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MINI-REVIEW

# Microbial L-asparaginase as a promising enzyme for treatment of various cancers

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## Abstract

Metabolic differences between normal and cancerous cells have been used as a point of view for developing anticancer drugs. Some degrading enzymes of certain amino acids have been regarded to kill cancerous cells. L-Asparaginase (ASNase) has shown an excellent therapeutic response to asparagine-auxotrophic cancers such as acute lymphoblastic leukemia (ALL). Some bacteria, yeasts, molds, plants, and animals produce ASNase. Bacterial ASNases from *Escherichia coli* and *Erwinia chrysanthemi* are the FDA-approved drugs for ALL treatment. Here, we review new natural prokaryotic and eukaryotic sources of ASNases, recent advances to introduce improvement strategies for the production of recombinant ASNases as well as their chemical modifications, immobilization, nanoencapsulation, and in silico studies to increase efficiency and decrease side effects. Recent studies for application of ASNases to treatment of asparagine-auxotrophic cancers, especially solid cancers, have been reviewed. Furthermore, challenges and future perspectives are discussed for this promising therapeutic enzyme.

### Key points

- *Review recent advances to introduce new sources of microbial L-asparaginases.*
- *Review improvement strategies for the development of stable and non-toxic L-asparaginases.*
- *Review microbial L-asparaginase application in various cancers' treatment.*

**Keywords** L-Asparaginase · Therapeutic enzyme · Anticancer drug · New microbial source · Improvement strategies

## Introduction

Enzymes are potent biocatalysts that are commercially utilized in various industrial functions, from clinical approaches to biofuels. Due to rapid growth in improvement techniques, expansion of enzyme application has attracted more attention over recent decades (Lukey et al. 2017). Tumor cells usually have unbalanced enzyme activity, which can be used as an effective strategy for therapeutic purposes. In other words, cancer is a consequence of perturbation of multiple different cellular pathways in which one or more enzymes lost their normal function (Baig et al. 2019). Cancer cells often have metabolically addictions to some extracellular essential substances because of either epigenetic silencing of enzyme genes or even genetic mutations in the enzyme's coding sequence. Therefore, many enzymes with different functions could target cancer cells and exert an anticancer effect. As an example, some cancer cells are dependent on exterior arginine amino acid and cannot synthesize their required arginine. Therefore, arginase can act as

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an anticancer drug by limiting arginine usage in these cancer cells (Garcia-Bermudez et al. 2020). As another example, PEGylated kynureninase degrades kynurenine (as an immunosuppressive metabolite) in the tumor microenvironment (TME) leading to an increase in the number of cytotoxic T cells ($CD8^+$ cells) and tumor cell death (Triplett et al. 2018). Enzyme therapy is a cost-effective and targeted approach that has fewer complications compared to conventional therapies such as surgery, radiotherapy, and chemotherapy (Baig et al. 2019). In addition, enzyme therapy approach offers several advantages relative to some novel treatments like DNA and mRNA cancer vaccines, which stimulate immune system against cancer cells. DNA and mRNA cancer vaccines need more various preparation laborious works such as the selection of target tumor antigen in comparison to enzyme therapy (Jahanafrooz et al. 2019). There have been many reported preclinical and clinical examples of enzyme application in cancer therapy. Some enzymes have been approved by the Food and Drug Administration (FDA); for instance, L-asparaginase (ASNase) or asparagine amidohydrolase (EC.3.5.1.1), as an anticancer drug (Dhankhar et al. 2020). In this review, recent information about various natural sources of ASNase and applied new improvement strategies, including chemical modifications, in silico studies, immobilization, and nanoencapsulation to provide more stable, less immunogenic, and more functional enzymes with high affinity to asparagine (Asn) are discussed. Then, we summarized the recent successes in the anticancer properties of ASNases in blood cancers. Furthermore, this review highlights the application of this enzyme as a promising bioactive molecule for the treatment of solid tumors.

## Historical background of L-asparaginase as an anticancer agent

At the beginning of the 1900s, the capacity of beef liver suspension and pig and horse tissues to hydrolyze Asn was revealed (Geddes and Hunter 1928). In 1922, it was found that the presence of ASNase is a type of biochemical adaptation to a vegetarian diet. Other experiments determined the distribution of ASNase in bacteria, yeast, plants, and other animals (Geddes and Hunter 1928). Evidence of tumor inhibitory characteristics of ASNase goes back to 1953, in which, for the first time cytotoxic effect of guinea pig serum on the cells of three transplantable mice and rat lymphomas in vivo was indicated. Later in 1961–1963, it was described that ASNase is the efficient component of guinea pig serum (Krishnapura et al. 2016; Mashburn and WRISTON Jr 1964). Further investigation showed that ASNase from *Escherichia coli* has similar anti-neoplastic activity to one extracted from guinea pig serum. Then large-scale production of ASNase from bacterial sources increased the availability of enzymes for therapeutic application. Around the 1970s, ASNase was introduced against acute lymphoblastic leukemia (ALL), and its anticancer effect has been proven against ALL. FDA approved ASNases from *E. coli* and *Erwinia chrysanthemi* in 1978 for use in ALL treatments (Krishnapura et al. 2016). So far, ASNase has been extracted from various origins, including bacteria, yeast, fungi, algae, plants, and animals (Ghasemian et al. 2019; Mazloum-Ravasan et al. 2020). Each source has its advantages and disadvantage with different physicochemical and kinetic properties such as molecular mass, optimum temperature and pH, $V_{max}$, and $K_m$ of the enzyme. Microorganisms are industrial sources for the production of ASNases (Mohideen 2020). There are two isoforms of bacterial ASNases with differences in structure and cellular localization. ASNase type I is a cytosolic enzyme with a lower affinity to Asn, and ASNase type II is a periplasmic enzyme with a higher affinity to Asn. ASNase type II is a promising enzyme in anticancer research (Mohideen 2020). Some recent studies about various microbial sources for ASNase production were summarized in Table 1. Compounds of culture media, fermentation, and purification processes are influencing factors on quality, stability, and activity of enzymes (Dias et al. 2016). ASNases from *E. coli*, *Er. chrysanthemi*, *Er. aroideae*, and *Serratia marcescens* were established and approved for cancer treatment. Immunogenicity of *Er. chrysanthemi* ASNase is lower than purified ASNase from *E. coli* (Duval et al. 2002). Recombinant ASNases have been investigated widely as a solution to decrease immunogenicity and glutaminase activity as well as increase stability and substrate affinity. Recombinant ASNases have been produced in safer and high-yield hosts (Table 2). The amino acid sequences can improve in recombinant ASNases via genetic engineering techniques such as site-directed mutagenesis (Brumano et al. 2019).

## Underlying anticancer mechanism of L-asparaginase

Asn auxotrophic state or low/no expression of ASNS is a required feature for a cancer cell to be sensitive to ASNase (Garcia-Bermudez et al. 2020). According to studies, increased expression of ASNS promotes cancer cell invasion and metastasis (Chiu et al. 2019). In addition to ASNS activity, the amount of glutamine (Gln) is another influencing host factor in ALL and other cancer (such as prostate cancer) cells response to ASNase, though, more resistance to ASNase is reported in cells with a normal Gln synthase, a high expression of glutamic acid (Glu) transporters, and a residual ASNS protein expression (Fig. 1) (Chiu et al. 2019). ASNase exerts anticancer effects at last in two perspectives. At first view, it hydrolyses non-essential Asn amino acid to

**Table 1** Selected studies for production of L-asparaginase as an anticancer agent from wild-type microorganisms

| Origin | Characteristics | References |
|---|---|---|
| Bacteria | | |
| *Erwinia chrysanthemi* | Marketed as Erwinaze® or Erwinase®<br>With low L-glutaminase activity | Emadi et al. (2018) |
| *Streptomyces fradiae* NEAE-82 | High stability<br>Higher catalytic activity over a wide range of pH and temperature<br>A potent anticancer agent<br>Molecular weight 53 kDa | El-Naggar et al. (2016) |
| *Streptomyces rochei* NEAE-K | A selective strong anti-proliferative effects on cervical epithelioid carcinoma (HeLa) and human liver cancer (HepG-2) cell lines<br>Molecular weight 64 kDa | El-Naggar and El-Shweihy (2020) |
| *Bacillus licheniformis* | L-Glutaminase free activity<br>Molecular weight 33.7 kDa<br>Cytotoxic activity against E6-1, MCF-7, and K-562 cell lines | Mahajan et al. (2014) |
| *Nocardiopsis alba* NIOT-VKMA08 | With no L-glutaminase activity<br>Very specific for L-asparagine | Meena et al. (2015a) |
| Molds | | |
| *Aspergillus oryzae* CCT3940 | No glutaminase activity<br>High stability under physiological condition<br>A more significant antitumor activity than commercial ASNases<br>Maximum enzyme activity 552.2 U $mL^{-1}$ | Dias et al. (2016) |
| *Aspergillus* sp. ALAA-2000 | Stable at 30 to 70 °C for 60 min<br>Maximum enzyme activity 30.64 U $mL^{-1}$<br>Molecular weight 25 kDa (type I)<br>Molecular weight 31 kDa (type II) | Abbas Ahmed et al. (2015) |
| *Curvularia* sp.<br>*Rhizopus* sp.<br>*Aspergillus* sp. | No glutaminase and urease activity<br>Maximum enzyme activity 33.59 U $mL^{-1}$ produced by *Aspergillus* sp. C7 strain | Doriya and Kumar (2016) |
| *Trichosporon asahii* IBBLA1 | Free from glutaminase and urease<br>Maximum enzyme activity 20.57 U $mL^{-1}$ | Ashok et al. (2019) |
| *Fusarium solani* AUMC8615 | High L-ASNaase production<br>Maximum enzyme activity 187.9 U $mL^{-1}$<br>Molecular weight 70 and 80 kDa | Isaac and Abu-Tahon (2016) |
| *Fusarium equiseti* AHMF4 | Without adverse effects related to commercial ASNase<br>Antiproliferative activity against Hela, epidermoid larynx carcinoma (Hep-2), hepatocellular carcinoma (HepG-2), colorectal carcinoma (HCT-116), and breast adenocarcinoma (MCF-7)<br>Maximum enzyme activity 40.78 U $mL^{-1}$<br>Molecular weight 45.7 kDa | El-Gendy et al. (2021) |
| Yeasts | | |
| *Yarrowia lipolytica* DSM3286 | With anticancer effects higher than commercial ASNase<br>Induction apoptosis and inhibition of growth in Burkitt's lymphoma Raji and acute lymphoblastic leukemia MOLT-4 cells<br>Inhibiting growth and migration of lung (A549) and breast (MCF7) solid cancer cells<br>Maximum enzyme activity 210 U $mL^{-1}$ | Darvishi et al. (2019); Darvishi and Shamsi (2018); Mazloum-Ravasan et al. (2021) |
| *Leucosporidium scottii* | High yield enzyme production<br>Co-production of lipids during the cultivation | Moguel et al. (2020) |

aspartic acid and ammonium ions ($NH_4^+$) that cause depletion of Asn in the plasma. Cancer cells mostly lose/reduce their asparagine synthetase (asparagine synthase, ASNS) and are dependent on plasma Asn to provide this amino acid for their protein, DNA, and RNA synthesis and metabolism; moreover, because of the negative charge of aspartic acid and

**Table 2** Selected studies for production of L-asparaginase by mutant and recombinant microorganisms

| Origin | Difference with wild-type ASNase | Advantages to wild-type ASNase | References |
|---|---|---|---|
| Bacteria | | | |
| *Erwinia chrysanthemi* | With E63Q mutation | Diminished ability to hydrolyze L-glutamine | Nguyen et al. (2016) |
| | With H240A mutation | Decreased immunogenicity | Yari et al. (2019) |
| *Escherichia coli* | Expressed in *PichiaPink*™<br>Humanized by glycosylation patterns | Better protein folding and processing<br>Efficient in evoking clinical efficiency | Sajitha et al. (2015) |
| | Expressed in *PichiaPink*™<br>With Man5GlcNAc2 glycans on its structure and triple mutant ASNase (3 M) | Less immunogenic | Lima et al. (2020) |
| *Acinetobacter soli* | Heterologously produced in *E. coli* | No activity toward L-Gln and D-Asn<br>Maximum enzyme activity 42 U $mL^{-1}$ | Jiao et al. (2020) |
| *Erwinia carotovora* | Heterologously produced in *E. coli*<br>Molecular weight 34.5 kDa | – | Roth et al. (2013) |
| *Streptomyces griseous* | Heterologously produced in *E. coli*<br>Molecular weight 33.7 kDa | No glutaminase activity<br>Maximum enzyme activity 123 U $mL^{-1}$ | Meena et al. (2015b) |
| *Pseudomonas fluorescens* | Heterologously produced in *E. coli* | Better activity at 37 °C<br>Better stability at 37 °C | Kishore et al. (2015) |
| Molds | | | |
| *Aspergillus terreus* | Heterologously produced in *E. coli*<br>Molecular weight 42 kDa | No activity toward L-Gln<br>Prolonged half-life | Saeed et al. (2018) |
| Yeasts | | | |
| *Saccharomyces cerevisiae* | Heterologously produced in *P. pastoris* | Less immunogenic<br>High yield enzyme production | Pillaca-Pullo et al. (2021); Rodrigues et al. (2019) |

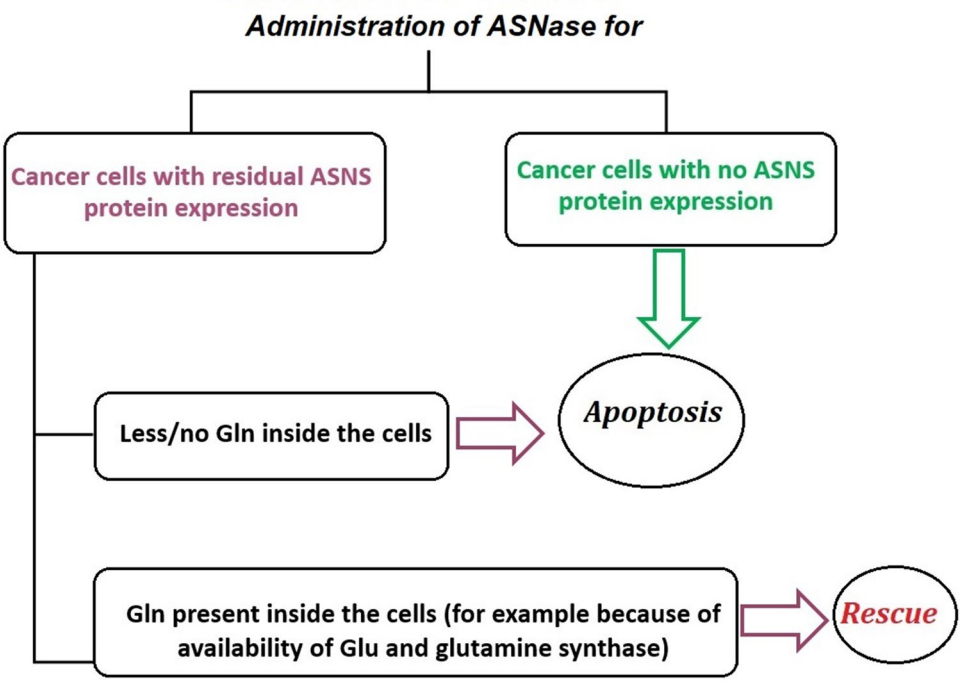


**Fig. 1** Effect of L-asparaginase (ASNase) therapy on cancer cell context. As indicated here, ASNase treatment in cancer cells with no asparagine synthase (ASNS) drives apoptosis. However, the absence or presence of glutamine (Gln), as an amine group donor, is a crucial factor in driving apoptosis or survival/rescue, respectively, in cancer cells with fewer ASNS

its impermeability to the plasma membrane, internal Asn is also the main precursor for aspartic acid in tumor cells. Therefore, Asn depletion automatically leads to aspartic acid depletion in cancer cells which results in decreased protein synthesis and possibly cell death. At the second view or non-canonical route, ASNase influences the cancer

cells directly; it was shown that ASNase affects the level of reactive oxygen species (ROS), cell cycle progression, autophagy, and apoptotic cell death (Song et al. 2015 and 2017; Costa-Silva et al. 2020). Inhibition of Akt/mTOR and Erk signaling pathways under ASNase treatment is another anticancer mechanism. Akt/mTOR and Erk signaling pathways are essential in cell growth and survival (Dhankhar et al. 2020). Akt/mTOR signaling pathway is also involved in autophagy induction. Administration of autophagy inhibitors in K562 chronic myeloid leukemia (CML) cells caused an increase in apoptotic cell death under ASNase treatment (Song et al. 2015). In addition, diffusion of ammonium ions into the cytosol by modifying pH leads to the activating of an apoptotic signaling pathway (Krishnapura et al. 2016).

## Chemically modified L-asparaginases

Like many other peptide drugs, various formulation strategies have been applied to reach more extended bioavailability or stability and decreased immunogenicity of ASNase. PEGylated *E. coli* ASNase pegaspargase (Oncaspar®) was approved by the FDA in 1994 and applied for the first-line treatment of ALL in 2006. In pegaspargase, 69–82 molecules of monomethoxy polyethylene glycol (PEG) chains are covalently attached to the cysteine amino acids side chains in ASNase (Lima et al. 2020). According to a comparative study, patients who received pegaspargase showed fewer side effects, less allergic response, and required fewer medical care visits which caused similar treatment overall cost to a native enzyme (Brumano et al. 2019). The half-life of pegaspargase (i.e., 5.5–7 days) is significantly higher than the native one (i.e., 26–30 h). Further, the required dosage of pegaspargase is reported as 2000–2500 IU/$m^2$ every 2 or 4 weeks that is less than for native ASNase, which is 6000 IU/$m^2$ thrice per week times/week (Dhankhar et al. 2020). Compared to native enzymes, PEGylated ASNase shows a decreased drug immunogenicity, increased water solubility, less peptide aggregation, increased pH and temperature resistance, and improved drug stability and efficiency. However, random attachment of chemical substances like PEG to ASNase causes batch-to-batch variation (Brumano et al. 2019). A novel more stable bioconjugate of ASNase and PEG named calaspargase pegol-mknl (CALASP) was approved in 2018 in which a recombinant *E. coli* ASNase conjugated with monomethoxy-PEG with a succinimidyl carbonate (SC) linker (Li et al. 2020). Intravenous injection of CALASP showed significantly longer serum asparaginase activity compared to pegaspargase which is critical to drug efficacy and successful treatment (Li et al. 2020). PASylation is an alternative to PEGylation in which proline/alanine-rich sequences (PAS) covalently bond with peptide drugs via genetic fusion or chemical coupling (Binder and Skerra 2017). PASylated ASNase, similar to PEGylated ASNase, introduced a stable form of ASNase with less/no immunogenicity in vivo (Brumano et al. 2019). Lactosylation and coupling with dextran are other chemical modifications that lead to a prolonged half-life of ASNase and increased resistance to thermal and protease cleavage (Muneer et al. 2020). It was reported that the involvement of some adjuvants such as sucrose and sorbitol in ASNase formulation could lead to an increase in the specific activity and stability, and an increase enzyme aggregation (Wlodarczyk et al. 2019).

## Immobilization and encapsulation of L-asparaginase

Different delivery methods have been applied to provide stability of peptide drugs under body temperature and pH. A promising approach for the cost-effective delivery of ASNase is its immobilization on various support materials, either physically or in a covalent attachment. In physical attachment, hydrogen bonds, van der Waals forces, and ionic interactions cause adsorption between enzymes and supports, which usually have a more negligible effect on the natural 3D structure of enzymes than covalent attachment (Brumano et al. 2019). According to one study, immobilization of ASNase on magnetic (i.e., $Fe_3O_4$) poly 2-hydroxyethyl methacrylate (HEMA), glycidyl methacrylate (GMA) nanoparticles decreased the $k_{cat}$ value of enzyme, which results in an increased affinity to a substrate. Also, the thermal stability and operational stability of immobilized ASNase on magnetic poly (HEMA-GMA) nanoparticles were significantly higher than Free-ASNase (Orhan and Aktaş Uygun 2020). Moreover, nanoparticles can be used for encapsulation of ASNase, which provides numerous advantages such as drug stability, reduced concentration of the drug, enhanced circulating time in body fluids, and decreased unwanted interaction between host proteins such as antibodies and drugs (Mu et al. 2020). Various biodegradable and biocompatible materials used in noncarrier forms, including liposomes, cationic polymers, cationic peptides, carbon nanotubes, and hollow nanospheres, are used widely to deliver peptide drugs such as ASNase (Brumano et al. 2019). Blackman et al. designed a polymeric vesicle known as polymersome or "nanobioreactor" encapsulated ASNase, which had some advantages compared to other nanocapsule-based ASNase because not only it could exert an anticancer effect without entering the cells but also protect enzyme against proteases and antibody recognition (Blackman et al. 2018). Both encapsulation and immobilization techniques not only increase enzyme stability and selectivity but also decrease the side effect of ASNase, including allergic response, blood coagulation, hepatic, pancreatitis,

and central nervous system toxicity. Moreover, the incorporation of bio-conjugate with cell surface-specific monoclonal cancer-specific antibodies could demonstrate an even more targetable function of ASNase in tumor cells (Poznansky et al. 1982). There is no nanoencapsulated FDA-approved ASNase so far. Size or mass heterogeneity of prepared nanostructures, less optimal biocompatibility, and a requirement to be decorated with targetable agents to reach specific binding to cancer cells are among the suggested drawbacks for the application of nanostructure-based ASNase, which can be solved by time (Brumano et al. 2019).

## In silico and molecular docking studies for improvement of L-asparaginase

Computer-aided techniques such as in silico studies have been used to analyze a collection of the previously reported experimental database to screen high-performance enzymes (Darvishi et al. 2019). Molecular docking, as more useful in silico studies, has been used for analyzing the interaction between both enzyme–substrate and receptor-ligand (Baral et al. 2020; Mohideen 2020). Indeed, docking has been used to select the better ASNase from a large group of ASNases from various sources. Baral et al. analyzed ASNases produced by different bacteria and archaea in a phylogenetic tree by homology modeling and bioinformatics tools to find the genus that synthesizes ASNase with a similar function and structure to *E. coli* ASNase as a template (Baral et al. 2020). It was proposed that enzymes with a different amino acid sequence are less immunogenic. The selected ASNase producers were at the most phylogenetic distance from the two commercially available genus producers ASNase type II (*E. coli* and *Erwinia*). Then, they evaluated the $K_m$, $k_{cat}$, binding energy, and active site interaction for selected ASNases by docking software and found ASNase from *Streptomyces griseus*, *Streptomyces collinus*, and *Streptomyces venezuelae* have better kinetics rather than currently commercially available ASNase (Baral et al. 2020). Another in silico study demonstrated that ASNase type I from *Vibrio campbellii* is a stable dimeric enzyme with a molecular weight of 36.9 kDa, which has a higher binding affinity to Asn and can be regarded as an alternative for commercially ASNase for treatment of ALL (Mohideen 2020). In addition, molecular docking studies have been used to predict the kinetic characteristics and glutaminase activity of various mutant versions of ASNase. The V27T mutant version of ASNase showed more stability and less glutaminase activity than the wild type with 100% retained activity (Ardalan et al. 2018). Also, N24S mutation was proposed as a protease-resistant and more stable ASNase by in silico studies (Maggi et al. 2017). Overall, molecular docking studies can suggest required modifications to improve ASNase for cancer treatment.

## L-Asparaginase as a potent anticancer agent

Unbalanced enzymes not only can be regarded as cancer biomarkers for cancer detection and validation but also can provide therapeutic targets in various approaches (Baig et al. 2019). The anti-proliferative effects of many natural agents are because of inhibiting enzymes or modulation of their expression. For instance, rice callus suspension culture by targeting and inhibiting lactate dehydrogenase leads to an increase in reactive oxygen species (ROS) and apoptosis induction in cancer cells (Baig et al. 2019). In addition, enzymes themselves have been used as therapeutic agents in cancer treatment. As normal cells can synthesize their required amino acids through their normal pathways, the application of depleting enzymes along with chemotherapy in auxotrophic tumors is reported as a targeted therapy in various cancers. Arginine deiminase, ANSase, methionase, lysine oxidase, glutaminase, and phenylalanine ammonia lyase are some microbial-depleting enzymes under investigation for cancer treatment (Dhankhar et al. 2020). ANSase causes depletion of Asn in TME, resulting in cancer cell death or growth arrest. ASNases from *E. coli* and *Er. chrysanthemi* have been approved and used globally in different brand names for ALL treatments. Further, its anticancer capacity in other blood cancers and solid cancers is promising, as discussed in the following (Ghasemian et al. 2019; Song et al. 2015).

## L-Asparaginase in treatment of hematological cancers

Blood cancers originate from blood cells or their precursors inside the bone marrow in various stages of differentiation. According to Fig. 2, blood cancers are divided based on their start location, subsequent tumor cell behavior, and originated cells; for example, acute myeloid leukemia (AML) originates from myeloid cells, which proliferate quickly (acute), or ALL starts in lymphocytes; if the cancerous state of lymphocytes begins in the lymph system, they will create lymphoma which according to their origin (T or B cells) and behavior are classified into a few subtypes. Radiation therapy, stem cell transplantation, chemotherapy, and targeted therapy are frequently used treatments for blood cancers (Chu et al. 2020). Some novel therapeutic approaches such as chimeric antigen receptor (CAR) T cell therapy have been also applied for the treatment of some leukemias (ALL and CLL) and large B cells non-Hodgkin's lymphoma. CAR T cell therapy is an exceptional therapy in

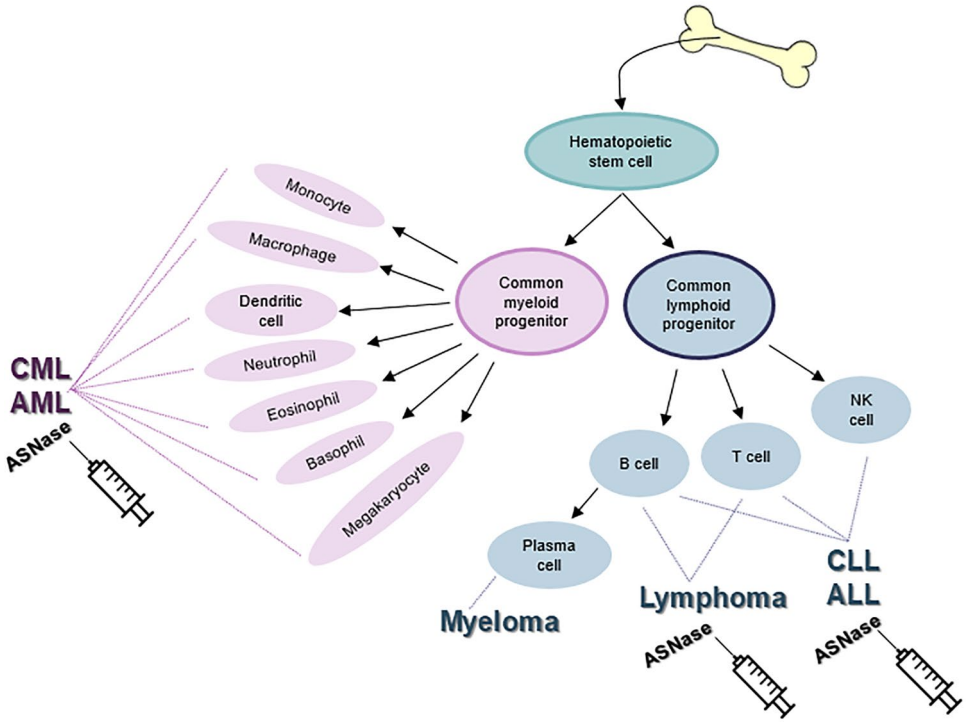


**Fig. 2** Various blood cancer groups and L-asparaginase (ASNase) therapy. As the hematopoietic stem cell divisions go through in the bone marrow, the progenitors become progressively more specialized in the range of cell types that they can give rise to, as indicated in this diagram. However, abnormalities in genetic, epigenist, and environmental factors mislead the normal differentiation of blood cells and convert them to malignant cells (red arrows), which are classified according to their starting cells and place (i.e., lymphoma starts in lymph nodes). ASNase has been approved for the treatment of acute lymphocytic leukemia (ALL) and has shown potential antineoplastic characteristics against chronic lymphocytic leukemia (CLL), adult acute myeloid leukemia (AML), chronic myeloid leukemia (CML), and lymphoma

which the patient's T cells are modified in the laboratory, so they can efficiently recognize and react against cancer cells. Today, researchers are working on expanding CAR therapy to cancers other than blood cancers (Lemal and Tournilhac 2019). CAR T cell therapy has some drawbacks, namely, a lack of determining cancer antigens approach, cytokine-derived toxicities, and immunosuppressive media of TME (Liu et al. 2017). Hematopoietic stem cell transplantation (HSCT), the oldest immunotherapy, is another cell therapy applied in pediatric ALL. Although HSCT can rescue children with high-risk ALL, scarcity of HLA-matched siblings or unrelated donors and conditioning regimens are some of the main obstacles to this type of therapy (Merli et al. 2019; Shem-Tov et al. 2020). Because of some severe side effects, CAR T cell therapy and HSCT, or both of them are primarily used for the treatment of relapsed ALL. Despite various sophisticated therapeutic approaches, easy, safe, and simple treatments are constantly attracting more attention, so enzymes, as a growing class of peptide drugs, are valuable agents for medicinal purposes.

ALL treatment response to ASNase has the most improved outcomes among blood cancers. Thus ALL cells are the most dependent cells to blood Asn for survival (Costa-Silva et al. 2020). T cell-derived ALL (T-ALL) showed more response to ASNase therapy among the hematological cancers (Dhankhar et al. 2020). As discussed in previous sections, investigation to find an improved form of *E. coli* and *Er. chrysanthemi* ASNase (e.g., recombinant, chemically modified, encapsulated, immobilized form) or finding alternative sources of ASNase is still ongoing. As an example, we reported that ASNase purified from *Yarrowia lipolytica* DSM3286 as a eukaryotic source because of its higher anticancer effects, no glutaminase activity is a promising alternative enzyme for ALL and Burkitt's lymphoma treatment (Mazloum-Ravasan et al. 2020). ASNase has shown anticancer characteristics in other types of blood cancer; for instance, because of low expression of ASNS in M0, M1, M4, and M5 subgroups of AML, they also showed sensitivity to ASNase. Moreover, the frequency of chromosome 7 monosomy is noticeable in AML patients because the gene encoding ASNS is located on chromosome 7 (Chiu et al. 2019).

## L-Asparaginase in treatment of non-hematological cancers

ASNase is a potent anticancer agent for ALL and Hodgkin's lymphoma. Given that, about 80% of human cancer belongs to carcinoma, the effectiveness of ASNase in this large group would be valuable. Carcinoma starts in epithelial cells throughout the body, which cover the outside and inside layers of the body (Lee-Six et al. 2019). Sarcoma cancers are another group of human cancers originating from connective tissues other than blood. Most successful anticancer agents are used for a few types of cancer, and one of the suggested reasons for their limited usage is the different nature of tumor cells and their strange TME (Jahanafrooz et al. 2020). As well as other anticancer agents, the anticancer activity of ASNase is also evaluated on several human cancer cell lines; for instance, the cervical cancer HeLa cell line showed reduced proliferation under the ASNase treatment isolated from *Pseudomonas aeruginosa* in a dose-depended manner (Fatima et al. 2019). In one study, ASNase from *Helicobacter pylori* inhibited cell cycle progression in fibroblasts and gastric cancer cell lines (Scotti et al. 2010). Interestingly, ASNase can disrupt several forms of peptide glycosylation in the endoplasmic reticulum (ER), including sialylation. Oligosaccharide is transferred to the side-chain $NH_2$ group of an Asn in the newly synthesized peptide. It was shown that distortion of the glycosylation pattern under ASNase treatment could inhibit the binding of ovarian cancer cells to the endothelial cell surface, thus inhibiting heterotypic cell–cell adhesion, which is needed for cancer cell dissemination (Yu et al. 2012).

Furthermore, phase I and II clinical trials have been performed to evaluate the effect of ASNase from *E. coli* in patients with pancreatic adenocarcinoma with null/low ASNS expression. In order to reduce toxicity, ASNase was applied in the form of erythrocyte-encapsulated ASNase (eryaspase) (ERYTECH Pharma, Lyon, France) or "cellular microbioreactor" in the phase I trial. In phase II clinical study in 2019, a combination of eryaspase with chemotherapy showed more improvements in patients' overall survival without progression. Notably, it was mentioned that a phase III clinical trial is underway (Bachet et al. 2015; Hammel et al. 2020). Table 3 summarizes the other examples of ASNase application for treating non-hematological cancers.

## Challenges and future perspectives in L-asparaginase application

ASNase is the intrinsic targetable drug for asparagine-auxotroph cancer cells. However, some undesirable aspects need to be considered to reach the best version of ASNase. Incorporation of non-myelosuppressive agents such as PEG with the enzyme has yielded better Asn depletion in adult ALL patients (Patel et al. 2017). The emergence of anti-PEG immunity in some patients, as well as the different number and architecture of attached PEG to peptide drugs like ASNase, are unsolved issues in the pharmaceutical market (Lima et al. 2020). It is worth mentioning that immunogenicity against ASNase itself is another possible undesirable effect of ASNase therapy, especially in adult patients. Therefore, recombinant glycosylated ASNase is somewhat preferred to PEGylated ASNase and non-glycosylated one (Lima et al. 2020). In addition, there are some other simple considerations to overcoming the previous barrier, including the amount of injected ASNase, administration route of a drug (intravenous or IV injection has more risk of allergic reaction than intramuscular or IM), injection times, and overall health/other health problems of cancer patients (Hasan et al. 2016). Moreover, the incorporation of ASNase with nano-carriers could palliate its immunogenicity or hypersensitivity (Hasan et al. 2016). In one in vitro study, selenium nanobiocomposites including fungal ASNases were synthesized, and its antiproliferative effect on human colon cancer, liver cancer, and osteosarcoma cell lines was evaluated (Baskar et al. 2019). Selenium nanoparticles selectively induce intrinsic apoptosis only in cancer cells and these nanobiocomposites of ASNase are suggested as a promising anticancer agent for colon cancer (Baskar et al. 2019).

The engineered mesenchymal stem cells (MSCs) involving the gene encoding ASNase could be used as a novel version of enzyme therapy (Lin et al. 2019). Furthermore, vectors carrying a gene encoding ASNase could be another way for enzyme therapy. Still, low transfection efficiency of the plasmids to cancer cells as well as a possible unnecessary response of the immune system to vectors are regarded as the main drawbacks of this strategy (Johansson and Ward 2017; Martino and Markusic 2020). The two mentioned methods have not been evaluated so far.

Stem cell-based cancer therapies are interesting and recommended for cancer treatment. Furthermore, MSC-derived membrane microvesicles (MVs) are also considered a drug delivery system. MVs are in a size range of 0.1–1 μm and loaded MVs with anticancer drugs demonstrated their promising potential in successful drug delivery (Chulpanova et al. 2018). Hence, MSC-derived MVs can be loaded with ASNase for effective cancer therapy.

**Table 3** Selected studies for treatment of asparagine-auxotroph solid cancers using L-asparaginase

| Cancer type | Study model | Results | ASNase origin | Form of ASNase | References |
|---|---|---|---|---|---|
| Gastric cancer | In vitro | Decrease in tumor cell growth and proliferation, induction of intrinsic apoptosis, and cell cycle arrest in AGS cells | *Pseudomonas fluorescens* | ASNase without any modification | Sindhu and Manonmani (2018) |
| Lewis lung carcinoma | BALB/c mice model | Significant decrease in tumor size and increase in a life span | *Pectobacterium carotovorum* | PEG-coated nano-liposomes conjugation | Do et al. (2019) |
| Breast cancer | BALB/c mice model | Inhibition of proliferation and growth of 4T1 tumor cells implanted to mice in a chrono-pharmacological manner | – | ASNase without any modification | Shiromizu et al. (2018) |
| Breast cancer | In vitro | Decrease in cell proliferation rate and clonogenicity of MCF-7 cells as well as initiating apoptosis along with overexpression of P53 and down-regulation of Bcl2 and promoting cell cycle arrest at the G2/M with no cytotoxic effect on normal cell line (WRL-68) | *E. coli* | Immobilization on PEGylated gold nanoparticles (GNPs) along with Arg-Gly-Asp (RGD) peptide | Al-Dulimi et al. (2020) |
| Breast cancer | In vitro | Apoptosis induction and inhibition of migration in MCF7 cells | *Yarrowia lipolytica* | ASNase without any modification | Mazloum-Ravasan et al. (2021) |
| Lung cancer | | Autophagy and apoptosis induction and inhibition of migration in A549 cells | | | |
| Ovarian cancer | In vitro | Inhibition of invasion of ovarian cancer cells and capillary tube formation | – | ASNase without any modification | Yu et al. (2012) |
| Liver cancer<br>Colon cancer<br>Osteosarcoma | In vitro | Significant toxicity effect on colon cancer and osteosarcoma cells | *A. terreus* | Selenium nanobiocomposites of ASNase | Baskar et al. (2019) |

Another caution in ASNase therapy is because of the side products that are produced during asparagine breakdown; agitation, confusion, and disorientation are some reported neurologic side effects as the result of a higher amount of circulating aspartic acid and ammonia (Vimal and Kumar 2017). For amelioration of the impact of an increasing amount of aspartic acid and ammonia, some combination therapy for purgation of aspartic acid and ammonia may be helpful. Liver and pancreas damage are also other reported toxicities following ASNase administration; in this regard, preclinical studies showed that application of glutaminase-free ASNase decreased its hepatotoxic effect because Gln is one of the ASNS substrates and the mentioned form of ASNase is not capable of inhibiting Asn synthesis in normal cells (Sahoo and Hart 2003). Other depleting strategies such as blocking Asn uptake by usually overexpressed transporters, for example, SLC transporters, on cancer cells is one of the alternatives suggested therapy to exploiting this goal; however, so far, they have not received FDA approval (Bhutia et al. 2014; Wang et al. 2015).

Finally, the production of ASNase in the eukaryotic host such as *S. cerevisiae* and *Y. lipolytica* could be a good substitution for the prokaryotic host because of the deletion of prokaryotic immunogenic epitopes and production of a glycosylated and more stable enzyme (Varsha et al. 2015; Darvishi 2014; Darvishi et al. 2018; Liu et al. 2021). ASNase from a human source is another alternative eukaryotic enzyme and would avoid the problems caused by the bacterial ASNase. Although the human ASNase did not have any glutaminase activity, its $K_m$ is significantly higher than bacterial ASNase. Therefore, studies to increase the affinity of the human ASNase to Asn and provide an effective anticancer enzyme are moving on it (Belviso et al. 2017). On the other hand, humanizing pig ASNase by generating chimeras with the human ASNase is another suggested solution to reach a low $K_m$ human-like enzyme. Human-like ASNases were a combination of the N-terminal domain of pig ASNase and the C-terminal domain of the human ASNase, which were cloned and expressed in *E. coli* and exerted in vitro ALL killing potential (Rigouin et al. 2017).

## Conclusions

Natural products have always played a crucial role in discovering new therapeutics. ASNase is a nature-provided enzyme and, as a bioactive molecule, influences only Asn-dependent cancer cells with no cytotoxic effect on normal cells; therefore, ASNase has the main characteristic of being a successful anticancer drug. In addition, this approach does not depend on prodrug conversion to be effective. As discussed above, not only ASNase considered a powerful chemotherapeutic anti-leukemic and anti-lymphoma drug, but also an excellent prospective drug for usage in the treatment of other cancers. Therefore, by minimizing its side effects and combination with commercial and novel strategies, this promising bio-compound could be a widespread anticancer drug in the future. As an essential point, the dependency content of cancer cells to Asn is a determinant factor in the responsiveness of cancer cells to ASNase therapy.

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## Declarations

**Ethics approval** This article does not contain any studies with human participants or animals.

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