase using CD spectroscopy. In this method, a difference in CD signaling between L-Asn and L-Asp is generated during the enzyme reaction. By measuring this difference, it is possible to continuously monitor the modified form of the natural enzyme.[97] Due to the presence of carboxyl and amide groups, L-Asn and L-Asp are optically active in the far-UV region (195 to 215 nm), while the molar ellipticity of the products is much higher than that of the substrate. The tests show that the dependence of the catalytic activity is proportional to the enzyme content in the concentration range from 0.001 to 0.015 mg ml^{-1} at a substrate concentration of 20 mM. In 2020, the same team proposed a reagent-free FTIR method to derive the kinetic parameters of L-ASNase as well as modified forms of different compositions in an aqueous solution and optically-dense multicomponent systems.[98] In this method, since the absorption bands of the L-Asn amide and carboxyl groups in L-Asp were well differentiated and easily identified from other absorption bands, they were the most analytically valuable for the determination of L-ASNase activity. The variation of the analyzed peak (at 1680 cm^{-1}, 1590 and 1390 cm^{-1}) intensities in the substrate concentration interval from 3 to 40 mM was linear over the first 5 min, allowing the reliable determination of the initial hydrolysis rate of L-ASNase in the V_{max} mode. This method showed a detection limit of 0.1–0.5 IU ml^{-1}, and it was also suitable for a variety of complex multi-component systems.

4.4 Other assays for L-ASNase detection

High performance liquid chromatography (HPLC), one of the common methods used for analysis and detection, is indispensable. Generally, HPLC methods (Fig. 4E) show more precise detection results than spectroscopy methods, though their detection processes are relatively complicated.[101]

Although HPLC is tedious and requires specialized and trained personnel, the results obtained with this method are relatively accurate and reliable. To achieve better detection results, Zhang *et al.* optimized a reverse-phase HPLC assay by improving the signal and stability of the analytes. Other important factors, including the precipitating reagent, derivatization conditions, and detection wavelengths, were also optimized.[104] However, the complexity of the operation and the high requirement of instrument use must be overcome for convenient detection.

Previously, Tagami *et al.* reported a simple kinetic method with an ammonia gas-sensing electrode to assay L-ASNase and L-Asn. This method was based on the deamination of L-Asn by L-ASNase from *E. coli*, which led to the formation of

Table 2 Summary of assays that can be used to determine L-ASNase activity

Assay	Substrate	Product analyzed	Characteristics	Ref.
Fluorescent probe	AspAMC	7-Amino-4-methylcoumarin (AMC)	A sensitive assay but this process needs to be performed with care, and it also possessed a K_m of 0.302 mM, with a detection limit of 100 U L^{-1} in neutral phosphate buffer	22
	TPAN-Asn	6-Naphthylamine-2,3-dicarboxylate (TPAN)	The probe has excellent accuracy, and has a detection limit of 264.4 U L^{-1} in neutral phosphate buffer. It also maintained specificity for 53 common interferents	23
Colorimetry	L-Asn	Ammonia	The reproducibility is good, but the method requires careful reagent handling, and Nessler's reagent is imprecise	85
	AHA	Indooxine	A relatively good detection limit but requires a demanding pH range	92
Spectrophotometry	L-Asn	Ammonia	This method is simple but dangerous and not sensitive enough to detect low L-ASNase concentrations	93
	L-Asn	L-Asp	High-throughput but complex	94
	L-Asn	Ammonia	Good linearity for detection of ammonia concentrations between 0 and 200 μM	95
	L-Asn	Ammonia	Good repeatability, detection range and accuracy. Protocol is demanding and dangerous	85
	L-Asn	L-Asp	High sensitivity and substrate selectivity	96
Circular dichroism spectroscopy	L-Asn	L-Asp	Direct and continuous method	97
FTIR spectroscopy	L-Asn	L-Asp	Direct detection without sample preparation. Wide complexity range and multicomponent systems	98
Kinetic enzymatic method (gas sensing electrode)	L-Asn	Ammonia	Direct quantification of ammonia. High cost but quantifies ammonia very accurately and instantaneously	99
Paper electrophoresis	L-Asn	L-Asp	Good detection sensitivity, but a cumbersome procedure	100
Chromatography	L-Asn	L-Asp	Capable of detecting picomolar levels of L-Asp but is time consuming and not continuous	101
Conductimetry	L-Asn or aspartate hydroxamate	Ammonia/aspartate	Relatively easy and fast assay method	102
	L-Asn	L-Asp	A fast response (30 s) and sensitive (LOD = 140 nM) detection method	103

ammonia. L-ASNase was detected to be in the concentration range of 0.1–1 mIU mL^{-1}. Accordingly, this method was deemed an accurate and instantaneous quantification method for ammonia; however, it is very expensive.[99] In addition, Drainas *et al.* reported a simple and instantaneous method of carrying out conductivity analysis based on the increase in conductivity while Frank *et al.* proposed a sensitive but time-consuming paper electrophoresis assay.[100,102] The latest related study showed that Gholami *et al.* immobilized L-ASNase on PANI/MWCNT/Nf nanocomposites to prepare a fast response (30 s) and sensitive detection (LOD = 140 nM) biosensor, which was successfully applied in the detection of patient blood samples.[103] In addition, we summarize all the above detection assays to show their characteristics (Table 2).

Among these methods, colorimetric methods are relatively simple to operate but also require complex reagents, and spectrophotometric methods are usually simple and fast but also cumbersome, while chromatographic methods usually require expensive equipment and long detection times. In addition, the fluorescent probe methods are fast, easy to operate and easy to observe, but there are some interference problems in humans. Therefore, we believe that the rapid and convenient colorimetric methods have better economic benefits in the production and purification processes, while the specificity and rapid response of the fluorescent probe methods will make them more promising in clinical testing.

5. Conclusions

L-ASNase has been extensively employed as a therapeutic agent for the treatment of ALL. This review summarized the structure, antitumor mechanisms, and side effects of L-ASNase, and evaluated the main detection of L-ASNase.

Although functional and structural studies have provided important references for exploring molecular signaling pathways and their response mechanisms, the specific molecular signaling pathway of L-ASNase therapy for ALL is still unclear. Most of the clinically available L-ASNase preparations are isolated from *E. coli*. The type II L-ASNase exhibits a high specific hydrolytic activity for L-Asn and displays anti-cancer activity. However, L-ASNase treats tumors through its cytotoxic effects with many side effects. Currently, the search for more microbial sources available for production (*e.g. Bacillus australimaris* NJB19) and the development of nanocarriers with better performance (*e.g.* magnetic nanoparticles) are effective means to improve the therapeutic efficacy of L-ASNase. While in clinical therapy, the detection of L-ASNase can help achieve accurate cancer treatments and reduce side effects. Spectrometry is the most widely reported activity detection method for L-ASNase since it is rapid and convenient. Chromatography is more sensitive and accurate. Fluorescence methods for determining the clinical activity of L-ASNase can achieve not only rapid and accurate detection *in vitro*, but also *in situ* and real-time evaluation in animal models.

To date, there has been no simple method to measure the activity of the toxic drug L-ASNase. Because of many interferences, indirect detection assays could not easily obtain accurate results from real tests. Furthermore, more accurate methods cannot achieve the level of simplicity and rapidity required in the clinic. Sensitive and convenient fluorescent probes that can directly interact with L-ASNase have been demonstrated to be more suitable for L-ASNase detection than other methods. In fact, their convenience and quick visualization capabilities could prove to be markedly significant during clinical diagnosis. Unfortunately, studies regarding fluorescent probes for L-ASNase detection are seldom reported. Finally, we believe that the mature and convenient Nessler's reagent method will continue to be developed and improved in the production assay of L-ASNase. For clinical detection, the unique advantages of the fluorescent probe method make it more promising, and we believe that probes with better specificity, reaction speed and optical properties will continue to be developed. We hope that in the future there will be better methods to improve the selectivity and sensitivity of clinical detection assays, which will bring more help to the health of humans.

Author contributions

N. Wang, W. Ji, Q. Wu, Dr. W. Du, and B. Ma presented the outline of this review. N. Wang wrote the Abstract, Introduction and Conclusions, "Structure and antitumor mechanism" section, and "Side effects" section. W. Ji, and N. Wang wrote the "Activity assay" section. W. Ji, N. Wang, L. Wang, W. Wu and W. Zhang made the graphic design and tables. W. Ji, Q. Wu, Dr. W. Du, B. Peng, B. Ma, and L. Li revised and edited the draft. Q. Wu, Dr. W. Du and B. Ma conceived and supervised this project. All the authors participated in the discussion of the draft and carefully revised the draft before the final submission.

Conflicts of interest

There are no conflicts to declare.

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