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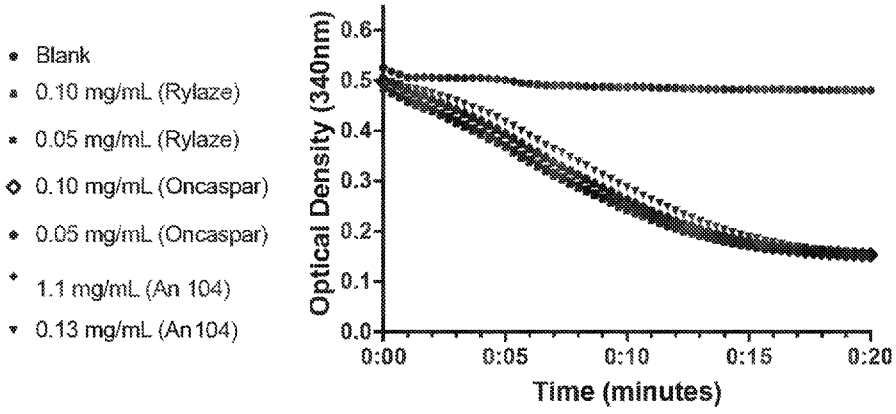

FIG. 2A

(57) Abstract: Disclosed herein are recombinant asparaginases with ancestral variant sequences for uses as therapeutics. In certain embodiments, it is contemplated that the ancestral variant asparaginases have reduced immunogenicity thereby preventing or reducing the risk of host inactivation and/or anaphylaxis. In certain embodiments, this disclosure relates to treating diseases associated with asparagine dependence comprising administering an effective amount of an ancestral variant asparaginase or conjugate disclosed herein to a subject in need thereof.

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RECOMBINANT ANCESTRAL VARIANT ASPARAGINASES AND USES IN MANAGING CANCER

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 63/472,192 filed June 9, 2023. The entirety of this application is hereby incorporated by reference for all purposes.

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED AS AN XML FILE VIA THE OFFICE ELECTRONIC FILING SYSTEM

The Sequence Listing associated with this application is provided in XML format and is hereby incorporated by reference into the specification. The name of the XML file containing the Sequence Listing is 22185PCT.xml. The XML file is 92 KB, was created on June 7, 2024, and is being submitted electronically via the USPTO patent electronic filing system.

BACKGROUND

Asparagine amidohydrolase (asparaginase) is a natural enzyme that catalyzes the breakdown of L-asparagine. Therapeutic asparaginases, also referred to as "L-asparaginase" or "L-ASNase" are commonly used in acute lymphoblastic leukemia (ALL) chemotherapy regimens and have played a significant role in improving these outcomes. However, many clinically approved asparaginases are derived from bacterial sequences. Patients often produce inactivating antibodies and are at risk of anaphylaxis among other adverse side effects such as hepatotoxicity, pancreatitis, coagulopathy, and thrombosis. Thus, there is a need to identify improvements.

Gupta et al. report data indicating asparaginase discontinuation results in poorer survival in high risk ALL patients. J Clin Oncol. 2020; 38(17):1897-1905.

Schalk et al. report identification and structural analysis of an L-asparaginase enzyme from guinea pig with putative tumor cell killing properties. J Biol Chem, 2014, 289(48): 33175–33186.

Rigouin et al. report discovery of human-like L-asparaginases with potential clinical use by directed evolution. Scientific Reports, 2017, 7: 10224. See U.S. Patent No. 11,578,315.

Vrooman et al. report efficacy and toxicity of pegaspargase and calaspargase pegol in childhood acute lymphoblastic leukemia. J Clin Oncol, 2021, 39:3496-350.

Fonseca et al. report strategies for circumventing the side effects of L-asparaginase. Biomed Pharmacother, 2021, 139:111616.

References cited herein are not an admission of prior art.

SUMMARY

This disclosure relates to asparaginases with ancestral variant sequences disclosed herein for uses as therapeutics. In certain embodiments, it is contemplated that the ancestral variant asparaginases have reduced immunogenicity thereby preventing or reducing the risk of host inactivation and/or anaphylaxis. In certain embodiments, this disclosure relates to treating diseases associated with asparagine dependence comprising administering an effective amount of an ancestral variant asparaginase or conjugate disclosed herein to a subject in need thereof.

In certain embodiments, this disclosure relates to recombinant ancestral variant asparaginase comprising an amino acid sequence selected from SEQ ID NO: 1-53 or variant thereof having 70%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity.

In certain embodiments, this disclosure relates to recombinant ancestral variant asparaginase comprising an amino acid sequence selected from SEQ ID NO: 54-63 or variant thereof having 70%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity.

In certain embodiments, this disclosure relates to methods of treating cancer comprising administering an effective amount of an ancestral variant asparaginase disclosed herein to a subject in need thereof. In certain embodiments, the cancer is a hematological cancer or a solid cancer.

In certain embodiments, the cancer is leukemia, lymphoma, acute lymphoblastic leukemia (ALL), lymphoblastic lymphoma (LBL), acute myelogenous leukemia (AML), chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma (SLL), chronic myelogenous leukemia, acute monocytic leukemia (AMOL), chronic myeloid leukemia (CML), B-cell acute lymphoblastic leukemia (B-ALL), myeloproliferative neoplasms (MPNs), and lymphomas, Hodgkin's lymphomas, and non-Hodgkin's lymphomas such as Burkitt lymphoma, B-cell lymphoma, or diffuse large B-cell lymphoma (DLBCL).

In certain embodiments, the cancer is a solid malignancy such as the cancer is a solid cancer ovarian cancer, ovarian clear cell carcinoma, pancreatic cancer, pancreatic ductal adenocarcinoma

(PDAC), colorectal cancer (CRC), breast cancer, metastatic breast cancer, glioblastoma, or hepatocellular carcinoma.

In certain embodiments, this disclosure relates to recombinant ancestral variant asparaginase is administered in combination with methotrexate, cytarabine, rapamycin, temozolomide, and/or 6-diazo-5-oxo-1-norleucine.

In certain embodiments, the recombinant ancestral variant asparaginase is conjugated to a biodegradable polymer. In certain embodiments, the biodegradable polymer comprises polyethylene glycol. In certain embodiments, the biodegradable polymer comprises an amide linking group connecting polyethylene glycol to the recombinant asparaginase. In certain embodiments, the biodegradable polymer comprises polyethylene glycol or monomethoxy polyethylene glycol. In certain embodiments, the biodegradable polymer comprises an amide linking group connecting polyethylene glycol or monomethoxy polyethylene glycol to the recombinant asparaginase through a lysine amino acid and/or to the N-terminal amino acid.

In certain embodiments, this disclosure relates to nucleic acids encoding a recombinant ancestral variant asparaginase disclosed herein in operable combination with a heterologous promoter. In certain embodiments, this disclosure relates to vectors encoding a nucleic acid encoding a recombinant ancestral variant asparaginase disclosed herein.

In certain embodiments, this disclosure relates to host cells comprising a nucleic acid or vector encoding a recombinant ancestral variant asparaginase disclosed herein. In certain embodiments, the nucleic acid is naked DNA, RNA, or mRNA.

In certain embodiments, this disclosure relates to pharmaceutical compositions comprising an ancestral variant asparaginase or conjugated as disclosed herein.

In certain embodiments, this disclosure relates to the use of a recombinant ancestral variant or conjugated as disclosed herein in the production or a medicament to treat conditions and diseases disclosed herein.

In certain embodiments, the recombinant asparaginases disclosed herein elicit a lower immunogenic response in a patient compared to other clinically approved L-asparaginases, e.g., E. coli L-asparaginase or Erwinia chrysanthemi L-asparaginase. In certain embodiments, significantly reduced immunogenicity is evidenced, e.g., by the reduction or elimination of an antibody response against the L-asparaginase preparation following administration or repeated administrations; and/or usefulness as a second-line therapy for patients who have developed

sensitivity to first-line therapies using, e.g., E. coli L-asparaginase or Erwinia chrysanthemi L-asparaginase or other non-E. coli-derived L-asparaginases. In certain embodiments, the recombinant asparaginases disclosed herein provide reduced immunogenicity and enhanced plasma half-life. In certain embodiments, the recombinant asparaginases disclosed herein are used in a method of treating patients with relapsed ALL who were previously treated with other asparaginase preparations, in particular those who were previously treated with E. coli-derived asparaginases.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Figure 1 shows a sequence comparison of human L-asparaginase (subject SEQ ID NO: 64) and an ancestral recombinant ancestral construct 104 "An 104" (query SEQ ID NO: 10). There are 508 out of 573 (89%) amino acid identities.

Figure 2A shows data indicating An-104 has asparaginase activity. L-asparaginase catalyzes the hydrolysis of L-asparagine to L-aspartate. Glutamic oxaloacetic transaminase subsequently catalyzes the transamination of L-aspartate and α-ketoglutarate to oxaloacetate and L-glutamate. Oxaloacetate is then reduced to malate in the presence of malic dehydrogenase with the concurrent oxidation of reduced β-nicotinamide adenine dinucleotide (NADH) to β-nicotinamide adenine dinucleotide (NAD+). The time-dependent change in absorbance of NADH at 340 nm is monitored on a microplate reader using the kinetic rate method. The change in absorbance over time is directly proportional to the rate at which the NADH is consumed, and it is directly related to the asparaginase activity of the sample.

Figure 2B shows data on asparaginase activity tested at an enzyme concentration of 0.1 mg/mL and an asparagine substrate concentration of 1 μM. Ancestral arginases reported herein typically have an IC_{50} of less than 1 μM. An-104 demonstrated desirable activity, within a comparable range to the clinically relevant E. coli and Erwinia asparaginases.

Figure 3A shows cytotoxicity data testing the ancestral L-ASNase candidates against a T-ALL cell line. CCRF-CEM IC_{50} values calculated using calorimetric MTT assay, correlating metabolic activity with number of viable cells. CCRF-CEM cells (100,000) were incubated with increasing concentrations of An-ASNase for 72 hours and IC_{50} values determined. An-107 demonstrated the highest cytotoxicity against CCRF-CEM cells.

Figure 3B shows data indicating anti-leukemia properties of ancestral L-asparaginases. MOLT-4 (T-ALL) cells (100,000) were incubated with increasing concentrations of An-ASNase for 72 hours and IC_{50} values determined using a trypan blue cell count.

Figure 4A shows constructs using domain swapping providing Ancestral Human Hybrids L-ASNases. Non-functional C-terminal ankyrin repeat domains are replaced with human sequence to increase sequence identity to human L-ASNase.

Figure 4B shows a sequence identify comparison of ancestral L-asparaginase-human hybrid sequences to human L-asparaginase.

Figure 5 shows results of in silico immunogenicity assessment of ancestral L-asparaginases. The calculations indicate that immunogenic epitope density to HLA-DRB1*07:01 in ancestral asparaginase candidates is lower when compared to current clinical bacterial asparaginases. The MHCII binding predictions were made using the IEDB analysis resource NetMHCIIpan (ver. 4.1) tool. HLA-DRB1*07:01 is associated with a higher risk of asparaginase allergies. See Fernandez et al. HLA-DRB1*07:01 is associated with a higher risk of asparaginase allergies. Blood. 2014, 124(8):1266-1276. See also Kaabinejadian et al. Accurate MHC Motif Deconvolution of Immunopeptidomics Data Reveals a Significant Contribution of DRB3, 4 and 5 to the Total DR Immunopeptidome. Front Immunol, 2022, 13:835454.

DETAILED DESCRIPTION

Before the present disclosure is described in greater detail, it is to be understood that this disclosure is not limited to particular embodiments described, and as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present disclosure will be limited only by the appended claims or as amended during prosecution.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present disclosure, the preferred methods and materials are now described.

All publications and patents cited in this specification are herein incorporated by reference as if each individual publication or patent were specifically and individually indicated to be

incorporated by reference and are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present disclosure is not entitled to antedate such publication by virtue of prior disclosure. Further, the dates of publication provided could be different from the actual publication dates that may need to be independently confirmed.

As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present disclosure. Any recited method can be carried out in the order of events recited or in any other order that is logically possible.

Embodiments of the present disclosure will employ, unless otherwise indicated, techniques of immunology, medicine, organic chemistry, biochemistry, molecular biology, pharmacology, physiology, and the like, which are within the skill of the art. Such techniques are explained fully in the literature.

It must be noted that, as used in the specification and the appended claims, the singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise. In this specification and in the claims that follow, reference will be made to a number of terms that shall be defined to have the following meanings unless a contrary intention is apparent.

As used herein, the term "about” means a range of values including the specified value, which a person of ordinary skill in the art would consider reasonably similar to the specified value. In embodiments, about means within a standard deviation using measurements generally acceptable in the art. In embodiments, about means a range extending to +/- a percentage of the specified value. In embodiments, about includes the specified value. In certain embodiments, the term “about” can include a 5 % or 10 % difference.

As used in this disclosure and claim(s), the words "comprising" (and any form of comprising, such as "comprise" and "comprises"), "having" (and any form of having, such as "have" and "has"), "including" (and any form of including, such as "includes" and "include") or "containing" (and any form of containing, such as "contains" and "contain") have the meaning

ascribed to them in U.S. Patent law in that they are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

"Consisting essentially of" or "consists of" or the like, have the meaning ascribed to them in U.S. Patent law in that when applied to methods and compositions encompassed by the present disclosure refers to the idea of excluding certain prior art element(s) as an inventive feature of a claim, but which may contain additional composition components or method steps, etc., that do not materially affect the basic and novel characteristic(s) of the compositions or methods, compared to those of the corresponding compositions or methods disclosed herein.

The term "comprising" in reference to a peptide having an amino acid sequence refers a peptide that may contain additional N-terminal (amine end) or C-terminal (carboxylic acid end) amino acids, i.e., the term is intended to include the amino acid sequence within a larger peptide. The term "consisting of" in reference to a peptide having an amino acid sequence refers to a peptide having the exact number of amino acids in the sequence and not more or having not more than a range of amino acids expressly specified in the claim. In certain embodiments, the disclosure contemplates that the "N-terminus of a peptide consists of an amino acid sequence," which refers to the N-terminus of the peptide having the exact number of amino acids in the sequence and not more or having not more than a rage of amino acids specified in the claim however the C-terminus may be connected to additional amino acids, e.g., as part of a larger peptide. Similarly, the disclosure contemplates that the "C-terminus of a peptide consists of an amino acid sequence," which refers to the C-terminus of the peptide having the exact number of amino acids in the sequence and not more or having not more than a range of amino acids specified in the claim however the N-terminus may be connected to additional amino acids, e.g., as part of a larger peptide.

A "subject" refers to any animal, preferably a human patient, livestock, or domestic pet.

As used herein, the terms "treat" and "treating" are not limited to the case where the subject (e.g., patient) is cured and the disease is eradicated. Rather, embodiments of the present disclosure also contemplate treatment that merely reduces symptoms, and/or delays disease progression.

As used herein, the terms "prevent" and "preventing" include the prevention of the recurrence, spread or onset. It is not intended that the present disclosure be limited to complete prevention. In some embodiments, the onset is delayed, or the severity of the disease is reduced.

As used herein, the term "combination with" when used to describe administration with an additional treatment means that the agent may be administered prior to, together with, or after the additional treatment, or a combination thereof.

As used herein, the term "therapeutically effective amount" refers to the amount of a protein (e.g., asparaginase or conjugate thereof), required to produce a desired therapeutic effect.

The term "nucleic acid" refers to a polymer of nucleotides, or a polynucleotide, e.g., RNA, DNA, or a combination thereof. The term is used to designate a single molecule, or a collection of molecules. Nucleic acids may be single stranded or double stranded and may include coding regions and regions of various control elements.

The term "encoding" refers to the inherent property of specific sequences of nucleotides in a polynucleotide, such as a gene, a cDNA, or an mRNA, to serve as templates for synthesis of other polymers and macromolecules in biological processes having either a defined sequence of nucleotides (e.g., rRNA, tRNA and mRNA) or a defined sequence of amino acids and the biological properties resulting therefrom. Thus, a gene, cDNA, or RNA, encodes a protein if transcription and translation of mRNA corresponding to that gene produces the protein in a cell or other biological system. Both the coding strand, the nucleotide sequence of which is identical to the mRNA sequence and is usually provided in sequence listings, and the non-coding strand, used as the template for transcription of a gene or cDNA, can be referred to as encoding the protein or other product of that gene or cDNA.

The terms "polypeptide," "peptide," and "protein" are used interchangeably herein to refer to polymers of amino acids of any length. The polymer can comprise modified amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component. Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids such as homocysteine, ornithine, p-acetylphenylalanine, D-amino acids, and creatine), as well as other modifications known in the art.

A "heterologous" nucleic acid sequence or peptide sequence refers to a nucleic acid sequence or a peptide sequence that does not naturally occur, e.g., because the whole sequence contains a segment from other plants, bacteria, viruses, other organisms, or joinder of two

sequences that occur the same organism but are joined together in a manner that does not naturally occur in the same organism or any natural state.

The term "recombinant" when made in reference to a nucleic acid molecule refers to a nucleic acid molecule which is comprised of segments of nucleic acid joined together by means of molecular biological techniques provided that the entire nucleic acid sequence does not occurring in nature, i.e., there is at least one mutation in the overall sequence such that the entire sequence is not naturally occurring even though separately segments may occur in nature. The segments may be joined in an altered arrangement such that the entire nucleic acid sequence from start to finish does not naturally occur. The term "recombinant" when made in reference to a protein or a peptide refers to a protein molecule that is expressed using a recombinant nucleic acid molecule.

The terms "vector" or " expression vector " refer to a recombinant nucleic acid containing a desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence in a particular host organism or expression system, e.g., cellular or cell-free expression system. Nucleic acid sequences necessary for expression in prokaryotes usually include a promoter, an operator (optional), and a ribosome binding site, often along with other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination and polyadenylation signals. In certain embodiments, this disclosure contemplates a vector encoding a peptide disclosed herein in operable combination with a heterologous promoter.

Protein "expression systems" refer to in vivo and in vitro (cell free) systems. Systems for recombinant protein expression typically utilize somatic cells transfected with a DNA expression vector that contains the template. The cells are cultured under conditions such that they translate the desired protein. Expressed proteins are extracted for subsequent purification. In vivo protein expression systems using prokaryotic and eukaryotic cells are well known. Proteins may be recovered using denaturants and protein-refolding procedures. In vitro (cell-free) protein expression systems typically use translation-compatible extracts of whole cells or compositions that contain components sufficient for transcription, translation, and optionally post-translational modifications such as RNA polymerase, regulatory protein factors, transcription factors, ribosomes, tRNA cofactors, amino acids, and nucleotides. In the presence of an expression vector, these extracts and components can synthesize proteins of interest. Cell-free systems typically do not contain proteases and enable labeling of the protein with modified amino acids. See, e.g., Shimizu et al., Cell-free translation reconstituted with purified components, 2001, Nat.

Biotechnol., 19, 751–755 and Asahara & Chong, Nucleic Acids Research, 2010, 38(13): e141, both hereby incorporated by reference in their entirety.

A "variant" refers to a chemically similar peptide sequence because of amino acid changes. In certain embodiments, a variant contains one or two, or more amino acid substitutions, deletions, or insertions. In certain embodiments, the substitutions are conserved substitutions. In certain embodiments, a variant contains one, two, or ten or more, or ten or less amino acid additions. In certain embodiments, the additions may be to the N-terminus or the C-terminus. The variant may be substituted with one or more chemical substituents.

A conservative amino acid substitution refers to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine. A variant may have "non-conservative" changes (e.g., replacement of a glycine with a tryptophan). Similar minor variations may also include amino acid deletions or insertions (in other words, additions), or both. Guidance in determining which and how many amino acid residues may be substituted, inserted, or deleted without abolishing biological activity may be found using computer programs well known in the art. Variants can be tested in functional assays. Certain variants have less than 10%, and preferably less than 5%, and still more preferably less than 2% changes (whether substitutions, deletions, and so on). Variants can be prepared for testing by mutating a vector to produce appropriate codon alternatives for peptide translation.

In certain embodiments, sequence "identity" refers to the number of exactly matching amino acids (expressed as a percentage) in a sequence alignment between two sequences of the alignment calculated using the number of identical positions divided by the greater of the shortest sequence or the number of equivalent positions excluding overhangs wherein internal gaps are counted as an equivalent position. In certain embodiments, any recitation of sequence identity expressed herein may be substituted for sequence similarity. Percent “similarity” is used to

quantify the similarity between two sequences of the alignment. This method is identical to determining the identity except that certain amino acids do not have to be identical to have a match. Amino acids are classified as matches if they are among a group with similar properties according to the following amino acid groups: Aromatic - F Y W; hydrophobic-A V I L; Charged positive: R K H; Charged negative - D E; Polar - S T N Q. The amino acid groups are also considered conserved substitutions.

As used herein, the term "conjugated" refers to linking molecular entities through covalent bonds, or by other specific binding interactions, such as due to hydrogen bonding or other van der Walls forces. The force to break a covalent bond is high, e.g., about 1500 pN for a carbon-to-carbon bond. The force to break a combination of strong protein interactions is typically a magnitude less, e.g., biotin to streptavidin is about 150 pN. Thus, a skilled artisan would understand that conjugation must be strong enough to bind molecular entities in order to implement the intended results.

A "linking group" refers to any variety of molecular arrangements that can be used to bridge to molecular moieties together. An example formula may be $-R_n-$ wherein R is selected individually and independently at each occurrence as: $-CR_nR_n-$, $-CHR_n-$, -CH-, -C-, $-CH_2-$, $-C(OH)R_n$, -C(OH)(OH)-, -C(OH)H, $-C(Hal)R_n-$, -C(Hal)(Hal)-, -C(Hal)H-, $-C(N_3)R_n-$, $-C(CN)R_n-$, -C(CN)(CN)-, -C(CN)H-, $-C(N_3)(N_3)-$, $-C(N_3)H-$, -O-, -S-, -N-, -NH-, $-NR_n-$, -(C=O)-, -(C=NH)-, -(C=S)-, $-(C=CH_2)-$, which may contain single, double, or triple bonds individually and independently between the R groups. If an R is branched with an R_n it may be terminated with a group such as $-CH_3$, -H, $-CH=CH_2$, -CCH, -OH, -SH, $-NH_2$, $-N_3$, -CN, or -Hal, or two branched Rs may form a cyclic structure. It is contemplated that in certain instances, each "n" may be individually and independently at each occurrence 1, 2, 3, 4, 5, or 6. It is contemplated that in certain instances, the total Rs or "n" may be less than 100 or 50 or 25 or 10. Examples of linking groups include bridging amide groups, alkyl groups, alkoxy groups, alkoxyalkyl groups, and combinations thereof.

The term "recombinant" when made in reference to a nucleic acid molecule refers to a nucleic acid molecule which is comprised of segments of nucleic acid joined together by means of molecular biological techniques. The term "recombinant" when made in reference to a protein or a polypeptide refers to a protein molecule which is expressed using a recombinant nucleic acid molecule.

The terms "vector" or " expression vector " refer to a recombinant nucleic acid containing a desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence in a particular host organism or expression system, e.g., cellular or cell-free. Nucleic acid sequences necessary for expression in prokaryotes usually include a promoter, an operator (optional), and a ribosome binding site, often along with other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination and polyadenylation signals.

Protein “expression systems” refer to in vivo and in vitro (cell free) systems. Systems for recombinant protein expression typically utilize cells, e.g., somatic cells, transfected with a DNA expression vector that contains the template. The cells are cultured under conditions such that they translate the desired protein. Expressed proteins are extracted for subsequent purification. In vivo protein expression systems using prokaryotic and eukaryotic cells are well known. Also, some proteins are recovered using denaturants and protein-refolding procedures. In vitro (cell-free) protein expression systems typically use translation-compatible extracts of whole cells or compositions that contain components sufficient for transcription, translation, and optionally post-translational modifications such as RNA polymerase, regulatory protein factors, transcription factors, ribosomes, tRNA cofactors, amino acids, and nucleotides. In the presence of an expression vectors, these extracts and components can synthesize proteins of interest. Cell-free systems typically do not contain proteases and enable labeling of the protein with modified amino acids. Some cell free systems incorporated encoded components for translation into the expression vector. See, e.g., Shimizu et al., Cell-free translation reconstituted with purified components, 2001, Nat Biotechnol, 19, 751–755 and Asahara & Chong, Nucleic Acids Research, 2010, 38(13): e141, both hereby incorporated by reference in their entirety.

A “selectable marker” is a nucleic acid introduced into a recombinant vector that encodes a polypeptide that confers a trait suitable for artificial selection or identification (report gene), e.g., beta-lactamase confers antibiotic resistance, which allows an organism expressing beta-lactamase to survive in the presence antibiotic in a growth medium. Another example is thymidine kinase, which makes the host sensitive to ganciclovir selection. It may be a screenable marker that allows one to distinguish between wanted and unwanted cells based on the presence or absence of an expected color. For example, the lac-z-gene produces a beta-galactosidase enzyme which confers a blue color in the presence of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). If

recombinant insertion inactivates the lac-z-gene, then the resulting colonies are colorless. There may be one or more selectable markers, e.g., an enzyme that can complement to the inability of an expression organism to synthesize a particular compound required for its growth (auxotrophic) and one able to convert a compound to another that is toxic for growth. URA3, an orotidine-5' phosphate decarboxylase, is necessary for uracil biosynthesis and can complement ura3 mutants that are auxotrophic for uracil. URA3 also converts 5-fluoroorotic acid into the toxic compound 5-fluorouracil. Additional contemplated selectable markers include any genes that impart antibacterial resistance or express a fluorescent protein. Examples include, but are not limited to, the following genes: ampr, camr, tetr, blasticidinr, neor, hygr, abxr, neomycin phosphotransferase type II gene (nptII), p-glucuronidase (gus), green fluorescent protein (gfp), egfp, yfp, mCherry, p-galactosidase (lacZ), lacZa, lacZAM15, chloramphenicol acetyltransferase (cat), alkaline phosphatase (phoA), bacterial luciferase (luxAB), bialaphos resistance gene (bar), phosphomannose isomerase (pmi), xylose isomerase (xylA), arabitol dehydrogenase (atlD), UDP-glucose:galactose-1-phosphate uridyltransferase (galT), feedback-insensitive α subunit of anthranilate synthase (OASA1D), 2-deoxyglucose (2-DOGR), benzyladenine-N-3-glucuronide, E. coli threonine deaminase, glutamate 1-semialdehyde aminotransferase (GSA-AT), D-amino acidoxidase (DAAO), salt-tolerance gene (rstB), ferredoxin-like protein (pflp), trehalose-6-P synthase gene (AtTPS1), lysine racemase (lyr), dihydrodipicolinate synthase (dapA), tryptophan synthase beta 1 (AtTSB1), dehalogenase (dhlA), mannose-6-phosphate reductase gene (M6PR), hygromycin phosphotransferase (HPT), and D-serine ammonialyase (dsdA).

Ancestral variant recombinant L-asparaginases

In certain embodiments, this disclosure relates to recombinant ancestral variant asparaginases comprising an amino acid sequence selected from SEQ ID NO: 1-53 or variant thereof having 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity or similarity. In certain embodiments, the recombinant ancestral variant asparaginase variant has 1 amino acid substitution. In certain embodiments, the recombinant ancestral variant asparaginase has 2 or 3 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 4 or 5 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 6 or 7 amino acid substitutions.

In certain embodiments, the recombinant ancestral variant asparaginase is a truncated version of SEQ ID NO: 1-53, e.g., comprising an amino acid sequence selected from amino acids 1 to 359 of SEQ ID NO: 1-53 or variant thereof having 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity to the truncated version, wherein the N-terminal methionine (M) is position 1. In certain embodiments, the recombinant ancestral variant asparaginase variant has 1 amino acid substitution. In certain embodiments, the recombinant ancestral variant asparaginase has 2 or 3 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 4 or 5 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 6 or 7 amino acid substitutions.

In certain embodiments, the recombinant ancestral variant asparaginase is a truncated version of SEQ ID NO: 1-53, e.g., comprising an amino acid sequence selected from amino acids 1 to 396 of SEQ ID NO: 1-53 or variant thereof having 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity to the truncated version. In certain embodiments, the recombinant ancestral variant asparaginase variant has 1 amino acid substitution. In certain embodiments, the recombinant ancestral variant asparaginase has 2 or 3 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 4 or 5 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 6 or 7 amino acid substitutions.

In certain embodiments, the recombinant ancestral variant asparaginase is a truncated versions of SEQ ID NO: 1-53, e.g., wherein the N-terminal methionine (M) is position 1, comprising an amino acid sequence selected from amino acids 10 to 573 of SEQ ID NO: 1-53, amino acids 1 to 563 of SEQ ID NO: 1-53, amino acids 20 to 573 of SEQ ID NO: 1-53, amino acids 1 to 553 of SEQ ID NO: 1-53, amino acids 30 to 573 of SEQ ID NO: 1-53, amino acids 1 to 543 of SEQ ID NO: 1-53, amino acids 40 to 573 of SEQ ID NO: 1-53, amino acids 1 to 533 of SEQ ID NO: 1-53, amino acids 50 to 573 of SEQ ID NO: 1-53, amino acids 1 to 523 of SEQ ID NO: 1-53, amino acids 60 to 573 of SEQ ID NO: 1-53, amino acids 1 to 513 of SEQ ID NO: 1-53, amino acids 70 to 573 of SEQ ID NO: 1-53, amino acids 1 to 503 of SEQ ID NO: 1-53, amino acids 20 to 563 of SEQ ID NO: 1-53, amino acids 10 to 553 of SEQ ID NO: 1-53, amino acids 30 to 563 of SEQ ID NO: 1-53, amino acids 10 to 543 of SEQ ID NO: 1-53, amino acids 40 to 563 of SEQ ID NO: 1-53, amino acids 10 to 533 of SEQ ID NO: 1-53, amino acids 50 to 563 of SEQ

ID NO: 1-53, amino acids 10 to 523 of SEQ ID NO: 1-53, amino acids 60 to 563 of SEQ ID NO: 1-53, amino acids 10 to 513 of SEQ ID NO: 1-53, amino acids 70 to 563 of SEQ ID NO: 1-53, amino acids 10 to 503 of SEQ ID NO: 1-53, amino acids 20 to 553 of SEQ ID NO: 1-53, amino acids 20 to 553 of SEQ ID NO: 1-53, amino acids 30 to 553 of SEQ ID NO: 1-53, amino acids 20 to 543 of SEQ ID NO: 1-53, amino acids 40 to 553 of SEQ ID NO: 1-53, amino acids 20 to 533 of SEQ ID NO: 1-53, amino acids 50 to 553 of SEQ ID NO: 1-53, amino acids 20 to 523 of SEQ ID NO: 1-53, amino acids 60 to 553 of SEQ ID NO: 1-53, amino acids 20 to 513 of SEQ ID NO: 1-53, amino acids 70 to 553 of SEQ ID NO: 1-53, amino acids 20 to 503 of SEQ ID NO: 1-53, amino acids 20 to 543 of SEQ ID NO: 1-53, amino acids 30 to 553 of SEQ ID NO: 1-53, amino acids 30 to 543 of SEQ ID NO: 1-53, amino acids 30 to 543 of SEQ ID NO: 1-53, amino acids 40 to 543 of SEQ ID NO: 1-53, amino acids 30 to 533 of SEQ ID NO: 1-53, amino acids 50 to 543 of SEQ ID NO: 1-53, amino acids 30 to 523 of SEQ ID NO: 1-53, amino acids 60 to 543 of SEQ ID NO: 1-53, amino acids 30 to 513 of SEQ ID NO: 1-53, amino acids 70 to 543 of SEQ ID NO: 1-53, amino acids 30 to 503 of SEQ ID NO: 1-53, or variant thereof having 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity to the truncated version. In certain embodiments, the recombinant ancestral variant asparaginase variant has 1 amino acid substitution. In certain embodiments, the recombinant ancestral variant asparaginase has 2 or 3 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 4 or 5 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 6 or 7 amino acid substitutions.

In certain embodiments, the recombinant ancestral variant asparaginase has SEQ ID NO: 10 (An 104) or a variant having 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity. In certain embodiments, the recombinant ancestral variant asparaginase variant has 1 amino acid substitution. In certain embodiments, the recombinant ancestral variant asparaginase has 2 or 3 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 4 or 5 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 6 or 7 amino acid substitutions.

In certain embodiments, the recombinant ancestral variant asparaginase has SEQ ID NO: 2 (An 69) or a variant having 98%, 99%, or greater sequence identity. In certain embodiments, the recombinant ancestral variant asparaginase variant has 1 amino acid substitution. In certain

embodiments, the recombinant ancestral variant asparaginase has 2 or 3 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 4 or 5 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 6 or 7 amino acid substitutions.

In certain embodiments, the recombinant ancestral variant asparaginase has SEQ ID NO: 4 (An 71) or a variant having 99% or greater sequence identity. In certain embodiments, the recombinant ancestral variant asparaginase variant has 1 amino acid substitution. In certain embodiments, the recombinant ancestral variant asparaginase has 2 or 3 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 4 or 5 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 6 or 7 amino acid substitutions.

In certain embodiments, the recombinant ancestral variant asparaginase has SEQ ID NO: 3 (An 70) or a variant having 99%, or greater sequence identity. In certain embodiments, the recombinant ancestral variant asparaginase variant has 1 amino acid substitution. In certain embodiments, the recombinant ancestral variant asparaginase has 2 or 3 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 4 or 5 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 6 or 7 amino acid substitutions.

In certain embodiments, the recombinant ancestral variant asparaginase has SEQ ID NO: 5 (An 72) or a variant having 98%, 99%, or greater sequence identity. In certain embodiments, the recombinant ancestral variant asparaginase variant has 1 amino acid substitution. In certain embodiments, the recombinant ancestral variant asparaginase has 2 or 3 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 4 or 5 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 6 or 7 amino acid substitutions.

In certain embodiments, the recombinant ancestral variant asparaginase has SEQ ID NO: 6 (An 85) or a variant having 97%, 98%, 99%, or greater sequence identity. In certain embodiments, the recombinant ancestral variant asparaginase variant has 1 amino acid substitution. In certain embodiments, the recombinant ancestral variant asparaginase has 2 or 3 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase

has 4 or 5 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 6 or 7 amino acid substitutions.

In certain embodiments, the recombinant ancestral variant asparaginase has SEQ ID NO: 7 (An 88) or a variant having 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity. In certain embodiments, the recombinant ancestral variant asparaginase variant has 1 amino acid substitution. In certain embodiments, the recombinant ancestral variant asparaginase has 2 or 3 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 4 or 5 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 6 or 7 amino acid substitutions.

In certain embodiments, the recombinant ancestral variant asparaginase has SEQ ID NO: 8 (An 93) or a variant having 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity. In certain embodiments, the recombinant ancestral variant asparaginase variant has 1 amino acid substitution. In certain embodiments, the recombinant ancestral variant asparaginase has 2 or 3 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 4 or 5 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 6 or 7 amino acid substitutions.

In certain embodiments, the recombinant ancestral variant asparaginase has SEQ ID NO: 12 (An 107) or a variant having 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity. In certain embodiments, the recombinant ancestral variant asparaginase variant has 1 amino acid substitution. In certain embodiments, the recombinant ancestral variant asparaginase has 2 or 3 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 4 or 5 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 6 or 7 amino acid substitutions.

In certain embodiments, the recombinant ancestral variant asparaginase has SEQ ID NO: 53 (An 108) or a variant having 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity. In certain embodiments, the recombinant ancestral variant asparaginase variant has 1 amino acid substitution. In certain embodiments, the recombinant ancestral variant asparaginase has 2 or 3 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 4 or 5 amino acid substitutions.

In certain embodiments, the recombinant ancestral variant asparaginase has 6 or 7 amino acid substitutions.

Human fusions

In certain embodiments, this disclosure relates to recombinant ancestral variant asparaginases comprising an amino acid sequence selected from SEQ ID NO: 54-63 or variant thereof having 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity or similarity. In certain embodiments, the recombinant ancestral variant asparaginase variant has 1 amino acid substitution. In certain embodiments, the recombinant ancestral variant asparaginase has 2 or 3 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 4 or 5 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 6 or 7 amino acid substitutions.

In certain embodiments, the recombinant ancestral variant asparaginase is a truncated version of SEQ ID NO: 54-63, e.g., comprising an amino acid sequence selected from amino acids 1 to 359 of SEQ ID NO: 54-63 or variant thereof having 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity to the truncated version, wherein the N-terminal methionine (M) is position 1. In certain embodiments, the recombinant ancestral variant asparaginase variant has 1 amino acid substitution. In certain embodiments, the recombinant ancestral variant asparaginase has 2 or 3 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 4 or 5 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 6 or 7 amino acid substitutions.

In certain embodiments, the recombinant ancestral variant asparaginase is a truncated version of SEQ ID NO: 54-63, e.g., comprising an amino acid sequence selected from amino acids 1 to 396 of SEQ ID NO: 54-63 or variant thereof having 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity to the truncated version. In certain embodiments, the recombinant ancestral variant asparaginase variant has 1 amino acid substitution. In certain embodiments, the recombinant ancestral variant asparaginase has 2 or 3 amino acid substitutions. In certain embodiments, the

recombinant ancestral variant asparaginase has 4 or 5 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 6 or 7 amino acid substitutions.

In certain embodiments, the recombinant ancestral variant asparaginase is a truncated versions of SEQ ID NO: 54-63, e.g., wherein the N-terminal methionine (M) is position 1, comprising an amino acid sequence selected from amino acids 10 to 573 of SEQ ID NO: 54-63, amino acids 1 to 563 of SEQ ID NO: 54-63, amino acids 20 to 573 of SEQ ID NO: 54-63, amino acids 1 to 553 of SEQ ID NO: 54-63, amino acids 30 to 573 of SEQ ID NO: 54-63, amino acids 1 to 543 of SEQ ID NO: 54-63, amino acids 40 to 573 of SEQ ID NO: 54-63, amino acids 1 to 533 of SEQ ID NO: 54-63, amino acids 50 to 573 of SEQ ID NO: 54-63, amino acids 1 to 523 of SEQ ID NO: 54-63, amino acids 60 to 573 of SEQ ID NO: 54-63, amino acids 1 to 513 of SEQ ID NO: 54-63, amino acids 70 to 573 of SEQ ID NO: 54-63, amino acids 1 to 503 of SEQ ID NO: 54-63, amino acids 20 to 563 of SEQ ID NO: 54-63, amino acids 10 to 553 of SEQ ID NO: 54-63, amino acids 30 to 563 of SEQ ID NO: 54-63, amino acids 10 to 543 of SEQ ID NO: 54-63, amino acids 40 to 563 of SEQ ID NO: 54-63, amino acids 10 to 533 of SEQ ID NO: 54-63, amino acids 50 to 563 of SEQ ID NO: 54-63, amino acids 10 to 523 of SEQ ID NO: 54-63, amino acids 60 to 563 of SEQ ID NO: 54-63, amino acids 10 to 513 of SEQ ID NO: 54-63, amino acids 70 to 563 of SEQ ID NO: 54-63, amino acids 10 to 503 of SEQ ID NO: 54-63, amino acids 20 to 553 of SEQ ID NO: 54-63, amino acids 20 to 553 of SEQ ID NO: 54-63, amino acids 30 to 553 of SEQ ID NO: 54-63, amino acids 20 to 543 of SEQ ID NO: 54-63, amino acids 40 to 553 of SEQ ID NO: 54-63, amino acids 20 to 533 of SEQ ID NO: 54-63, amino acids 50 to 553 of SEQ ID NO: 54-63, amino acids 20 to 523 of SEQ ID NO: 54-63, amino acids 60 to 553 of SEQ ID NO: 54-63, amino acids 20 to 513 of SEQ ID NO: 54-63, amino acids 70 to 553 of SEQ ID NO: 54-63, amino acids 20 to 503 of SEQ ID NO: 54-63, amino acids 20 to 543 of SEQ ID NO: 54-63, amino acids 30 to 553 of SEQ ID NO: 54-63, amino acids 30 to 543 of SEQ ID NO: 54-63, amino acids 30 to 543 of SEQ ID NO: 54-63, amino acids 40 to 543 of SEQ ID NO: 54-63, amino acids 30 to 533 of SEQ ID NO: 54-63, amino acids 50 to 543 of SEQ ID NO: 54-63, amino acids 30 to 523 of SEQ ID NO: 54-63, amino acids 60 to 543 of SEQ ID NO: 54-63, amino acids 30 to 513 of SEQ ID NO: 54-63, amino acids 70 to 543 of SEQ ID NO: 54-63, amino acids 30 to 503 of SEQ ID NO: 54-63, or variant thereof having 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity to the truncated version. In certain embodiments, the recombinant ancestral variant asparaginase variant has 1

amino acid substitution. In certain embodiments, the recombinant ancestral variant asparaginase has 2 or 3 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 4 or 5 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 6 or 7 amino acid substitutions.

In certain embodiments, the recombinant ancestral variant asparaginase has SEQ ID NO: 54 (An-69 h_c) or a variant having 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity. In certain embodiments, the recombinant ancestral variant asparaginase variant has 1 amino acid substitution. In certain embodiments, the recombinant ancestral variant asparaginase has 2 or 3 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 4 or 5 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 6 or 7 amino acid substitutions.

In certain embodiments, the recombinant ancestral variant asparaginase has SEQ ID NO: 55 (An-70 h_c) or a variant having 98%, 99%, or greater sequence identity. In certain embodiments, the recombinant ancestral variant asparaginase variant has 1 amino acid substitution. In certain embodiments, the recombinant ancestral variant asparaginase has 2 or 3 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 4 or 5 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 6 or 7 amino acid substitutions.

In certain embodiments, the recombinant ancestral variant asparaginase has SEQ ID NO: 56 (An-71 h_c) or a variant having 99% or greater sequence identity. In certain embodiments, the recombinant ancestral variant asparaginase variant has 1 amino acid substitution. In certain embodiments, the recombinant ancestral variant asparaginase has 2 or 3 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 4 or 5 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 6 or 7 amino acid substitutions.

In certain embodiments, the recombinant ancestral variant asparaginase has SEQ ID NO: 57 (An-72 h_c) or a variant having 99%, or greater sequence identity. In certain embodiments, the recombinant ancestral variant asparaginase variant has 1 amino acid substitution. In certain embodiments, the recombinant ancestral variant asparaginase has 2 or 3 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 4 or 5 amino acid

substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 6 or 7 amino acid substitutions.

In certain embodiments, the recombinant ancestral variant asparaginase has SEQ ID NO: 58 (An-85 h_c) or a variant having 98%, 99%, or greater sequence identity. In certain embodiments, the recombinant ancestral variant asparaginase variant has 1 amino acid substitution. In certain embodiments, the recombinant ancestral variant asparaginase has 2 or 3 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 4 or 5 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 6 or 7 amino acid substitutions.

In certain embodiments, the recombinant ancestral variant asparaginase has SEQ ID NO: 59 (An-88 h_c) or a variant having 97%, 98%, 99%, or greater sequence identity. In certain embodiments, the recombinant ancestral variant asparaginase variant has 1 amino acid substitution. In certain embodiments, the recombinant ancestral variant asparaginase has 2 or 3 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 4 or 5 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 6 or 7 amino acid substitutions.

In certain embodiments, the recombinant ancestral variant asparaginase has SEQ ID NO: 60 (An-93 h_c) or a variant having 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity. In certain embodiments, the recombinant ancestral variant asparaginase variant has 1 amino acid substitution. In certain embodiments, the recombinant ancestral variant asparaginase has 2 or 3 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 4 or 5 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 6 or 7 amino acid substitutions.

In certain embodiments, the recombinant ancestral variant asparaginase has SEQ ID NO: 61 (An-104 h_c) or a variant having 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity. In certain embodiments, the recombinant ancestral variant asparaginase variant has 1 amino acid substitution. In certain embodiments, the recombinant ancestral variant asparaginase has 2 or 3 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 4 or 5 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 6 or 7 amino acid substitutions.

In certain embodiments, the recombinant ancestral variant asparaginase has SEQ ID NO: 62 (An-107 h_c) or a variant having 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity. In certain embodiments, the recombinant ancestral variant asparaginase variant has 1 amino acid substitution. In certain embodiments, the recombinant ancestral variant asparaginase has 2 or 3 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 4 or 5 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 6 or 7 amino acid substitutions.

In certain embodiments, the recombinant ancestral variant asparaginase has SEQ ID NO: 63 (An-108 h_c) or a variant having 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity. In certain embodiments, the recombinant ancestral variant asparaginase variant has 1 amino acid substitution. In certain embodiments, the recombinant ancestral variant asparaginase has 2 or 3 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 4 or 5 amino acid substitutions. In certain embodiments, the recombinant ancestral variant asparaginase has 6 or 7 amino acid substitutions.

In certain embodiments, the ancestral variant asparaginases as disclosed herein can be produced by any commonly used method. Typical examples include the recombinant expression in suitable host systems, e.g., cell, mammalian cell, bacteria, or yeast. In general, the ancestral variant asparaginases may be produced by living host cells that have been genetically engineered to produce the polypeptide. Methods of genetically engineering cells to produce proteins are well known in the art. See e.g., Ausubel et al., eds. (1990), Current Protocols in Molecular Biology (Wiley, New York). Such methods include introducing nucleic acids that encode and allow expression of the polypeptide into host cells. These host cells can be bacterial cells, fungal cells, or animal cells grown in culture. In one embodiment, ancestral variant asparaginases are produced in mammalian cells. Typical mammalian host cells for expressing the asparaginase include Chinese Hamster Ovary (CHO cells), lymphocytic cell lines, e.g., NS0 myeloma cells, SP2 cells, COS cells.

In addition to the nucleic acid sequences encoding the ancestral variant asparaginase, the recombinant expression vectors may carry additional sequences, such as sequences that regulate replication of the vector in host cells (e.g., origins of replication) and selectable marker genes. The selectable marker gene facilitates selection of host cells into which the vector has been introduced

(see e.g., U.S. Pat. Nos. 4,399,216; 4,634,665; and 5,179,017). For example, typically the selectable marker gene confers resistance to drugs, such as G418, hygromycin, or methotrexate, on a host cell into which the vector has been introduced.

Standard molecular biology techniques can be used to prepare the recombinant expression vector, transfect the host cells, select for transformants, culture the host cells and recover the ancestral variant asparaginases or cells coated with the peptide from the culture medium. For example, the ancestral variant asparaginases or cells can be isolated by affinity chromatography.

In certain embodiments, this disclosure relates to nucleotide sequences or nucleic acids that encode ancestral variant asparaginases as disclosed herein, genetic constructs that include nucleotide sequences or nucleic acids and one or more elements for genetic constructs known per se. In certain embodiments, this disclosure relates to hosts or host cells that contain such nucleotide sequences or nucleic acids, and/or that express (or are capable of expressing) ancestral variant asparaginases disclosed herein.

In certain embodiments, this disclosure relates to methods for preparing ancestral variant asparaginases or cells expressing the asparaginases using constructs disclosed herein, which method comprises cultivating or maintaining a host cell under conditions such that said host cell produces or expresses the ancestral variant asparaginases as disclosed herein.

In certain embodiments, the disclosure relates to recombinant ancestral variant asparaginases comprising sequences disclosed herein or variants or fusions thereof wherein the interior amino acid sequence, the amino terminal end, or the carbon terminal end of the amino acid sequence are optionally attached to a heterologous amino acid sequence, label, or reporter molecule.

In certain embodiments, the disclosure relates to the recombinant vectors comprising a nucleic acid encoding an ancestral variant asparaginase as disclosed herein. In certain embodiments, the recombinant vector optionally comprises a mammalian, human, insect, viral, bacterial, bacterial plasmid, yeast associated origin of replication or gene such as a gene or retroviral gene or lentiviral LTR, TAR, RRE, PE, SLIP, CRS, and INS nucleotide segment or gene selected from tat, rev, nef, vif, vpr, vpu, and vpx or structural genes selected from gag, pol, and env. In certain embodiments, the recombinant vector optionally comprises a gene vector element (nucleic acid) such as a selectable marker region, lac operon, a CMV promoter, a hybrid chicken B-actin/CMV enhancer (CAG) promoter, tac promoter, T7 RNA polymerase promoter, SP6 RNA

polymerase promoter, SV40 promoter, internal ribosome entry site (IRES) sequence, cis-acting woodchuck post regulatory element (WPRE), scaffold-attachment region (SAR), inverted terminal repeats (ITR), c-myc tag coding region, metal affinity tag coding region, streptavidin binding peptide tag coding region, polyHis tag coding region, HA tag coding region, MBP tag coding region, GST tag coding region, polyadenylation coding region, SV40 polyadenylation signal, SV40 origin of replication, Col E1 origin of replication, f1 origin, pBR322 origin, or pUC origin, TEV protease recognition site, loxP site, Cre recombinase coding region, or a multiple cloning site such as having 5, 6, or 7 or more restriction sites within a continuous segment of less than 50 or 60 nucleotides or having 3 or 4 or more restriction sites with a continuous segment of less than 20 or 30 nucleotides.

In certain embodiments, the recombinant ancestral variant asparaginase is conjugated, e.g., through a linking group, to a biodegradable polymer such as polyethylene glycol, mono-methoxy-polyethylene glycol, a fatty acid, or combinations thereof. In certain embodiments, this disclosure relates to a conjugate of an ancestral variant asparaginase as disclosed herein having substantial L-asparagine amidohydrolase activity and polyethylene glycol, wherein the polyethylene glycol has a molecular weight less than or equal to or about 100, 500, 1000, 2000, 3000, 4000, or 5000 Da. In certain embodiments, the ancestral variant asparaginase conjugate has a sequence as reported herein and has an in vitro activity of at least 60%, 65%, 70%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% as compared to the ancestral variant asparaginase when not conjugated to a biodegradable polymer, e.g., polyethylene glycol.

In certain embodiments, the recombinant asparaginases disclosed herein elicit a lower immunogenic response in a patient compared to other clinically approved L-asparaginases, e.g., E. coli L-asparaginase or Erwinia chrysanthemi L-asparaginase. In certain embodiments, significantly reduced immunogenicity, is evidenced, e.g., by the reduction or elimination of an antibody response against the L-asparaginase preparation following administration or repeated administrations; and/or usefulness as a second-line therapy for patients who have developed sensitivity to first-line therapies using, e.g., E. coli L-asparaginase or Erwinia chrysanthemi L-asparaginase or other non-E. coli-derived L-asparaginases. In certain embodiments, the recombinant asparaginases disclosed herein provide reduced immunogenicity and enhanced plasma half-life. In certain embodiments, the recombinant asparaginases disclosed herein are used

in methods of treating patients with relapsed ALL who were previously treated with other asparaginase preparations, in particular those who were previously treated with E. coli-derived asparaginases.

In certain embodiments, the conjugate has a longer in vivo circulating half-life compared to the ancestral variant asparaginase when not conjugated to a biodegradable polymer, e.g., polyethylene glycol. In certain embodiments, the biodegradable polymer, e.g., polyethylene glycol, is covalently linked to one or more amino groups (wherein "amino groups" includes lysine residues and/or the N-terminus) of the ancestral variant asparaginase. In certain embodiments, the biodegradable polymer, e.g., polyethylene glycol, is covalently linked to the one or more amino groups by an amide bond. In certain embodiments, the biodegradable polymer, e.g., polyethylene glycol, is covalently linked to at least from about 40% to about 100% of the accessible amino groups (e.g., lysine residues and/or the N-terminus of the protein) or at least from about 40% to about 90% of total amino groups (e.g., lysine residues and/or the N-terminus of the protein).

In certain embodiments, the ancestral variant asparaginase or conjugate is administered at a dose of 4000 IU/m^2 intravenously. In certain embodiments, the ancestral variant asparaginase or conjugate is administered at a dose of 3000 IU/m^2 intravenously. In certain embodiments, the ancestral variant asparaginase or conjugate is administered at a dose of 2500 IU/m^2 intravenously. In certain embodiments, the ancestral variant asparaginase or conjugate is administered at a dose of 2000 IU/m^2 intravenously. In certain embodiments, the ancestral variant asparaginase or conjugate is administered at a dose of 1500 IU/m^2 intravenously. In certain embodiments, the ancestral variant asparaginase or conjugate is administered at a dose of 1000 IU/m^2 intravenously.

In certain embodiments, the ancestral variant asparaginase or conjugate depletes blood or plasma asparagine levels to between or less than 0.05–0.4 IU/mL, or less than 0.02 IU/mL, or an undetectable level for at least about 12, 24, 48, 96, 108, or 120 hours.

In certain embodiments, this disclosure relates to recombinant ancestral variant asparaginases or conjugate that provide a reduced risk of anaphylaxis, urticaria, itching, bronchospasm, hepatotoxicity, pancreatitis, coagulopathy, hyperammonemia, neurotoxicity, hemorrhage, thrombosis, and or high titers of serum immunoglobulin G (IgG) antibodies, e.g., when compared to pegaspargase (SS-PEG asparaginase), calaspargase pegol (SC-PEG asparaginase, E. coli L-asparaginase), asparaginase Erwinia chrysanthemi, or crisantaspase (recombinant asparaginase Erwinia chrysanthemi).

In certain embodiments, this disclosure relates to recombinant ancestral variant asparaginases or conjugate that provide a reduced glutaminase activity, e.g., 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90%, e.g., when compared to pegaspargase (SS-PEG asparaginase), calaspargase pegol (SC-PEG asparaginase, E. coli L-asparaginase), asparaginase Erwinia chrysanthemi, or crisantaspase (recombinant asparaginase Erwinia chrysanthemi).

Ancestral sequence reconstructed L-asparaginases for used in the treatment of hematologic and solid malignancies

L-asparaginase (L-ASNase) is a component of the chemotherapy armamentarium used to treat acute lymphoblastic leukemia (ALL). L-ASNase plays a significant role in improving ALL survival outcomes. Preclinical data indicated that L-ASNase is beneficial in several solid malignancies such as pancreatic cancer, colorectal cancer, and metastatic breast cancer. However, because existing clinical L-ASNases are bacterial in origin, e.g., derived from either *Escherichia coli* or *Erwinia chrysanthemi*. Thus, they are sometimes highly immunogenic, with reactions ranging from silent inactivation to severe anaphylaxis, mediated by the development of anti-L-ASNase IgG and IgE antibodies. Additionally, significant liver and pancreatic toxicity is seen in adults treated with L-ASNase, thus limiting its widespread use beyond ALL, especially in adult solid malignancies. Emerging data indicates that this toxicity is related to the concurrent glutaminase activity seen in bacterial L-ASNases. Thus, experiments were performed to identify less immunogenic L-ASNase with reduced glutaminase activity.

Enzymatically, L-ASNase catalyzes the production of free L-aspartic acid from the amino acid L-asparagine, thereby depleting the circulating pool of L-asparagine. ALL and other tumors that lack asparagine synthase activity are dependent on the extrinsic supply of asparagine for protein synthesis and are therefore extremely sensitive to L-ASNase. The anti-tumor properties of L-ASNase were reported to be first discovered when mice treated with guinea pig serum demonstrated regression in subcutaneous lymphomas. This response was later confirmed to be because of guinea pig L-ASNase. Difficulty in manufacturing guinea pig L-ASNase for clinical use led to researchers utilizing bacterial sources from L-ASNase production. Patients with allergic reactions to *E. coli* derived L-ASNase now receive crisantaspase, the L-ASNase native to *E. chrysanthemi*. A recombinant form of crisantaspase called Rylaze™ received FDA approval in 2021. However, there remains no L-ASNase substitute for patients with reactions to crisantaspase.

Ancestral sequence reconstruction (ASR) is a platform to identify L-ASNase variants. ASR utilizes extant sequences to predict ancient DNA and protein sequences thereby providing higher-resolution mapping through comparisons of sequential phylogeny branches. Deeper characterization has now confirmed the significantly favorable anti-leukemic and enzymatic properties of guinea pig L-ASNase compared to human L-ASNase. Furthermore, guinea pig L-ASNase shares approximately 70% sequence identity with human L-ASNase compared to the approximately 30% identity shared by bacterial L-ASNases. Additionally, guinea pig L-ASNase has reduced glutaminase activity which may result in lower toxicity in adults. It is contemplated that ancestral sequence reconstruction is a viable platform to bioengineer variant recombinant L-asparaginases with reduced toxicities and improved enzymatic specificity. Experiments were performed to identify and characterize ancient DNA sequences along the human and guinea pig lineage to allow for the mapping of functional residues, with the goal of developing an enhanced, less immunogenic, therapeutic L-ASNase candidate.

To utilize ASR to engineer and characterize variant L-ASNase therapeutic candidates. A phylogenic tree was constructed utilizing extant L-ASNase sequences and ancestral sequences along the human and guinea pig lineage were identify. cDNA sequences for ancestral L-ASNase (An-ASNase) variants are ligated into a His6-SUMO-pET14b vector and transformed into E. coli BL21(DE3) cells for protein expression. Following purification, kinetic properties of An-ASNase variants are determined and compared to bacterial, guinea pig and human L-ASNase controls. Crystal structures of lead candidates are evaluated. Combining the functional and structural data gained, variant bioengineered human L-ASNase variants are designed and tested. To measure the chemotherapeutic potential of the ancestral L-ASNase candidates in hematologic and solid cancer models, anti-leukemia cytotoxicity studies are performed against multiple ALL cell lines and primary patient samples. Patient-derived xenograft models from ALL patients who successfully tolerated L-ASNase therapy may be used to validate the new drug candidates. A metastatic breast cancer model using the 4T1 cell line may be utilized to study the therapeutic potential of An-ASNase candidates in a solid cancer.

Methods of managing cancer

In certain embodiments, this disclosure relates to methods of treating a disease or condition such as cancer comprising administering a recombinant ancestral variant asparaginase or conjugate

as disclosed herein to a subject in need thereof. In certain embodiments, the recombinant ancestral variant asparaginase or conjugate comprises an amino acid sequence selected from SEQ ID NO: 1-53 or variant thereof having 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity.

In certain embodiments, this disclosure relates to methods of treating a disease or condition such as cancer comprising administering a recombinant ancestral variant asparaginase or conjugate as disclosed herein to a subject in need thereof. In certain embodiments, the recombinant ancestral variant asparaginase or conjugate comprises an amino acid sequence selected from SEQ ID NO: 54-63 or variant thereof having 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity.

In certain embodiments, this disclosure relates to a method for treating acute lymphoblastic leukemia (ALL) comprising administering to a patient in need of a therapeutically effective amount of an ancestral variant asparaginase or conjugate as disclosed herein. In certain embodiments, treatment with an ancestral variant asparaginase or conjugate is administered as a first line therapy or second line therapy in patients, particularly patients with ALL, where objective signs of allergy or hypersensitivity, including "silent hypersensitivity," have developed to other asparaginase preparations, e.g., subject is "antibody positive" for an asparaginase enzyme. In certain embodiments, administration is about twice a week to about once a month, typically once per week or once every other week, as a single agent (e.g., monotherapy) or as part of a combination of chemotherapy drugs, including, but not limited to glucocorticoids, corticosteroids, anticancer compounds or other agents, including, but not limited to methotrexate, dexamethasone, prednisone, prednisolone, vincristine, cyclophosphamide, and anthracycline. In certain embodiments, patients with ALL will be administered the ancestral variant asparaginase or conjugate as disclosed herein as a component of multi-agent chemotherapy during 1, 2, or 3 chemotherapy phases including induction, consolidation or intensification, and maintenance. In certain embodiments, the patient has relapsed ALL. In certain embodiments, the patient was previously treated with other asparaginase preparations, e.g., bacterial or E. coli derived asparaginases.

In certain embodiments, the recombinant asparaginases disclosed herein elicit a lower immunogenic response in a patient compared to other clinically approved L-asparaginases, e.g., E. coli L-asparaginase or Erwinia chrysanthemi L-asparaginase. In certain embodiments,

significantly reduced immunogenicity, is evidenced, e.g., by the reduction or elimination of an antibody response against the L-asparaginase preparation following administration or repeated administrations; and/or usefulness as a second-line therapy for patients who have developed sensitivity to first-line therapies using, e.g., E. coli L-asparaginase or Erwinia chrysanthemi L-asparaginase or other non-E. coli-derived L-asparaginases. In certain embodiments, the recombinant asparaginases disclosed herein provide reduced immunogenicity and enhanced plasma half-life. In certain embodiments, the recombinant asparaginases disclosed herein are used in methods of treating patients with relapsed ALL who were previously treated with other asparaginase preparations, in particular those who were previously treated with E. coli-derived asparaginases.

In certain embodiments, the ancestral variant asparaginase or conjugate is administered at a dose of 4000 IU/m^2 intravenously. In certain embodiments, the ancestral variant asparaginase or conjugate is administered at a dose of 3000 IU/m^2 intravenously. In certain embodiments, the ancestral variant asparaginase or conjugate is administered at a dose of 2500 IU/m^2 intravenously. In certain embodiments, the ancestral variant asparaginase or conjugate is administered at a dose of 2000 IU/m^2 intravenously. In certain embodiments, the ancestral variant asparaginase or conjugate is administered at a dose of 1500 IU/m^2 intravenously. In certain embodiments, the ancestral variant asparaginase or conjugate is administered at a dose of 1000 IU/m^2 intravenously.

In certain embodiments, the ancestral variant asparaginase or conjugate depletes plasma asparagine levels to between 0.05–0.4 IU/mL, or less than 0.05 IU/mL, or less than 0.04 IU/mL, or less than 0.03 IU/mL, or less than 0.02 IU/mL, or an undetectable level for at least about 12, 24, 48, 96, 108, or 120 hours.

"Cancer" refers to any of various cellular diseases with malignant neoplasms characterized by the proliferation of cells. It is not intended that the diseased cells must actually invade surrounding tissue and metastasize to new body sites. Cancer can involve any tissue of the body and has many different forms in each body area. Within the context of certain embodiments, whether "cancer is reduced" may be identified by a variety of diagnostic manners known to one skill in the art including, but not limited to, observation the reduction in size or number of tumor masses or if an increase of apoptosis of cancer cells observed, e.g., if more than a 5 % increase in apoptosis of cancer cells is observed for a sample compound compared to a control without the

compound. It may also be identified by a change in relevant biomarker or gene expression profile, such as PSA for prostate cancer, HER2 for breast cancer, or others.

In certain embodiments, the cancer is a hematological cancer or a solid cancer. In certain embodiments, the cancer is leukemia, lymphoma, acute lymphoblastic leukemia (ALL) lymphoblastic lymphoma (LBL), acute myelogenous leukemia (AML), chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma (SLL), chronic myelogenous leukemia, acute monocytic leukemia (AMOL), chronic myeloid leukemia (CML), B-cell acute lymphoblastic leukemia (B-ALL), myeloproliferative neoplasms (MPNs), and lymphomas, Hodgkin's lymphomas, and non-Hodgkin's lymphomas such as Burkitt lymphoma, B-cell lymphoma, or diffuse large B-cell lymphoma (DLBCL).

In certain embodiments, treatment is done in combination with chemotherapy in combination with radiation therapy or a stem cell transplant, e.g., hematopoietic stem cell transplant.

In certain embodiments, the recombinant ancestral variant asparaginase or conjugate is administered in combination with another anticancer agent. A "chemotherapy agent," "chemotherapeutic," "anti-cancer agent," or the like, refer to molecules that are recognized to aid in the treatment of a cancer. Contemplated examples include the following molecules or derivatives such as abemaciclib, abiraterone acetate, methotrexate, paclitaxel, adriamycin, acalabrutinib, brentuximab vedotin, ado-trastuzumab emtansine, aflibercept, afatinib, netupitant, palonosetron, imiquimod, aldesleukin, alectinib, alemtuzumab, pemetrexed disodium, copanlisib, melphalan, brigatinib, chlorambucil, amifostine, aminolevulinic acid, anastrozole, apalutamide, aprepitant, pamidronate disodium, exemestane, nelarabine, arsenic trioxide, ofatumumab, atezolizumab, bevacizumab, avelumab, axicabtagene ciloleucel, axitinib, azacitidine, carmustine, belinostat, bendamustine, inotuzumab ozogamicin, bevacizumab, bexarotene, bicalutamide, bleomycin, blinatumomab, bortezomib, bosutinib, brentuximab vedotin, brigatinib, busulfan, irinotecan, capecitabine, fluorouracil, carboplatin, carfilzomib, ceritinib, daunorubicin, cetuximab, cisplatin, cladribine, cyclophosphamide, clofarabine, cobimetinib, cabozantinib-S-malate, dactinomycin, crizotinib, ifosfamide, ramucirumab, cytarabine, dabrafenib, dacarbazine, decitabine, daratumumab, dasatinib, defibrotide, degarelix, denileukin diftitox, denosumab, dexamethasone, dexrazoxane, dinutuximab, docetaxel, doxorubicin, durvalumab, rasburicase, epirubicin, elotuzumab, oxaliplatin, eltrombopag olamine, enasidenib, enzalutamide, eribulin,

vismodegib, erlotinib, etoposide, everolimus, raloxifene, toremifene, panobinostat, fulvestrant, letrozole, filgrastim, fludarabine, flutamide, pralatrexate, obinutuzumab, gefitinib, gemcitabine, gemtuzumab ozogamicin, glucarpidase, goserelin, propranolol, trastuzumab, topotecan, palbociclib, ibritumomab tiuxetan, ibrutinib, ponatinib, idarubicin, idelalisib, imatinib, talimogene laherparepvec, ipilimumab, romidepsin, ixabepilone, ixazomib, ruxolitinib, cabazitaxel, palifermin, pembrolizumab, ribociclib, tisagenlecleucel, lanreotide, lapatinib, olaratumab, lenalidomide, lenvatinib, leucovorin, leuprolide, lomustine, trifluridine, olaparib, vincristine, procarbazine, mechlorethamine, megestrol, trametinib, temozolomide, methylnaltrexone bromide, midostaurin, mitomycin C, mitoxantrone, plerixafor, vinorelbine, necitumumab, neratinib, sorafenib, nilutamide, nilotinib, niraparib, nivolumab, tamoxifen, romiplostim, sonidegib, omacetaxine, pegaspargase, ondansetron, osimertinib, panitumumab, pazopanib, interferon alfa-2b, pertuzumab, pomalidomide, mercaptopurine, regorafenib, rituximab, rolapitant, rucaparib, siltuximab, sunitinib, thioguanine, temsirolimus, thalidomide, thiotepa, trabectedin, valrubicin, vandetanib, vinblastine, vemurafenib, vorinostat, zoledronic acid, or combinations thereof such as cyclophosphamide, methotrexate, 5-fluorouracil (CMF); doxorubicin, cyclophosphamide (AC); mustine, vincristine, procarbazine, prednisolone (MOPP); adriamycin, bleomycin, vinblastine, dacarbazine (ABVD); cyclophosphamide, doxorubicin, vincristine, prednisolone (CHOP); bleomycin, etoposide, cisplatin (BEP); epirubicin, cisplatin, 5-fluorouracil (ECF); epirubicin, cisplatin, capecitabine (ECX); methotrexate, vincristine, doxorubicin, cisplatin (MVAC).

In certain embodiments, the anticancer agent is cytarabine (ara-C) and an anthracycline drug. In certain embodiments, the anthracycline drug is daunorubicin or idarubicin. In certain embodiments, the anticancer agent is cladribine, fludarabine, or etoposide.

In certain embodiments, the anticancer agent is prednisone, dexamethasone, vincristine, daunorubicin, doxorubicin, cyclophosphamide, methotrexate, cytarabine, 6-mercaptopurine, 6-thioguanine or nelarabine.

In certain embodiments, the subject is diagnosed with leukemia cells having an *FLT3* gene mutation and the ancestral variant asparaginase or conjugate is administered in combination with administering midostaurin.

In certain embodiments, the subject is diagnosed with leukemia cells having a high expression of CD33 and the ancestral variant asparaginase or conjugate is administered in combination with administering gemtuzumab ozogamicin.

In certain embodiments, the chemotherapy agent is an anti-PD-1, anti-PD-L1 anti-CTLA4 antibody or combinations thereof, such as an anti-CTLA4 (e.g., ipilimumab, tremelimumab) and anti-PD1 (e.g., nivolumab, pembrolizumab, cemiplimab) and anti-PD-L1 (e.g., atezolizumab, avelumab, durvalumab).

In certain embodiments, the ancestral variant asparaginase or conjugate is administered before or after receiving bone marrow transplant or blood stem cell transplant and optionally radiation therapy.

In certain embodiments, the method of administration is in a subject with a lymphodepleted environment due to prior or concurrent administration of lymphodepleting agents. In certain embodiments, lymphodepleting agents (e.g., cyclophosphamide and fludarabine).

In certain embodiments, the subject is a human patient.

In certain embodiments, the cancer is selected from bladder cancer, lung cancer, breast cancer, colon cancer, rectal cancer, endometrial cancer, pancreatic cancer, kidney cancer, prostate cancer, thyroid cancer, brain cancer, multiple myeloma, lymphoma, or leukemia.

In certain embodiments, the disclosure also provides for the use of an ancestral variant asparaginase or conjugate disclosed herein for the preparation of a medicament. Such preparations are contemplated for the treatment of a cancer or neoplasm in a human patient or other mammal. In certain embodiment, this disclosure relates to methods for the treatment a subject at risk of, exhibiting symptoms of, suspected of, or diagnosed with a cancer or neoplasm selected from skin cancer, melanoma, Barret's adenocarcinoma; biliary tract carcinomas; breast cancer; cervical cancer; cholangiocarcinoma; central nervous system tumors including primary CNS tumors such as glioblastomas, astrocytomas (including glioblastoma multiforme) and ependymomas, and secondary CNS tumors (i.e., metastases to the central nervous system of tumors originating outside of the central nervous system), colorectal cancer, including large intestinal colon carcinoma; gastric cancer; carcinoma of the head and neck including squamous cell carcinoma of the head and neck; hematologic cancers including leukemias and lymphomas such as acute lymphoblastic leukemia, acute myelogenous leukemia (AML), myelodysplastic syndromes, chronic myelogenous leukemia, Hodgkin's lymphoma, non-Hodgkin's lymphoma, megakaryoblastic leukemia, multiple myeloma and erythroleukemia; hepatocellular carcinoma; lung cancer including small cell lung cancer and non-small cell lung cancer; ovarian cancer; endometrial

cancer; pancreatic cancer; pituitary adenoma; prostate cancer; renal cancer; sarcoma; and thyroid cancers.

In certain embodiments, this disclosure relates to treating diseases associated with asparagine dependence comprising administering an effective amount of an ancestral variant asparaginase or conjugate disclosed herein to a subject in need thereof.

In certain embodiments, the disease is a non-malignant hematologic disease which respond to asparagine depletion include immune system-mediated blood diseases, e.g., infectious diseases such as those caused by HIV infection (i.e., AIDS).

In certain embodiments, non-hematologic diseases associated with asparagine dependence include autoimmune diseases, for example rheumatoid arthritis, SLE, autoimmune, collagen vascular diseases, AIDS, etc.

In certain embodiments, the disease is an autoimmune disease such as osteoarthritis, psoriasis, insulin dependent diabetes mellitus, multiple sclerosis, sclerosing panencephalitis, systemic lupus erythematosus, rheumatic fever, inflammatory bowel disease (e.g., ulcerative colitis and Crohn's disease), chronic active hepatitis, glomerulonephritis, myasthenia gravis, pemphigus vulgaris, and Graves' disease.

In certain embodiments, the ancestral variant asparaginase or conjugate disclosed herein can be used alone in the treatment of each of the foregoing conditions or can be used to provide additive or potentially synergistic effects with certain existing chemotherapies, radiation, biological or immunotherapeutics (including monoclonal antibodies) and vaccines. The ancestral variant recombinant asparaginase or conjugate disclosed herein may be useful for restoring effectiveness of certain existing chemotherapies and radiation and or increasing sensitivity to certain existing therapies, chemotherapies, and/or radiation.

In certain embodiments, the subject is a human subject is 2, 12, or 16 years old or older. In certain embodiments, the subject is a human subject is 2, 12, or 15 years old or less than 2, 12, or 16 years old. In certain embodiments, the subject is a human subject is 55 or 65 years old or older.

In certain embodiments, the subject is a human subject is an infant, e.g., from one month to two years of age. In certain embodiments, the subject is a human subject is a child, e.g., from one two to twelve years of age. In certain embodiments, the subject is a human subject is an adolescent, e.g., from twelve to sixteen years of age. In certain embodiments, the subject is a human subject sixteen years of age or older.

Pharmaceutical compositions

In certain embodiments, this disclosure relates to pharmaceutical compositions comprising ancestral variant asparaginase or conjugate disclosed herein and optionally a pharmaceutically acceptable excipient. In certain embodiments, the pharmaceutical composition comprises a recombinant ancestral variant or conjugated as disclosed herein which is in a vial in lyophilized form. In certain embodiments, the pharmaceutical composition comprises a recombinant ancestral variant or conjugated as disclosed herein which is in an aqueous pH buffered isotonic solution.

In certain embodiments, this disclosure relates to pharmaceutical compositions comprising ancestral variant asparaginase or conjugate disclosed herein contained in a vial as a lyophilized powder to be reconstituted with a solvent. In another embodiment, the pharmaceutical composition is a "ready to use" solution enabling, further to an appropriate handling, an administration through, e.g., intramuscular, intravenous (infusion and/or bolus), intra-cerebro-ventricular (icv), or subcutaneous routes.

Pharmaceutical compositions comprising ancestral variant asparaginase or conjugate disclosed herein can be administered to a patient using standard techniques. Techniques and formulations generally may be found in Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co., Easton, Pa., 1990 (herein incorporated by reference). Suitable dosage forms, in part, depend upon the use or the route of entry, for example, oral, transdermal, transmucosal, or by injection (parenteral). Such dosage forms should allow the therapeutic agent to reach a target cell or otherwise have the desired therapeutic effect. For example, pharmaceutical compositions injected into the blood stream preferably are soluble. In certain embodiments, ancestral variant asparaginase or conjugate disclosed herein can be formulated as pharmaceutically acceptable salts and complexes thereof.

Pharmaceutically acceptable carriers and/or excipients can also be incorporated into a pharmaceutical composition to facilitate administration of the particular ancestral variant asparaginase, or conjugate disclosed herein. Examples of carriers include calcium carbonate, calcium phosphate, various sugars such as lactose, glucose, or sucrose, or types of starch, cellulose derivatives, gelatin, vegetable oils, polyethylene glycols, and physiologically compatible solvents. Examples of physiologically compatible solvents include sterile solutions of water for injection (WFI), saline solution and dextrose.

In certain embodiments, the ancestral variant asparaginase or conjugate disclosed herein may be formulated into conventional oral dosage forms such as capsules, tablets, pills, microparticles, and liquid preparations such as syrups, elixirs, and concentrated drops. Pharmaceutical compositions according to the invention can be administered by different routes, including intravenous, intraperitoneal, subcutaneous, intramuscular, oral, topical (transdermal), or transmucosal administration.

In certain embodiments, injection (parenteral administration) may be used, e.g., intramuscular, intravenous, intraperitoneal, and subcutaneous injection. For injection, pharmaceutical compositions are formulated in liquid solutions, preferably in physiologically compatible buffers or solutions, such as saline solution, Hank's solution, or Ringer's solution. In addition, the ancestral variant asparaginase or conjugate disclosed herein may be formulated in solid form and redissolved or suspended immediately prior to use. For example, lyophilized forms of the conjugate can be produced. In certain embodiments, the conjugate is administered intramuscularly. In certain embodiments, the conjugate is administered intravenously.

Systemic administration can also be accomplished by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are well known in the art, and include, for example, transmucosal administration, bile salts, and fusidic acid derivatives. In addition, detergents may be used to facilitate permeation. Transmucosal administration, for example, may be through nasal sprays, inhalers (for pulmonary delivery), or rectal suppositories. For topical administration, compounds can be formulated into ointments, salves, gels, or creams.

The amounts of the conjugate to be delivered will depend on many factors, for example, the biological half-life of the compound, the age, size, weight, and physical condition of the patient, and the disease or disorder to be treated. The importance of these and other factors to be considered are well known to those of ordinary skill in the art. Generally, the amount of the conjugate to be administered will range from about 10 International Units per square meter of the surface area of the patient's body (IU/m^2) to 50,000 IU/m^2, with a dosage range of about 1,000 IU/m^2 to about 15,000 IU/m^2, and a range of about 6,000 IU/m^2 to about 15,000 IU/m^2, and a range of about 10,000 to about 15,000 IU/m^2 (about 20-30 mg protein/m^2) to treat a malignant hematologic disease, e.g., leukemia. Typically, these dosages are administered via intramuscular or intravenous injection at an interval of about once per week or once every other week during the course of

therapy, or 3 times weekly, to about once per month. Other dosages and/or treatment regimens may be employed as determined by the attending physician.

Ancestral sequence reconstruction (ASR) to engineer and characterize novel L-ASNase therapeutic candidates

ASR is an in-silico method for studying the predicted molecular evolution of a specific protein. The process involves comparing DNA and/or protein sequences of orthologs and inferring evolutionary ancestors of extant species using a bioinformatics pipeline. By custom synthesizing the ancestral cDNA sequences and expressing them in cell culture, recombinant versions of the predicted ancient proteins can be isolated and studied in the laboratory with the goal of identifying amino acid substitutions that contribute to enhanced functionality.

ASR will be performed utilizing publicly available extant mammalian L-ASNase sequences including the human and guinea pig LASNase sequence. Guinea pig L-ASNase demonstrating improved enzyme kinetic properties despite a 69.8% sequence identity and 88.6% homology with human L-ASNase suggest one can build a phylogenic tree common to ancestors of both human and guinea pig. L-ASNase sequences were aligned using MUSCLE and an evolutionary phylogeny tree. Ancestral L-ASNase sequences were identified for analysis which have an identity ranging from about 70%-99% when compared to human L-ASNase.

Table 1 shows a comparison of amino acid identities of ancient L-Asparaginase to human L-Asparaginase.

L-Asparaginase	Identity to Human ASNase	Amino Acid Replacements
Human	100	0
Guinea Pig	69	175
An-62	75	143
An-69	98	7
An-71	98	10
An-70	98	11
An-72	97	14
An-85	96	21

An-88	81	108
An-93	90	56
An-103	86	76
An-104	88	65
An-107	88	68

To express and purify ancestral variant L-asparaginase candidates one can use a bacterial protein expression platform. Complementary DNA (cDNA) sequences were generated using an E. coli codon optimization algorithm and synthesized de novo to contain 5' NdeI and 3' BamHI restriction sites for subcloning into a His6-SUMO-pET14b expression vector. Sequences along the lineage where human and guinea pig converge are the nodes designated for resurrection. Plasmids with correctly inserted genes are transformed into E. coli BL21(DE3) cells for protein expression. Cells are grown at 37 °C in Terrific Broth (TB) until optical density of 0.6–0.8 is reached. Expression of target gene is induced for expression by 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), followed by incubation at 18 °C overnight growth. After lysis by sonification, the An-ASNase candidates are purified on a nickel column and N-terminal His6 tags are cleaved. Sequence comparison of An 104 and a human consensus sequence is shown in figure 1.

An-ASNase 104

MARAAGPERRLLAIYTGGTIGMRSERGVLVPGRGLAAVLRTLPMFHDEEHARACGLPE
DTLVLPPASPDQRVIYTVLECQPLFDSSDMTITEWVQIAQTIERHYEQYHGFVVIHGTDT
MAFAASVLSFVLENLQKTVILTGAQVPIHALWNDGRENLLGALLMAGQYVIPEVCLFFQ
NQLFRGNRTTKVDARRFAAFCSPNLPPLATVGADVTINRELVRKVSGKDRLVVHSSME
RDVGLLRLYPGIPAALVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRAAAERGLVIV
NCTHCLQGAVTSDYAAGMAMAGAGIVSGFDMTSEAALAKLSYVLGQPGLSLDDRKEL
LAKDLRGEMTLPAVDEHRPSLQGSTLGRGVAWLLSLSGSQEADAVRDALMPSLACAA
AHAGDLEALQALVELGSDLSLEDFNGQTPLHAAARGGHAGVVTMLLQRGVDVNARDQ
DGLSPLLLAVRGRHQGVIGLLRAAGACLSPQELEDAGTELCRLASRADSEGLRAWWQA

GADLGQPGYDGRSALHVAEAAGNLEVVALLQSLEGGVGAQAPGPEVLPGV (SEQ ID NO: 10)

An-ASNase 62

MARAGGPERRLLAIYTGGTIGMRSQGGVLVPGRNLAAALRKLPMFHDEEYAREHQLPA
DTLVLPLTSQNQRIIYTVLECQPLFDSSDMTINEWVKIAKDIERNYEQYHGFVVIHGTDT
MAFAASMLSFMLENLQKTVILTGAQVPIHALWNDGRENLLGSLLMAGQYVIPEVCLFF
QNQLFRGNRVTKVDSRRFAAFCSPNLPPLATVGADITINHELILKANMKKQLVVHSNME
RNVGVLRLYPGITAAMVRAFLQPPMKGVVMETFGSGNGPTNPDLLQELKKATERGMVI
VNCTHCLQGSVTSDYAAGMALAGAGIISGSDMTSEAALAKLSYVLGRPGLSLEDKKKL
LTKNLRGEMTLPLPEDYRVSLRDSKFVQVVAKYLSLSCSQELDAVRDALTPSLACAAAR
TGDLEALEALLELGGDLSQGDFDGRTPLHVAARGGHVGVVQRLLRHGAKVNARDRDG
ASPLLMAVKGRHRDVIGLLRAAGAHLSPQELQDAGTELCRLAASGDVDGLIAWRQAGA
DLGQPGYDGQSPLQVAEAAGNQEVVNLLRTLRGGTGAQSSGLP (SEQ ID NO: 1)

An-ASNase 69

MARAVGPERRLLAVYTGGTIGMRSELGVLVPGTGLAAILRTLPMFHDEEHARARGLSE
DTLVLPPASRNQRILYTVLECQPLFDSSDMTIAEWVRVAQTIERHYEQYHGFVVIHGTDT
MAFAASMLSFMLENLQKTVILTGAQVPIHALWSDGRENLLGALLMAGQYVIPEVCLFF
QNQLFRGNRTTKVDARRFAAFCSPNLLPLATVGADITINRELVRKVDGKAGLVVHSSME
QDVGLLRLYPGIPAALVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRVATERGLVIV
NCTHCLQGAVTTDYAAGMAMAGAGVISGFDMTSEAALAKLSYVLGQPGLSLDDRKEL
LTKDLRGEMTPPSVEERRPSLQGNTLGRGVSWLLSLSGSQEADALRNALVPSLACAAAH
AGDLEALQALVELGSDLGLVDFNGQTPLHAAARGGHTEAVTMLLQRGVDVNTRDTDG
FSPLLLAVRGRHPGVIGLLREAGASLSTQELEEAGTELCRLAYRADLEGLQVWWQAGA
DLGQPGYDGHSALHVAEAAGNLAVVAFLQSLEGAVGAQAPCPEVLPGV (SEQ ID NO: 2)

An-ASNase 70

MARAVGPERRLLAVYTGGTIGMRSELGVLVPGTGLAAILRTLPMFHDEEHARARGLSE
DTLVLPPASRNQRILYTVLECQPLFDSSDMTIAEWVRVAQTIERHYEQYHGFVVIHGTDT

MAFAASMLSFMLENLQKTVILTGAQVPIHALWSDGRENLLGALLMAGQYVIPEVCLFF
QNQLFRGNRTTKVDARRFAAFCSPNLPPLATVGADITINRELVRKVDGKAGLVVHSSME
QDVGLLRLYPGIPAALVRAFLQPPLKGMVMETFGSGNGPTKPDLLQELRVATERGLVIV
NCTHCLQGAVTTDYAAGMAMAGAGVISGFDMTSEAALAKLSYVLGQPGLSLDDRKEL
LTKDLRGEMTPPSVEERRPSLQGNTLGRGVSWLLSLSGSQEADALRNALMPSLACAAA
HAGDLEALQALVELGSDLGLVDFNGQTPLHAAARGGHAEAVTMLLQRGVDVNTRDTD
GFSPLLLAVRGRHPGVIGLLREAGASLSTQELEEAGTELCRLAYRADLEGLQVWWQAG
ADLGQPGYDGHSALHVAEAAGNLAVVAFLQSLEGAVGAQAPCPEVLPGV (SEQ ID NO: 3)

An-ASNase 71

MARAVGPERRLLAVYTGGTIGMRSELGVLVPGTGLAAILRTLPMFHDEEHARARGLSE
DTLVLPPASRNQRILYTVLECQPLFDSSDMTIAEWVRVAQTIERHYEQYHGFVVIHGTDT
MAFAASMLSFMLENLQKTVILTGAQVPIHALWSDGRENLLGALLMAGQYVIPEVCLFF
QNQLFRGNRTTKVDARRFAAFCSPNLLPLATVGADITINRELVRKVDGKAGLVVHSSME
QDVGLLRLYPGIPAALVRAFLQPPLKGMVMETFGSGNGPTKPDLLQELRVATERGLVIV
NCTHCLQGAVTTDYAAGMAMAGAGVISGFDMTSEAALAKLSYVLGQPGLSLDDRKEL
LTKDLRGEMTPPSVEERRPSLQGNTLGRGVSWLLSLSGSQEADALRNALMPSLACAAA
HAGDLEALQALVELGSDLGLVDFNGQTPLHAAARGGHAEAVTMLLQRGVDVNTRDTD
GFSPLLLAVRGRHPGVIGLLREAGASLSTQELEEAGTELCRLAYRADLEGLQVWWQAG
ADLGQPGYDGHSALHVAEAAGNLAVVAFLQSLEGAVGAQAPCPEVLPGV (SEQ ID NO: 4)

An-ASNase 72

MARAVGPERRLLAVYTGGTIGMRSELGVLVPGTGLAAILRTLPMFHDEEHARARGLSE
DTLVLPPASPNQRILYTVLECQPLFDSSDMTIAEWVRVAQTIERHYEQYHGFVVIHGTDT
MAFAASMLSFMLENLQKTVILTGAQVPIHALWSDGRENLLGALLMAGQYVIPEVCLFF
QNQLFRGNRTTKVDARRFAAFCSPNLPPLATVGADVTINRELVRKVGGKAGLVVHSSM
EQDVGLLRLYPGIPAALVRAFLQPPLKGMVMETFGSGNGPTKPDLLQELRVATERGLVI
VNCTHCLQGAVTTDYAAGMAMAGAGVISGFDMTSEAALAKLSYVLGQPGLSLDDRKE
LLTKDLRGEMTPPSVEERRPSLQGNTLGRGVSWLLSLSGSQEADALRNALMPSLACAA

AHAGDLEALQALVELGSDLGLVDFNGQTPLHAAARGGHAEAVTMLLQRGVDVNTRDT DGFSPLLLAVRGRHPGVIGLLREAGASLSTQELEEAGTELCRLAYRADLEGLQVWWQA GADLGQPGYDGHSALHVAEAAGNLAVVAFLQSLEGAVGAQAPCPEVLPGV (SEQ ID NO: 5)

An-ASNase 85

MARAVGPERRLLAVYTGGTIGMRSELGVLVPGTGLAAILRTLPMFHDEEHARARGLPE DTLVLPPASPNQRILYTVLECQPLFDSSDMTIAEWVRVAQTIERHYEQYHGFVVIHGTDT MAFAASVLSFMLENLQKTVILTGAQVPIHALWSDGRENLLGALLMAGQYVIPEVCLFFQ NQLFRGNRTTKVDARRFAAFCSPNLPPLATVGADVTINRELVRKVGGKAGLVVHSSME QDVGLLRLYPGIPAALVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRVATERGLVIV NCTHCLQGAVTTDYAAGMAMAGAGVISGFDMTSEAALAKLSYVLGQPGLSLDDRKEL LAKDLRGEMTPPSVEERRPSLQGNTLGRGVSWLLSLSGSQEADALRNALMPSLACAAA HAGDLEALQALVELGSDLSLVDFNGQTPLHAAARGGHAEAVTMLLQRGVDVNTRDTD GLSPLLLAVRGRHPGVIGLLRAAGASLSTQELEEAGTELCRLASRADLEGLQVWWQAG ADLGQPGYDGHSALHVAEAAGNLAVVAFLQSLEGAVGAQAPGPEVLPGV (SEQ ID NO: 6)

An-ASNase 88

MARAAGPERRLLLIYTGGTLGMRSERGVLVPGPGLAAVLRTLPMFHDEEHARAQGLPD DTLVLPPASPGPRVIYTVLECQPLLDSSDMTITEWVQIAQTIERHYEQYHGFVVIHGTDT MAFAASVLSFVLENLHKPVILTGAQVPIHALWNDSRENLLGALLMAGQYIIPEVCLFIQN QLFRGNRVTKVDTQRFGAFCSPNLPPLATVGADVTIARELVRKASWKDALVVHSSMER DVGLLRLYPGIPASLVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRAAAERGLIIVNC SHCLRGPVTPGYASGLAMAGASIVSGFDMTSEAALAKLSYVLGQPGLSLDHRKELLAK DLRGEMTLPTVDKHQSSLQGSTLGRGVAWLLSLSGGQEVDAVRDAVMPSLALAAAHA GDLEALQALVELGSDLCLEDSNGQTPLHVAARRGHEGVVTMLLHRGVDVNARDQDGL SPLLLAVQGRHQGTIGLLRTAGACLSPQDLEDAGTELCRLASRADTEGLRAWWQAGAD LQQPGYDGRSALCVAEAAGNLEVVALLRSLEGAVGAQASGPEVLPGVA (SEQ ID NO: 7)

An-ASNase 93

MARAAGPERRLLAIYTGGTIGMRSELGVLVPGRGLAAVLRTLPMFHDEEHARACGLPE
DTLVLPPASPDQRVIYTVLECQPLFDSSDMTITEWVQIAQTIERHYEQYHGFVVIHGTDT
MAFAASVLSFVLENLQKTVILTGAQVPIHALWSDGRENLLGALLMAGQYVIPEVCLFFQ
NQLFRGNRTTKVDARRFAAFCSPNLPPLATVGADVTINRELVRKVGGKEGLVVHSSME
RDVGLLRLYPGIPAALVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRAAAERGLVIV
NCTHCLQGAVTSDYAAGMAMAGAGIISGFDMTSEAALAKLSYVLGQPGLSLDDRKELL
AKDLRGEMTPPAVDEHRPSLQGSTLGRGVAWLLSLSGSQEADAVRDALMPSLACAAA
HAGDLEALQALVELGSDLSLEDFNGQTPLHAAARGGHAGAVTMLLQRGVDVNARDQD
GLSPLLLAVRGRHQGVIGLLRAAGACLSPQELEDAGTELCRLASRADSEGLQAWWQAG
ADLGQPGYDGHSALHVAEAAGNLEVVALLQSLEGAVGAQAPGPEVLPGV (SEQ ID NO: 8)

An-ASNase 103

MARAAGPERRLLAIYTGGTIGMRSEGGVLVPGRGLAAVLRRLPMFHDEEYARACQLPE
DTLVLPPASPDQRVIYTVLECQPLFDSSDMTITEWVQIAQTIERHYEQYHGFVVIHGTDT
MAFAASMLSFVLENLQKTVILTGAQVPIHALWNDGRENLLGALLMAGQYVIPEVCLFF
QNQLFRGNRVTKVDSRRFAAFCSPNLPPLATVGADITINRELVRKVRGKERLVVHSSME
RDVGVLRLYPGIPAALVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRAAAERGLVIV
NCTHCLQGAVTSDYAAGMALAGAGIVSGFDMTSEAALAKLSYVLGQPGLSLDDRKELL
AKDLRGEMTLPAVDEHRPSLRGSTLGRGVAQFLSLSGSQELDAVRDALMPSLACAAAH
AGDLEALQALVELGSDLSLEDFNGQTPLHAAARGGHAGVVTMLLQRGVDVNARDRDG
LSPLLLAVRGRHQGVIGLLRAAGACLSPQELEDAGTELCRLASRADSEGLRAWWQAGA
DLGQPGYDGRSALHVAEAAGNLEVVTLLQSLQGGAGAQAPGPEVLPGV (SEQ ID NO: 9)

An-ASNase 106

MARAAGPERRLLAIYTGGTIGMRSERGVLVPGRGLAAVLRTLPMFHDEEHARACGLPE
DTLVLPPASPDQRVIYTVLECQPLFDSSDMTITEWVQIAQTIERHYEQYHGFVVIHGTDT
MAFAASVLSFVLENLQKTVILTGAQVPIHALWNDGRENLLGALLMAGQYVIPEVCLFFQ
NQLFRGNRVTKVDARRFAAFCSPNLPPLATVGADVTINRELVRKVRGKDRLVVHSSME
RDVGLLRLYPGIPAALVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRAAAERGLVIV

NCTHCLQGAVTSDYAAGMAMAGAGIVSGFDMTSEAALAKLSYVLGQPGLSLDDRKEL
LAKDLRGEMTLPAVDEHRPSLQGSTLGRGVAQLLSLSGSQEADAVRDALMPSLACAAA
HAGDLEALQALVELGSDLSLEDFNGQTPLHAAARGGHAGVVTMLLQRGVDVNARDQD
GLSPLLLAVRGRHQGVIGLLRAAGACLSPQELEDAGTELCRLASRADSEGLRAWWQAG
ADLGQPGYDGRSALHVAEAAGNLEVVTLLQSLQGGAGAQAPGPEVLPGV (SEQ ID NO: 11)

An-ASNase 107

MARAAGPERRLLAIYTGGTIGMRSERGVLVPGRGLAAVLRTLPMFHDEEHARACGLPE
DTLVLPPASPDQRVIYTVLECQPLFDSSDMTITEWVQIAQTIERHYEQYHGFVVIHGTDT
MAFAASVLSFVLENLQKPVILTGAQVPIHALWNDGRENLLGALLMAGQYVIPEVCLFFQ
NQLFRGNRTTKVDARRFAAFCSPNLPPLATVGADVTINRELVRKASGKDRLVVHSSME
RDVGLLRLYPGIPAALVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRAAAERGLVIV
NCTHCLQGAVTSDYAAGMAMAGAGIVSGFDMTSEAALAKLSYVLGQPGLSLDDRKEL
LAKDLRGEMTLPAVDEHRPSLQGSTLGRGVAWLLSLSGSQEADAVRDALMPSLACAA
AHAGDLEALQALVELGSDLSLEDFNGQTPLHAAARGGHAGVVTMLLQRGVDVNARDQ
DGLSPLLLAVRGRHQGVIGLLRAAGACLSPQELEDAGTELCRLASRADSEGLRAWWQA
GADLGQPGYDGRSALHVAEAAGNLEVVALLQSLEGGVGAQAPGPEVLPGVA (SEQ ID NO: 12)

An-ASNase 56

MTEEAERAGGLALSYREPAPELGGGCGPGAEARVLVINTGGTIGMVPQPEGLVPEAKKL
AGALKKMPILHDATYAKETQLYNRLGADTLVLPISSQNKRIIYTILELSPLLDSSNMTPSE
WDKIARCLEANYEKYDGFVILHGTDTMAYTSSALSFMCENLGKTVILTGSQVPIYELRN
DGRDNLLGSLLIAGQFVIPEVCLYFHNKLYRGNRVTKVDAGSFSAFSSPNLPPLANAEVD
IIINWETIWRANTTKKFAIHKNMDSNVGLLRLFPGITATAVRAFLQSPMKGIVLETYGSG
NAPNNCPELLEELRKATDRGVVILNCTQCLRGSVSSVYATGKTLMDAGVIPGGDMTPE
AALAKLSYVLSKPGLTLDEKKKMLNKNLRGEMTVVPIGTKITLKDSKFIQEIAKYLFISC
KEELAAVRDALTPSLACAAAKNGDLDALKALQDLGGNLSSGDYDGRTPLHVASCEGNL
EVVRHLLKSGATVYAKDRLGATPLLNAIKFRHHDVIELLRTTGAHLSSQELVNVGTELC

SLAFNGDLDGLMAWHLAGVDLNQPGYDGNTPANVAKAAGHAKVIEFLNKLEAG (SEQ ID NO: 13)

An-ASNase 57

MEEPGGPVRRLLAIYTGGTIGMRSQGGVLVPGQNLVAALRKLPMFHDEEFSRGHNLPA DTLALPLTSQNKRIVYTVLECQPLFDSSDMTIKEWTKIAKDIEANYDTYHGFVVIHGTDT MAFATSMLSFMLGNLQKTVILTGAQVPIHALWNDGRENLLGSLLMAGQFVIPEVCLFFQ NQLFRGNRVTKVDSRKFAAFCSPNLPPLALIGADITINYELMMRPNLKKQLLVHTNMER NVGVLRLYPGITAAMVKGFLQPPMRGMVMETFGTGNGPTARDLLQELKDATERGMIII NCTHCLQGHVTSDYAAGLAIAGTGIISGSDMTSEAALAKLSYVLGRADLSLEGKKKLLR KNLRGEMTPLPEDYRVSLRDSKFVQVVAKYLGLSSSQELDAVRDALTPSLACAAARTG DLEALDAILDMGGDLSQGDFDGRTPLHVAAREGHLGAVQRLLRHGARVGATDRDGAS PLLAAVKGRHLDVIDVLRAAGAELSPQELQGVGTELCRLAASGDIDGLIVWRQAGADW AEVGYTGQSPLQVAEAAGNQEVVNVLRTLGLGSGGQSCGSH (SEQ ID NO: 14)

An-ASNase 58

MARSGDPVRRLLAIYTGGTIGMRSQEGVLVPGSNLLTALRKLPMFNDEEYSREHQLPAD TLALPLTSQNQRIIYTILEYDPLFDSSDMTINEWIRIAKDIERNYEQYFGFVIIHGTDTMAF ASSMLSFILGNLQKTVILTGAQVPIHTLWNDGRENLLGSLLMAGQYVIPEVCLFFQNQLY RGNRVTKVDSRRFAAFCSPNLPPLATVGADITVNHELILKASMNKQLVVHSNMERNVG VLRLYPGITAAMVRGFLQPPMKGVVMETFGTGNGPTNPDLLWELKKATERGMVIVNCT HCLQGSVTSDYAAGMALSGIGIISGSDMTSEAALAKLSYVLGRDDLSLEDKKKLLTKNL RGEMTPLPEDFRVSLRDSKFVQLIANLLYLNCSQDLDAVRDSLTPTLACAAARMGDME ALEAIIELGGDLSESDFDGRTPLHMAARGGHVGAVQCLLRNGAKVNSWDGDRSSPLLM AVKGRHRDVIRLLRDAGAHLSPQELQGAGTELCRLAASGDRDGLIAWQQAGVDLFQV GYDGQTPLQVAEAAGNQELVNLLRAHRMETGTRSDILP (SEQ ID NO: 15)

An-ASNase 59

MSRATGPERRLLAIYTGGTIGMRSEQGVLLPGRGLANVLRTLPMFHDEAYAQACGLPE DTLVLPPTHANQRVIYTVLECQPLFDSSDMTITEWVQIAQTIETHYEQYHGFVILHGTDT MAFAASALAFVLENLQKTVILTGSQVPIHALWSDGRENLLGALLMAGQYVIPEVCLFFQ

NQLFRGTRVTKVDTRRFAAFRSPNLPPLAVVGADVIINRELVRRARGQGRLVVRGGME
RDVGLPRLYPGIPATPVRAFLQPPLKGVVTETFGSGNGPTKPDLLQELRAAAERGLIILNC
THCLQGSVTSHYAAGMAAAGAGIVSGFDMTSEAALAKLSYVLGRPGLSVDGRKELLAR
DLRGEMTLPAVDEGPPPLCGSTLRLGVAQFLSLSQESDAVRDTLTPSLACAAARAGDLQ
AWQVLASLQGTDLSLEDFNGQTPLHAAARGGRAGVVAMLLQKGVNVDARDEDGLSPL
LLAVRGRRPSVIGLLRAAGACLSPQELEEAGTELCRLASRADGEGLQAWWQAGADLGR
PGYDGRSALLVAEAAGNLEVMRQLQSLQGGAGGLALVGLPGRADHASEELPALRLDPG
LGDASKGQA (SEQ ID NO: 16)

An-ASNase 60

MARSGGPVRRLLAIYTGGTIGMRSQGGVLVPGQNLVAALRKLPMFHDEEYSREHQLPA
DTLALPLTSQNQRIIYTVLECQPLFDSSDMTINEWIKIAKDIERNYEQYHGFVVIHGTDTM
AFASSMLSFMLGNLQKTVILTGAQVPIHALWNDGRENLLGSLLMAGQYVIPEVCLFFQN
QLFRGNRVTKVDSRRFAAFCSPNLPPLATVGADITINHELILKANMKKQLVVHSNMERN
VGVLRLYPGITAAMVRGFLQPPMKGVVMETFGTGNGPTNPDLLQELKKATERGMVIVN
CTHCLQGSVTSDYAAGMALAGTGIISGSDMTSEAALAKLSYVLGRADLSLEDKKKLLT
KNLRGEMTPLPEDYRVSLRDSKFVQVVAKYLSLSCSQELDAVRDALTPSLACAAARTG
DLEALEAILELGGDLSQGDFDGRTPLHVAARGGHVGAVQRLLRHGAKVNARDRDGASP
LLMAVKGRHRDVIDLLRAAGAHLSPQELQGAGTELCRLAASGDVDGLIAWRQAGADL
AQVGYDGQSPLQVAEAAGNQEVVNLLRTLRMGTGAQSSGLP (SEQ ID NO: 17)

An-ASNase 61

MARALGPERRLLAIYTGGTIGMRREDGVLVPGRGLAAVLKTMPMFHDAEHAQACGLP
EDTLLLPPASPSQRVIYTVLECQRLFDSSDMTITEWVQIAQTIERHYEQYHGFVVIHGTDT
MAFAASVLSFVLENLEKPVILTGAQVPIHALQSDGRENLLGALLMASQFVIPEVCLFFQN
QLFRGNRTTKVDARRFAAFCSPNLLPLATVGADVTISWELVRRASRQGRLVVHSGMER
DVGLLRLYPGIPAALVRAFLQPPLKGVVMETFGSGNGPSAPDLLQELRAASARGLLIVN
CTHCLQGTVTSDYAAGTAMAGTGIVSGFDMTSEAALAKLSYVLGQPGLSLDQRKELMT
TDLRGEMTLPAIDRCPRSPQGSVLGRGLAWLLGLGNNQEADAVRDALVPSLACAVAHS
GDLEALQVLMELGSDLCLKDFRGQSPLHAAARAGQAGVVATLLQRGVQVDAHDQDG
YSPLLLAVRGRHEGVIRLLRAANARLSPQELEDAGTELCRLAARGDSAGLRAWVQAGA

DLGQPGYDGRSALQVARAAGNWEVVAFLQGPESTVDAQSPDPEVLPGVP (SEQ ID NO: 18)

An-ASNase 63

MSRAVEPERRLLAIYTGGTIGMRIERGVLVPGRGLAAALRTLPMFHDEDHARALGLPED TLVLPPASPDQRVIYTVLECQPLFDSSDMTITEWVQIAQTIERHYGQYHGFVVIHGTDTM AFAASVLSFVLENLQKTVILTGAQVPIHALWNDGRENLLGALLMAGQYVIPEVCLFFQN QLFRGNRVTKVDSRRFAAFCSPNLPPLAVVGADVILNRELVRKVRGKERLVVHSSVERD VGLLRLYPGIPAALVRAFLQPPLRGVVMETFGCGNGPTKPDLLREFRAATERGLLIVNCT HCLQGTVTSGYAAGMAVAGAGIVSGFDMTSEAAMAKLSYVLGQPGLSLDSRKQLLAR DLRGEVTLPSGDEHQPSLTCSTLGRGVAQLLSLSQEADAVREALTPGLACAAAHAGDLD VLQALVELGSDLSQENFNGQTPLHAAARGGHPEVVTMLLQRGVGVSARDEDGLSPLLL AVKGRHQDIIGLLRAAGACLSPQELEDAGTELCRLASRADLEGLQSWWRAGADLACPG YDGRSALLVAEAAGNLEVVTFLQNLQGGAAVQAPGPATLP (SEQ ID NO: 19)

An-ASNase 64

MARAVGSERRLLAVYTGGTIGMRSELGVLVPGTGLAAILRTLPMFHDEEHARACGLSE DILVLPPATPNQRILYTVLECQPLFDSSDMTIAEWVRVAQTIERHYKQYHGFVVIHGTDT MAFAASMLSFMLENLQKTVILTGAQVSIHALWSDGRENLLGALLMAGQYVIPEVCLFF QNQLFRGNRTTKVDARRFAAFCSPNLPPLATVGADVTVNRELVRKVGGKAGLVVHSS MEQDVGLLRLYPGIPAALVRAFLQPPLKGMVMETFGSGNGPTKPDLLQELQMATERGL VIVNCTHCLQGTVTTDYAAGMAMEGAGVISGFDMTSEAALAKLSYVLGQPGLSLDDRK ELLTKDLRGEMTPPSVEECQPSLQGNTLGRGVSWLLSLSGSQEADALRNALMPSLACAA AHAGNLEVLQVLVELGSDLGLVDFNGQTPLHAAARGGHAEAVTMLLQGGVDVNTRDT DGFSPLLLAVRGRHSGVIGLLREAGASLSTQELEEAGTELCRLAYRADLEGLQVWWQA GADLGQPGYDGHSALHVAEAAGNLAVVAFLQSLEGAVGAQVPCPEVLPGV (SEQ ID NO: 20)

An-ASNase 65

MARATGPERRLLAIYTGGTIGMRSEGGVLVPGRGLAAVLKTLHMFHDEEYARAHSLPE DTLVLPPASPDQRIIYTVLECQPLFDSSDMTITEWVQIAQTIERHYAQYQGFVVIHGTDT

MAFAASVLSFMLENLQKPVVLTGAQVPIHALWSDGRENLLGALLMAGQYVIPEVCLFF
QNQLFRGNRTTKVDARRFAAFCSPNLPPLATVGADVTINRELVRKACGKNHLVVHSSM
EPDVGLLRLYPGIPASLVRTFLQPPLKGVVMETFGSGNGPTKPDLLQELRVAAEQGLIIV
NCTHCLQGAVTSDYASGMAMAGAGIVSGFDMTSEAALAKLSYVLGQPGLSLNDRKKL
LAKDLRGEMTLPATDVLLQDGMLGCRVAWLLSMNGSQEADTMKDVLLPGLALAAAH
AGDLDTLQAFVELGRDLNLKDYSGQTPLHVAARRGHAAVVTMLLQRGADVDARNED
GQSPLLLAVRGRHQSVIGLLRAAGARLSPQELEDVGTELCRLASRADSEGLRAWWQAG
ADLGQPDYDGHCALQVAEAAGNADVVALLQSFKDRVCAQPQTPH (SEQ ID NO: 21)

An-ASNase 66

MARATEPERRLLAVYTGGTIGMRSERGVLIPGRGLADVLRTLPMFHDEEHARACDLPED
TLVLPPASPEQRVIYTVLECQPLFDSSDMTITEWVQIAQIIERHYEQYHGFVVIHGTDTMA
FAASVLSFVLENLQKTVVLTGAQVPIHALWNDGRENLLGALLMAGQYVIPEVCLFFQN
QLFRGNRVTKVDARRFAAFCSPNLPPLAIVGPDVTINQELVRKVRGMERLVVHSSMEHD
VGLLRLYPGIPAALVRAFLQHPLKGVVMETFGSGNGPTKPDLLQELRAATERGLIIVNCT
HCLQGAVTSDYAAGMAMAGAGIVSGFDMTSEAALAKLSYVLGQPGLSLEGRKELLAR
DLRGEMTLPAVDERRPSLQRSVLGHGVAQLLRQSQGADAVRDTLMPSLACAGARAGD
LEALQALVELGSDLSLEDCSGQTPLHAAARGGHVEVLAMLLQRGVNVNARDQDGLTPL
LLAVRGRHQGVIELLRAAGACLSPEELEDAGTELCRLASRGDCEGLRAWWQAGADLGR
PGYDGRSALLIAEAAGDLEVVTVLQSLQGGVGAQALGPEVLPAVA (SEQ ID NO: 22)

An-ASNase 67

MARAVGSERRLLAVYTGGTIGMRSELGVLVPGTGLAAILRTLPMFHDEEHARARGLSE
DILVLPPATPNQRILYTVLECQPLFDSSDMTIAEWVRVAQTIERHYKQYHGFVVIHGTDT
MAFAASMLSFMLENLQKTVILTGAQVPIHALWSDGRENLLGALLMAGQYVIPEVCLFF
QNQLFRGNRTTKVDARRFAAFCSPNLPPLATVGADVTVNRELVRKVGGKAGLVVHSS
MEQDVGLLRLYPGIPAALVRAFLQPPLKGMVMETFGSGNGPTKPDLLQELQMATERGL
VIVNCTHCLQGAVTTDYAAGMAMEGAGVISGFDMTSEAALAKLSYVLGQPGLSLDDR
KELLTKDLRGEMTPPSVEECRPSLQGNTLGRGVSWLLSLSGSQEADALRNALMPSLACA
AAHAGNLEVLQALVELGSDLGLVDFNGQTPLHAAARGGHAEAVTMLLQGGVDVNTR
DTDGFSPLLLAVRGRHPGVIGLLREAGASLSTQELEEAGTELCRLAYRADLEGLQVWW

QAGADLGQPGYDGHSALHVAEAAGNLAVVAFLQSLEGAVGAQVPCPEVLPGV (SEQ ID NO: 23)

An-ASNase 68

MARAVGPERRLLAVYTGGTIGMRSELGVLVPGTGLAAILRTLPMFHDEEHARTRGLSED TLVLPPASHNQRILYTVLECQPLFDSSDMTIAEWVRVAQTIERHYEQYHGFVVIHGTDT MAFAASMLSFMLENLQKTVILTGAQVPIHALWSDGRENLLGALLMAGQYVIPEVCLFF QNQLFRGNRTTKVDARRFAAFCSPNLPPLATVGADITINRELVRKVDGKAGLVVHSSME QDVGLLRLYPGIPAALVRAFLQPPLKGMVMETFGSGNGPTKPDLLQELRVATERGLVIV NCTHCLQGAVTTAYAAGMAMAGAGVISGFDMTSEAALAKLSYVLGQPGLSLDDRKEL LTKDLRGEMTPPSVEERRPSLQGNTPGRGVSWLLSLSGSQEADALRNALMPSLACAAA HAGDLEALQALVELGSELGLVDFNGQTPLHAAARGGHAEAVTMLLQRGVDVNTRDTD GFSPLLLAVRGRHPGVIGLLREAGASLSTQELEEAGTELCRLAYRADLEGLQVWWQAG ADLGQPGYDGHSALHVAEAAGNLAVVAFLQSLEGAVGAQAPCPEVLPGV (SEQ ID NO: 24)

An-ASNase 73

MARATGPERRLLAIYTGGTIGMRSEGGVLVPGRGLAAVLRTLHMFHDEEYARAHSLPE DTLVLPPASPDQRIIYTVLECQPLFDSSDMTITEWVQIAQTIERHYTQYQGFVVIHGTDTM AFAASVLSFMLENLQKPVILTGAQVPIHALWSDGRENLLGALLMAGQYVIPEVCLFFQN QLFRGNRTTKVDARRFAAFCSPNLPPLATVGADVTINRELVRKASGKNHLVVHSSMEPD VGLLRLYPGIPASLVRTFLQPPLKGVVMETFGSGNGPTKPDLLQELRVAAEQGLIIVNCT HCLQGAVTSDYASGMAMAGAGIVSGFDMTSEAALAKLSYVLGQPGLSLNDRKKLLAK DLRGEMTLPATDELLQDGMLGCRVAWLLSMNGSQEADAMKDVLLPGLALAAAHAGD LDTLQAFVELGRDLNLKDYSGQTPLHVAARRGHAAVVTMLLQRGVDVDARNEDGQSP LLLAVRGRHQSVIGLLRAAGARLSPQELEDVGTELCRLASRADSEGLRAWWQAGADLG QPDYDGHCALQVAEAAGNADVVALLQSFKDRVSAQPQTPH (SEQ ID NO: 25)

An-ASNase 74

MARATGPERRLLAIYTGGTIGMRSEGGVLVPGRGLAAVLRTLHMFHDEEYARAHHLPE DTLVLPPASPDQRILYTVLECQPLFDSSDMTITEWVQIAQTIERHYTQYQGFVVIHGTDT

MAFAASVLSFMLENLQKPVILTGAQVPIHALWNDGRENLLGALLMAGQYIIPEVCLFFQ
NQLFRGNRTTKVDARRFAAFCSPNLPPLATVGADVTINRELVRKASGKDRLVVHSSME
RDVGLLRLYPGIPASLVRTFLQPPLKGVVMETFGSGNGPTKPDLLQELRVAAEQGLIIVN
CTHCLQGAVTSDYASGMAMAGAGIVSGFDMTSEAALAKLSYVLGQPGLSLDDRKKLL
AKNLRGEMTLPVTDEHQSSLQDGMLGRRVAWLLSLNGSQEADAMQDVLMPSLALAA
AHAGDLDTLQAFVELGRDLNLKDCSGQTPLHVAARRGHAAVVTMLLQKGVDVNARN
EDGHSPLLLAVRGRHQGVIGLLRAAGACLSPQELEDVGTELCRLASRADSEGLRAWWQ
AGADLGQLDYDGHCALQVAEAAGNADVVALLQSLKDRVSAQPQAPH (SEQ ID NO: 26)

An-ASNase 75

MARATGPERRLLAIYTGGTIGMRSEGGVLVPGRGLAAVLRTLHMFHDEEYARAHSLPE
DTLVLPPASPDQRIIYTVLECQPLFDSSDMTITEWVQIAQTIERHYTQYQGFVVIHGTDTM
AFAASVLSFMLENLQKPVILTGAQVPIHALWSDGRENLLGALLMAGQYVIPEVCLFFQN
QLFRGNRTTKVDARRFAAFCSPNLPPLATVGADVTINRELVRKASGKNHLVVHSSMEPD
VGLLRLYPGIPASLVRTFLQPPLKGVVMETFGSGNGPTKPDLLQELRVAAEQGLIIVNCT
HCLQGAVTSDYASGMAMAGAGIVSGFDMTSEAALAKLSYVLGQPGLSLNDRKKLLAK
DLRGEMTLPATDELRSSLQDGMLGCRVAWLLSMNGSQEADAMKDVLLPGLALAAAH
AGDLDTLQAFVELGRDLNLKDCSGQTPLHVAARRGHAAVVTMLLQRGVDVDARNED
GQSPLLLAVRGRHQSVIGLLRAAGARLSPQELEDVGTELCRLASRADSEGLRAWWQAG
ADLGQPDYDGHCALQVAEAAGNADVVALLQSFKDRVSAQPQTPH (SEQ ID NO: 27)

An-ASNase 76

MARATGPERRLLAIYTGGTIGMRSEGGVLVPGRGLAAVLRTLHMFHDEEYARAHHLPE
DTLVLPPASPDQRILYTVLECQPLFDSSDMTITEWVQIAQTIERHYTQYQGFVVIHGTDT
MAFAASVLSFMLENLQKPVILTGAQVPIHALWNDGRENLLGALLMAGQYIIPEVCLFFQ
NQLFRGNRTTKVDARRFAAFCSPNLPPLATVGADVTINRELVRKASRKDRLVVHSSMER
DVGLLRLYPGIPASLVRTFLQPPLKGVVMETFGSGNGPTKPDLLQELRVASEQGLVIVNC
THCLQGAVTSDYASGMAMAGAGIVSGFDMTSEAALAKLSYVLGQPGLSLDDRKKLLA
KNLRGEMTLPVTDEHQSSLQDGMLGRRVAWLLSLNGSQEADAMQDVLMSSLALAAA
HAGDLDTLQAFVELGRDLNLKDCSGQTPLHVAARRGHAAVVTMLLQKGVDVNAHNE

DGHSPLLLAVRGRHQGVIGLLRAAGACLSPQELEDVGTELCRLASRADSEGLRAWWQA
GADLGQLDYDGHCALQVAEAAGNADVVALLQSLKDRVSAQPQPH (SEQ ID NO: 28)

An-ASNase 77

MARATGPERRLLAIYTGGTIGMRSEGGVLVPGRGLAAVLRTLHMFHDEEYARAHSLPE
DTLVLPPASPDQRIIYTVLECQPLFDSSDMTITEWVQIAQTIERHYTQYQGFVVIHGTDTM
AFAASVLSFMLENLQKPVILTGAQVPIHALWNDGRENLLGALLMAGQYIIPEVCLFFQN
QLFRGNRTTKVDARRFAAFCSPNLPPLATVGADVTINRELVRKASGKDRLVVHSSMER
DVGLLRLYPGIPASLVRTFLQPPLKGVVMETFGSGNGPTKPDLLQELRVAAEQGLIIVNC
THCLQGAVTSDYASGMAMAGAGIVSGFDMTSEAALAKLSYVLGQPGLSLDDRKKLLA
KDLRGEMTLPATDEHRSSLQDGMLGRRVAWLLSLNGSQEADAMQDVLMPSLALAAA
HAGDLDTLQAFVELGRDLNLKDCSGQTPLHVAARRGHAAVVTMLLQRGVDVNARNE
DGHSPLLLAVRGRHQGVIGLLRAAGACLSPQELEDVGTELCRLASRADSEGLRAWWQA
GADLGQPDYDGHCALQVAEAAGNADVVALLQSLKDRVSAQPQAPH (SEQ ID NO: 29)

An-ASNase 78

MARAAGPERRLLAIYTGGTIGMRSERGVLVPGKGLAAVLRTLPMFHDEGHAQACGLPE
DTLVLPPACPEQRVMYTVLECQPLFDSSDMTITEWVQIAQTIERHYGQYHGFVVIHGTD
TMAFAASVLSFMLENLQKTVILTGAQVPIHALWNDGRENLLGALLLAGQYVIPEVCLFF
QNQLFRGNRVTKVDTRRFAAFCSPNLPPLATVGADVTINRELVRKVRGQGRLVVHGGM
ERDVGLLRLYPGIPATLVRVFLQPPLKGVVLETFGSGNGPTKPDLLQELREAAERGLVIV
NCTHCLQGAVTSDYGAGTALAGAGTISGSDMTSEAALAKLSYVLGLPGLSLDGRKELL
ARDLRGEMTLPATDELRPSLRDSTLGRGVAQLLSLSQEAEDVRDALVPSLACAAAHAG
DLEALQVLAELGSDLSLEDFGGQTPLHSAARGGQAGVVTMLLQRGLDVNARDRDGLSP
LLLAVRGRHQGVIGLLRAAGACLSPQELEDSGTELCRLASRADCEGLRAWWQAGADLR
QPGYDGRSALHVAEAAGNLEVVALLQSLQGGVGDQALGPVSPPPPCGVGPGGDCVLGT
EPV (SEQ ID NO: 30)

An-ASNase 79

MARAAGPERRLLAIYTGGTIGMRSERGVLVPGKGLAAVLRTLPMFHDEGHAQACGLPE
DTLVLPPACPDQRVMYTVLECQPLFDSSDMTITEWVQIAQTIERHYGQYHGFVVIHGTD

TMAFAASVLSFMLENLQKTVILTGAQVPIHALWNDGRENLLGALLLAGQYVIPEVCLFF
QNQLFRGNRVTKVDTRRFAAFCSPNLPPLATVGADVTINRELVRKVRGQGRLVVHGSM
ERDVGLLRLYPGIPAALVRVFLQPPLKGVVMETFGSGNGPTKPDLLQELREAAERGLVI
VNCTHCLQGAVTSDYGAGTALAGAGTISGSDMTSEAALAKLSYVLGLPGLSLDGRKEL
LARDLRGEMTLPATDELRPSLRGSTPGRGVAQLLSLSQEAEDVRDALVPSLACAAAHA
GDLEALQVLAELGSDLSLEDFGGQTPLHSAARGGQAGVVTMLLQRGLDVNARDKDGL
SPLLLAVRGRHQGVIGLLRAAGACLSPQDLEDSGTELCRLASRADCEGLRAWWQAGAD
LRQPGYDGRSALHVAEAAGNLEVVALLQSLQGGVGDQALGPVSPLPPCGVGPGGDCVL
GTEPV (SEQ ID NO: 31)

An-ASNase 80

MARAAGPERRLLAIYTGGTIGMRSERGVLVPGKGLAAVLRTLPMFHDEGHAQACGLPE
DTLVLPPACPDQRVMYTVLECQPLFDSSDMTITEWVQIAQTIERHYGQYHGFVVIHGTD
TMAFAASVLSFMLENLQKTVILTGAQVPIHALWNDGRENLLGALLLAGQYVIPEVCLFF
QNQLFRGNRVTKVDTRRFAAFCSPNLPPLATVGADVTINRELVRKVRGQGRLVVHGSM
ERDVGLLRLYPGIPAALVRVFLQPPLKGVVMETFGSGNGPTKPDLLQELREAAERGLVI
VNCTHCLQGAVTSDYGAGTALAGAGTISGSDMTSEAALAKLSYVLGLPGLSLDGRKEL
LARDLRGEMTLPATDELRPSLRGSTLGRGVAQLLSLSQEAEDVRDALVPSLACAAAHA
GDLEALQVLAELGSDLSLEDFGGQTPLHSAARGGQAGVVTMLLQRGLDVNARDKDGL
SPLLLAVRGRHQGVIGLLRAAGACLSPQELEDSGTELCRLASRADCEGLRAWWQAGAD
LRQPGYDGRSALHVAEAAGNLEVVALLQSLQGGVGDQALGPVSPLPPCGVGPGGDCVL
GTEPV (SEQ ID NO: 32)

An-ASNase 81

MARAAGPERRLLAVYTGGTIGMRSERGVLVPGRGLAAVLKMLPMFHDEEHARARGLP
EDTLVLPPASPDQRVIYTVLECQPLFDSSDMTITEWVQIAQTIESHYARYDGFVVIHGTDT
MAFAASVLSFVLENLQKTVILTGAQVPIHALWNDGRENLLGALLLAGQYVIPEVCLFFQ
NQLFRGNRVTKVDARRFAAFCSPNLPPLATVGADVTINQELVRRVRGQGRLVVHSSME
RNVGLLRLYPGIPASLVRAFLQPPMKGVVMETFGSGNGPTKPDLLQELRAAAERGLVIV
NCTHCLQGAVTSDYGAGMALSGAGTVSGFDMTSEAALAKLSYVLGLPGLSLDGRKELL
ARDLRGEMTPPAVDEPRPSLRGGVLGRGVAQLLSLRQEADEVWDALVPSLACAAAYV

GDLEALQVLVELGSDRSLEDFSGQTPLHAAARAGQAGAVTMLLQRGLDVNARDKGGL
SPLLLAVGGRHRDVIGLLRAAGACLSPQELEDSGTELCRLASRADCEGLRAWGQAGAD
LRQPGYDGRSALHVAEAAGNLEVVALLQNLRGGAGDQAPGPEVLPGV (SEQ ID NO: 33)

An-ASNase 82

MARAAGPERRLLAVYTGGTIGMRSERGVLVPGRGLAAVLRMLPMFHDEEHARACGLP
EDTLVLPPASPDQRVFYTVLECQPLFDSSDMTITEWVQIAQIIESHYARYDGFVVIHGTDT
MAFAASVLSFVLENLQKTVILTGAQVPIHALWNDGRENLLGALLLAGQYVIPEVCLFFQ
NQLFRGNRVTKVDARRFAAFCSPNLPPLAAVGADVTINRELVCRVRGQGRLVVHSSME
RNVGLLRLYPGIPASLVRAFLQPPMKGVVMETFGSGNGPTKPDLLQELRAAAERGLVIV
NCTHCLQGAVTSDYGAGVALSGAGTVPGFDMTSEAALAKLSYVLGLPGLSLDGRKELL
ARDLRGEMTPPAADEPRPSLQGSVLGRGVAQLLSLRQEADEVWDALVPSLACAAAYA
GDLEALQVLVELGSDQSLEDFSGQTPLHAAARAGQAGAVTMLLQRGLDVNARDKGGL
SPLLLAVGGRHRGVIGLLRAAGACLSPQELEDSGTELCRLASRADCEGLRAWGQAGAD
LRQPGYDGRSALHVAEAAGNLEVVALLQNLRGGAGDQALGPEVLPGV (SEQ ID NO: 34)

An-ASNase 83

MARAAGPERRLLAVYTGGTIGMRSERGVLVPGRGLAAVLRMLPMFHDEEHARACGLP
EDTLVLPPASPDQRVIYTVLECQPLFDSSDMTITEWVQIAQTIESHYARYDGFVVIHGTDT
MAFAASVLSFVLENLQKTVILTGAQVPIHALWNDGRENLLGALLLAGQYVIPEVCLFFQ
NQLFRGNRVTKVDARRFAAFCSPNLPPLATVGADVTINRELVRRVRGQGRLVVHSSME
RNVGLLRLYPGIPASLVRAFLQPPMKGVVMETFGSGNGPTKPDLLQELRAAAERGLVIV
NCTHCLQGAVTSDYGAGMALSGAGTVSGFDMTSEAALAKLSYVLGLPGLSLDGRKELL
ARDLRGEMTPPAVDEPRPSLQGSVLGRGVAQLLSLRQEADEVWDALVPSLACAAAYA
GDLEALQVLVELGSDQSLEDFSGQTPLHAAARAGQAGAVTMLLQRGLDVNARDKGGL
SPLLLAVGGRHRGVIGLLRAAGACLSPQELEDSGTELCRLASRADCEGLRAWGQAGAD
LRQPGYDGRSALHVAEAAGNLEVVALLQNLRGGAGDQALGPEVLPGV (SEQ ID NO: 35)

An-ASNase 84

MARAPGPERRLLLIYTGGTLGMRSERGVLVPGPGLAAVLRTLPMFHDEEHARAQGLPD
DTLVLPPASPGPRVIYTVLQCQPLLDSSDMTITEWVQIAQTIERHYEQYHGFVVIHGTDT

MAFAASVLSFVLENLHKPVILTGAQVPIHALWNDSRENLLGALLMAGQYIIPEVCLFIQN
QLFRGNRVTKVDTQRFGAFCSPNLPPLATVGADVTVARELVRTASWKDALVVHSSMER
DVGLLRLYPGIPASLVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRAAAERGLIIVNC
SHCLRGPVTPGYASGLAEAGASIVSGFDMTSEAALAKLSYVLGQPGLSLDHRKELLAKD
LRGEMTLPTVDKHQSSLQGSALGRSVAWLLGLSGGQEVDAVRDAVMPSLALAAAHAG
DLEALQALVELGSDLCLEDSNGQTPLHVAARRGHEGVVTMLLHRGVDVNARDQDGLS
PLLLAVQGRHQGTIGLLRTAGACLSPQDLEDAGTELCRLASRADTEGLRAWWQAGADL
KQPGYDGRSALCVAEAAGNLEVVALLRSLEGAVGARASGPEVLPGVA (SEQ ID NO: 36)

An-ASNase 86

MSRALEPERRLLAIYTGGTIGMRNERGVLVPGRGLAAVLRTLPMFHDEDHARALGLPE
DTLVLPPASPDQRVIYTVLECQPLFDSSDMTITEWVQIAQTIERHYGQYHGFVVIHGTDT
MAFAASVLSFVLENLQKTVILTGAQVPIHALWNDGRENLLGALLMAGQYVIPEVCLFFQ
NQLFRGNRVTKVDSRRFAAFCSPNLPPLAVVGADVTLNRELVRKVRGKDRLVVHSSME
RDVGLLRLYPGIPAALVRAFLQPPLKGVVMETFGSGNGPTKPDLLRELRAATERGLIIVN
CTHCLQGTVTSGYAAGMAVAGAGIVSGFDMTSEAAMAKLSYVLGQPGLSLDSRKQLL
ARDLRGEMTLPSGDEHRPSLTCSTLGRGVAQLLSLSQEADAVRDALTPGLACAAAHAG
DLDVLQALVELGSDLSQENFNGQTPLHAAARGGHPEVVTMLLQRGVDVSARDEDGLSP
LLLAVKGRHQDVIGLLRAAGACLSPQELEDAGTELCRLASRADLEGLQSWWRAGADLG
CPGYDGRSALLVAEAAGNLEVVTFLQNLQGGAGVQAPGPATLPGSA (SEQ ID NO: 37)

An-ASNase 87

MARAAGPERRLLAIYTGGTIGMRSEGGVLVPGRGLAAVLRRLPMFHDEEYAQACQLPE
DTLVLPPASPDQRVIYTVLEWQPLLDSSDMTMAEWVQIAQTIERHYEQYHGFVVIHGTD
TMAFAASMLSFVLENLQKPVILTGAQVPIHALWNDGRENLLGALLMAGQYLIPEVCLFF
QNQLFRGNRATKVDSRRFAAFCSPNLPPLATVGADITINRELVRKVRGKEPLVVHSSME
RDVGVLRLYPGIPASLVRAFLQPPLKGVVLETFGSGNGPTKPELLQELRAAAGRGLLILN
CTHCLQGAVTSDYAAGTALAGAGLVSGFDMTSEAALAKLAYVLGQPGLSLDDRKQLL
AQDLRGEMTLPAADEHRPSLRGSSLGRQVAQFLSLSGSQELDAVRDALLPSLACAAAH
AGDLEALQALVEVGSDLSMEDFNGQTPLHAAARGGHAGVVSMLLQRGVDVNARDRD
GLSPLLLAVRGRHQGVIGLLRAAGACLSPQELEDAGTELCRLASRADAEGLQAWWQAG

ADLGQPGYDGRNALHVAESAGNLEVVTLLQSLQGRASAQAPGPEGIPGV (SEQ ID NO: 38)

An-ASNase 89

MARALGPERRLLAVYTGGTIGMRSEQGVLVPGSGLAAVLRTLPMFHDEEHARACGLPE DTLVLPPASPDQRVIYTVLECQPLFDSSDMTITEWVQIAQTIERHYGQYDGFVVIHGTDT MAFAASVLSFVLENLQKTVILTGAQVPIHALWNDGRENLLGALLMAGQYVIPEVCLFFQ NQLFRGNRVTKVDSRRFAAFCSPNLPPLAVVGTDVTINRELVRKARGKDPLVVHSSMER DVGLLRLYPGIPAALVRAFLQPPLKGVVMETFGSGNGPTKPDLLRELQAAAERGLVIVN CTHCLQGAVTSDYAAGMAMAGAGIVSGFDMTSEAALAKLSYVLGQPGLSLDGRKELL ARDLRGEMTLPTGAERRPSLSCSTLGRGVAQLLTLSQEADAVRDALMPSLACAAAHAG NLEVLQALVELGSDLSLENFNGQTPLHAAARGGQAGAVTMLLRRGVDVDPRDEDGLSP LLLAVKGRHRGVIELLRAAGACLSPRELEDAGTELCRLASRADLEGLQSWWQAGADLG RPGYDGRSALLVAEAAGNLEVVTFLQNLQGGAGAQAPGPEVLPGVQ (SEQ ID NO: 39)

An-ASNase 90

MARALGPERRLLAVYTGGTIGMRSEQGVLVPGSGLAAVLRTLPMFHDEEHARACGLPE DTLVLPPASPDQRVIYTVLECQPLFDSSDMTITEWVQIAQTIERHYGQYDGFVVIHGTDT MAFAASVLSFVLENLQKTVILTGAQVPIHALWNDGRENLLGALLMAGQYVIPEVCLFFQ NQLFRGNRVTKVDSRRFAAFCSPNLPPLAVVGTDVTINRELVRKARGKDRLVVHSSME RDVGLLRLYPGIPAALVRAFLQPPLKGVVMETFGSGNGPTKPDLLRELQAAAERGLVIV NCTHCLQGAVTSDYAAGMAMAGAGIVSGFDMTSEAALAKLSYVLGQPGLSLDGRKEL LARDLRGEMTLPTGAERRPSLSCSTLGRGVAQLLTLSQEADAVRDALMPSLACAAAHA GNLEVLQALVELGSDLSLENFNGQTPLHAAARGGQAGAVTMLLRRGVDVDPRDEDGL SPLLLAVKGRHRGVIELLRAAGACLSPRELEDAGTELCRLASRADLEGLRSWWQAGAD LGRPGYDGRSALLVAEAAGNLEVVTFLQNLQGGAGAQAPGPEVLPGVQ (SEQ ID NO: 40)

An-ASNase 91

MARALGPERRLLAVYTGGTIGMRSERGVLVPGRGLAAVLRTLPMFHDEEHARACGLPE DTLVLPPASPDQRVIYTVLECQPLFDSSDMTITEWVQIAQTIERHYEQYHGFVVIHGTDT

MAFAASVLSFVLENLQKTVILTGAQVPIHALWNDGRENLLGALLMAGQYVIPEVCLFFQ
NQLFRGNRVTKVDSRRFAAFCSPNLPPLAVVGTDVTINRELVRKARGKDRLVVHSSME
RDVGLLRLYPGIPAALVRAFLQPPLKGVVMETFGSGNGPTKPDLLRELQAAAERGLVIV
NCTHCLQGAVTSDYAAGMAMAGAGIVSGFDMTSEAALAKLSYVLGQPGLSLDGRKEL
LARDLRGEMTLPTGDERRPSLSCSTLGRGVAQLLTLSQEADAVRDALMPSLACAAAHA
GNLEVLQALVELGSDLSLENFNGQTPLHAAARGGQAGAVTMLLQRGVDVDARDEDGL
SPLLLAVKGRHRGVIGLLRAAGACLSPRELEDAGTELCRLASRADLEGLRSWWQAGAD
LGRPGYDGRSALLVAEAAGNLEVVTFLQNLQGGAGAQAPGPEVLPGVE (SEQ ID NO: 41)

An-ASNase 92

MARAAGPERRLLAIYTGGTIGMRSQRGVLVPGRGLAAVLRTLPMFHDEEHARACGLPE
DTLVLPPASPDQRVIYTVLECQPLFDSSDMTITEWVQIAQTIERHYEQYHGFVVIHGTDT
MAFAASVLSFVLENLQKTVILTGAQVPIHALWNDGRENLLGALLMAGQYVIPEVCLFFQ
NQLFRGTRVTKVDTRRFAAFCSPNLPPLAVVGADVTINRELVRKVRGKDPLVVHSSME
RDVGLLRLYPGIPATLVRAFLQPPLKGVVMETFGSGNGPTKPDLLRELRAAAERGLIILN
CTHCLQGAVTSDYAAGMATAGAGIVSGFDMTSEAALAKLSYVLGQPGLSLDGRKELLA
RDLRGEMTLPRVEEHRPSLQSGTLGLGVAQLLSLSQETDAVRDALTPSLACAAAHAGD
LEALQALAELGSDLSLQDFSGQTPLHVAARRGHAGVVTMLLQRGVDVNARDEDGLSPL
LLAVRGRHQGVIGLLRAAGACLSPQELEDAGTELCRLASRADSEGLRAWWQAGADLG
QPGYDGRSALLVAEAAGNLEVVTLLQSLQGGAGVLAPGPEVLPGVAACSGSSGA (SEQ ID NO: 42)

An-ASNase 94

MARALGPERRLLAIYTGGTIGMRSERGVLVPGRGLAAVLRTLPMFHDEEHARACGLPE
DTLVLPPASPDQRVIYTVLECQPLFDSSDMTITEWVQIAQTIERHYEQYHGFVVIHGTDT
MAFAASVLSFVLENLQKTVILTGAQVPIHALWNDGRENLLGALLMAGQYVIPEVCLFFQ
NQLFRGNRVTKVDSRRFAAFCSPNLPPLAVVGADVTINRELVRKVRGKDRLVVHSSME
RDVGLLRLYPGIPAALVRAFLQPPLKGVVMETFGSGNGPTKPDLLRELRAAAERGLVIV
NCTHCLQGAVTSDYAAGMAMAGAGIVSGFDMTSEAALAKLSYVLGQPGLSLDGRKEL
LARDLRGEMTLPAGDEHRPSLSCSTLGRGVAQLLSLSQEADAVRDALMPSLACAAAHA

GDLEVLQALVELGSDLSLENFNGQTPLHAAARGGHAGVVTMLLQRGVDVNARDEDGL SPLLLAVKGRHQGVIGLLRAAGACLSPQELEDAGTELCRLASRADLEGLRSWWQAGAD LGRPGYDGRSALLVAEAAGNLEVVTFLQNLQGGAGAQAPGPEVLPGVA (SEQ ID NO: 43)

An-ASNase 95

MARAAEPERRLLAVYTGGTIGMRSERGVLIPGRGLAAVLRTLPMFHDEEHARACGLPE DTLVLPPASPDQRVIYTVLECQPLFDSSDMTITEWVQIAQTIERHYEQYHGFVVIHGTDT MAFAASVLSFVLENLQKTVILTGAQVPIHALWNDGRENLLGALLMAGQYVIPEVCLFFQ NQLFRGNRVTKVDARRFAAFCSPNLPPLAVVGADVTINRELVRKVRGKERLVVHSSME RDVGLLRLYPGIPAALVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRAAAERGLVIV NCTHCLQGAVTSDYAAGMAMAGAGIVSGFDMTSEAALAKLSYVLGQPGLSLDGRKEL LARDLRGEMTLPAVDEHRPSLQGSTLGHGVAQLLSLSQGADAVRDALMPSLACAAAH AGDLEALQALVELGSDLSLEDFNGQTPLHAAARGGHAGVVTMLLQRGVDVNARDEDG LSPLLLAVRGRHQGVIGLLRAAGACLSPQELEDAGTELCRLASRGDSEGLRAWWQAGA DLGRPGYDGRSALLVAEAAGNLEVVTLLQSLQGGVGAQAPGPEVLPAVA (SEQ ID NO: 44)

An-ASNase 96

MARAAGPERRLLAIYTGGTIGMRSERGVLVPGRGLAAVLRTLPMFHDEEHARACGLPE DTLVLPPASPDQRVIYTVLECQPLFDSSDMTITEWVQIAQTIERHYEQYHGFVVIHGTDT MAFAASVLSFVLENLQKTVILTGAQVPIHALWNDGRENLLGALLLAGQYVIPEVCLFFQ NQLFRGNRVTKVDARRFAAFCSPNLPPLATVGADVTINRELVRRVRGQGRLVVHSSME RDVGLLRLYPGIPAALVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRAAAERGLVIV NCTHCLQGAVTSDYGAGMALAGAGTVSGFDMTSEAALAKLSYVLGLPGLSLDGRKEL LARDLRGEMTPPAVDELRPSLQGSTLGRGVAQLLSLSQEADEVRDALVPSLACAAAHA GDLEALQVLVELGSDLSLEDFSGQTPLHAAARGGQAGVVTMLLQRGLDVNARDKDGL SPLLLAVRGRHQGVIGLLRAAGACLSPQELEDSGTELCRLASRADCEGLRAWWQAGAD LRQPGYDGRSALHVAEAAGNLEVVALLQSLQGGAGDQALGPEVLPGV (SEQ ID NO: 45)

An-ASNase 97

MARAAGPERRLLAIYTGGTIGMRSERGVLVPGRGLAAVLRTLPMFHDEEHARARGLPE
DTLVLPPASPDQRVVYTVLECQPLFDSSDMTITEWVQIAQTIERHYEQYHGFVVIHGTDT
MAFAASVLSFVLENLQKTVILTGAQVPIHALWNDGRENLLGALLLAGQYVIPEVCLFFQ
NQLFRGNRVTKVDARRFAAFCSPNLPPLATVGADVTINRELVRRVRGKGRLVVHSSME
RDVGLLRLYPGIPAALVRAFLQPPLKGVVLETFGSGNGPTKPDLLQELRAAAERGLVIV
NCTHCLQGAVTSDYGAGMAMAGAGTVSGFDMTSEAALAKLSYVLGLPGLSLDGRKEL
LARDLRGEMTPPAVDELWPSLQGSTLGRGVAQLLSLSQEADAVRDALAPSLACAAAHA
GDLEALQVLVELGSDLSLEDFSGQTPLHAAARGGQAGVVTMLLQRGLDVNARDKDGL
SPLLLAVRGRHQGVIGLLRAAGACLSPQELEDSGTELCRLASRADCEGLRAWWQAGAD
LRQPGYDGRSALHIAEAAGNLEVVTLLQSLQGGAGAQALGPEVLPGV (SEQ ID NO: 46)

An-ASNase 98

MARAAGPERRLLAIYTGGTIGMRSERGVLVPGRGLAAVLRTLPMFHDEEHARACGLPE
DTLVLPPASPDQRVIYTVLECQPLFDSSDMTITEWVQIAQTIERHYEQYHGFVVIHGTDT
MAFAASVLSFVLENLQKTVILTGAQVPIHALWNDGRENLLGALLLAGQYVIPEVCLFFQ
NQLFRGNRVTKVDARRFAAFCSPNLPPLATVGADVTINRELVRRVRGKGRLVVHSSME
RDVGLLRLYPGIPAALVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRAAAERGLVIV
NCTHCLQGAVTSDYGAGMAMAGAGTVSGFDMTSEAALAKLSYVLGLPGLSLDGRKEL
LARDLRGEMTPPAVDELRPSLQGSTLGRGVAQLLSLSQEADAVRDALVPSLACAAAHA
GDLEALQVLVELGSDLSLEDFSGQTPLHAAARGGQAGVVTMLLQRGLDVNARDKDGL
SPLLLAVRGRHQGVIGLLRAAGACLSPQELEDSGTELCRLASRADCEGLRAWWQAGAD
LRQPGYDGRSALHVAEAAGNLEVVTLLQSLQGGAGAQALGPEVLPGV (SEQ ID NO: 47)

An-ASNase 99

MARAAGPERRLLAIYTGGTIGMRSERGVLVPGRGLAAVLRTLPMFHDEEHARACGLPE
DTLVLPPASPDQRVIYTVLECQPLFDSSDMTITEWVQIAQTIERHYEQYHGFVVIHGTDT
MAFAASVLSFVLENLQKTVILTGAQVPIHALWNDGRENLLGALLMAGQYVIPEVCLFFQ
NQLFRGNRVTKVDARRFAAFCSPNLPPLAVVGADVTINRELVRKVRGKDRLVVHSSME
RDVGLLRLYPGIPAALVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRAAAERGLVIV
NCTHCLQGAVTSDYAAGMAMAGAGIVSGFDMTSEAALAKLSYVLGQPGLSLDGRKEL

LARDLRGEMTLPAVDEHRPSLQGSTLGRGVAQLLSLSQEADAVRDALMPSLACAAAHA
GDLEALQALVELGSDLSLEDFNGQTPLHAAARGGHAGVVTMLLQRGVDVNARDEDGL
SPLLLAVRGRHQGVIGLLRAAGACLSPQELEDAGTELCRLASRADSEGLRAWWQAGAD
LGRPGYDGRSALLVAEAAGNLEVVTLLQSLQGGAGAQAPGPEVLPGVA (SEQ ID NO: 48)

An-ASNase 100

MARAAGPERRLLAIYTGGTIGMRSERGVLVPGRGLAAVLRTLPMFHDEEHARACGLPE
DTLVLPPASPDQRVIYTVLECQPLFDSSDMTITEWVQIAQTIERHYEQYHGFVVIHGTDT
MAFAASVLSFVLENLQKTVILTGAQVPIHALWNDGRENLLGALLMAGQYVIPEVCLFFQ
NQLFRGTRVTKVDTRRFAAFCSPNLPPLAVVGADVTINRELVRKVRGKDRLVVHSSME
RDVGLLRLYPGIPATLVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRAAAERGLIILN
CTHCLQGAVTSDYAAGMATAGAGIVSGFDMTSEAALAKLSYVLGQPGLSLDGRKELLA
RDLRGEMTLPAVDEHRPSLQGSTLGLGVAQLLSLSQEADAVRDALTPSLACAAAHAGD
LEALQALAELGSDLSLEDFNGQTPLHAAARGGHAGVVTMLLQRGVDVNARDEDGLSPL
LLAVRGRHQGVIGLLRAAGACLSPQELEDAGTELCRLASRADSEGLRAWWQAGADLG
QPGYDGRSALLVAEAAGNLEVVTLLQSLQGGAGALAPGPEVLPGVAACSGSSGA (SEQ ID NO: 49)

An-ASNase 101

MARAAGPERRLLAIYTGGTIGMRSERGVLVPGRGLAAVLRTLPMFHDEEHARACGLPE
DTLVLPPASPDQRVIYTVLECQPLFDSSDMTITEWVQIAQTIERHYEQYHGFVVIHGTDT
MAFAASVLSFVLENLQKTVILTGAQVPIHALWNDGRENLLGALLMAGQYVIPEVCLFFQ
NQLFRGNRVTKVDARRFAAFCSPNLPPLAVVGADVTINRELVRKVRGKDRLVVHSSME
RDVGLLRLYPGIPAALVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRAAAERGLVIV
NCTHCLQGAVTSDYAAGMAMAGAGIVSGFDMTSEAALAKLSYVLGQPGLSLDGRKEL
LARDLRGEMTLPAVDEHRPSLQGSTLGRGVAQLLSLSQEADAVRDALMPSLACAAAHA
GDLEALQALVELGSDLSLEDFNGQTPLHAAARGGHAGVVTMLLQRGVDVNARDEDGL
SPLLLAVRGRHQGVIGLLRAAGACLSPQELEDAGTELCRLASRADSEGLRAWWQAGAD
LGQPGYDGRSALLVAEAAGNLEVVTLLQSLQGGAGAQAPGPEVLPGVA (SEQ ID NO: 50)

An-ASNase 102

MARAAGPERRLLAIYTGGTIGMRSERGVLVPGRGLAAVLRTLPMFHDEEHARACGLPE
DTLVLPPASPDQRVIYTVLECQPLFDSSDMTITEWVQIAQTIERHYEQYHGFVVIHGTDT
MAFAASVLSFVLENLQKTVILTGAQVPIHALWNDGRENLLGALLMAGQYVIPEVCLFFQ
NQLFRGNRVTKVDARRFAAFCSPNLPPLATVGADVTINRELVRKVRGKDRLVVHSSME
RDVGLLRLYPGIPAALVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRAAAERGLVIV
NCTHCLQGAVTSDYAAGMAMAGAGIVSGFDMTSEAALAKLSYVLGQPGLSLDGRKEL
LARDLRGEMTLPAVDEHRPSLQGSTLGRGVAQLLSLSQEADAVRDALMPSLACAAAHA
GDLEALQALVELGSDLSLEDFNGQTPLHAAARGGHAGVVTMLLQRGVDVNARDQDGL
SPLLLAVRGRHQGVIGLLRAAGACLSPQELEDAGTELCRLASRADSEGLRAWWQAGAD
LGQPGYDGRSALHVAEAAGNLEVVTLLQSLQGGAGAQAPGPEVLPGV (SEQ ID NO: 51)

An-ASNase 105

MARAAGPERRLLAIYTGGTIGMRSERGVLVPGRGLAAVLRTLPMFHDEEHARACSLPE
DTLVLPPASPDQRIIYTVLECQPLFDSSDMTITEWVQIAQTIERHYEQYHGFVVIHGTDTM
AFAASVLSFMLENLQKPVILTGAQVPIHALWNDGRENLLGALLMAGQYIIPEVCLFFQN
QLFRGNRTTKVDARRFAAFCSPNLPPLATVGADVTINRELVRKASGKDRLVVHSSMER
DVGLLRLYPGIPASLVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRAAAERGLVIVN
CTHCLQGAVTSDYASGMAMAGAGIVSGFDMTSEAALAKLSYVLGQPGLSLDDRKKLL
AKDLRGEMTLPAVDEHRSSLQGSTLGRGVAWLLSLSGSQEADAVRDALMPSLALAAA
HAGDLEALQALVELGSDLSLEDSNGQTPLHVAARRGHAGVVTMLLQRGVDVNARDQD
GLSPLLLAVRGRHQGVIGLLRAAGACLSPQELEDAGTELCRLASRADSEGLRAWWQAG
ADLGQPGYDGRSALHVAEAAGNLEVVALLQSLEGGVGAQAPGPE (SEQ ID NO: 52)

An-ASNase 108

MARAAGPERRLLAIYTGGTIGMRSERGVLVPGRGLAAVLRTLPMFHDEEHARACGLPE
DTLVLPPASPDQRVIYTVLECQPLFDSSDMTITEWVQIAQTIERHYEQYHGFVVIHGTDT
MAFAASVLSFVLENLQKPVILTGAQVPIHALWNDGRENLLGALLMAGQYIIPEVCLFFQ
NQLFRGNRTTKVDARRFAAFCSPNLPPLATVGADVTINRELVRKASGKDRLVVHSSME
RDVGLLRLYPGIPASLVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRAAAERGLVIV

NCTHCLQGAVTSDYASGMAMAGAGIVSGFDMTSEAALAKLSYVLGQPGLSLDDRKEL
LAKDLRGEMTLPAVDEHRSSLQGSTLGRGVAWLLSLSGSQEADAVRDALMPSLALAAA
HAGDLEALQALVELGSDLSLEDSNGQTPLHVAARRGHAGVVTMLLQRGVDVNARDQD
GLSPLLLAVRGRHQGVIGLLRAAGACLSPQELEDAGTELCRLASRADSEGLRAWWQAG
ADLGQPGYDGRSALHVAEAAGNLEVVALLQSLEGGVGAQAPGPEVLPGVA (SEQ ID NO: 53)

Ancestral Human Chimeras (An h_c) – Utilizing 365-573 of human L-ASNase protein as the C terminal consisting of ankyrin repeat.

An-69 h_c

MARAVGPERRLLAVYTGGTIGMRSELGVLVPGTGLAAILRTLPMFHDEEHARARGLSE
DTLVLPPASRNQRILYTVLECQPLFDSSDMTIAEWVRVAQTIERHYEQYHGFVVIHGTDT
MAFAASMLSFMLENLQKTVILTGAQVPIHALWSDGRENLLGALLMAGQYVIPEVCLFF
QNQLFRGNRTTKVDARRFAAFCSPNLLPLATVGADITINRELVRKVDGKAGLVVHSSME
QDVGLLRLYPGIPAALVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRVATERGLVIV
NCTHCLQGAVTTDYAAGMAMAGAGVISGFDMTSEAALAKLSYVLGQPGLSLDDRKEL
LTKDLRGEMTPPSVEERRPSLQGNTLGGGVSWLLSLSGSQEADALRNALVPSLACAAA
HAGDVEALQALVELGSDLGLVDFNGQTPLHAAARGGHTEAVTMLLQRGVDVNTRDTD
GFSPLLLAVRGRHPGVIGLLREAGASLSTQELEEAGTELCRLAYRADLEGLQVWWQAG
ADLGQPGYDGHSALHVAEAAGNLAVVAFLQSLEGAVGAQAPCPEVLPGV (SEQ ID NO: 54)

An-70 h_c

MARAVGPERRLLAVYTGGTIGMRSELGVLVPGTGLAAILRTLPMFHDEEHARARGLSE
DTLVLPPASRNQRILYTVLECQPLFDSSDMTIAEWVRVAQTIERHYEQYHGFVVIHGTDT
MAFAASMLSFMLENLQKTVILTGAQVPIHALWSDGRENLLGALLMAGQYVIPEVCLFF
QNQLFRGNRTTKVDARRFAAFCSPNLPPLATVGADITINRELVRKVDGKAGLVVHSSME
QDVGLLRLYPGIPAALVRAFLQPPLKGMVMETFGSGNGPTKPDLLQELRVATERGLVIV
NCTHCLQGAVTTDYAAGMAMAGAGVISGFDMTSEAALAKLSYVLGQPGLSLDDRKEL
LTKDLRGEMTPPSVEERRPSLQGNTLGGGVSWLLSLSGSQEADALRNALVPSLACAAA

HAGDVEALQALVELGSDLGLVDFNGQTPLHAAARGGHTEAVTMLLQRGVDVNTRDTD
GFSPLLLAVRGRHPGVIGLLREAGASLSTQELEEAGTELCRLAYRADLEGLQVWWQAG
ADLGQPGYDGHSALHVAEAAGNLAVVAFLQSLEGAVGAQAPCPEVLPGV (SEQ ID NO:
55)

An-71 h_c

MARAVGPERRLLAVYTGGTIGMRSELGVLVPGTGLAAILRTLPMFHDEEHARARGLSE
DTLVLPPASRNQRILYTVLECQPLFDSSDMTIAEWVRVAQTIERHYEQYHGFVVIHGTDT
MAFAASMLSFMLENLQKTVILTGAQVPIHALWSDGRENLLGALLMAGQYVIPEVCLFF
QNQLFRGNRTTKVDARRFAAFCSPNLLPLATVGADITINRELVRKVDGKAGLVVHSSME
QDVGLLRLYPGIPAALVRAFLQPPLKGMVMETFGSGNGPTKPDLLQELRVATERGLVIV
NCTHCLQGAVTTDYAAGMAMAGAGVISGFDMTSEAALAKLSYVLGQPGLSLDDRKEL
LTKDLRGEMTPPSVEERRPSLQGNTLGGGVSWLLSLSGSQEADALRNALVPSLACAAA
HAGDVEALQALVELGSDLGLVDFNGQTPLHAAARGGHTEAVTMLLQRGVDVNTRDTD
GFSPLLLAVRGRHPGVIGLLREAGASLSTQELEEAGTELCRLAYRADLEGLQVWWQAG
ADLGQPGYDGHSALHVAEAAGNLAVVAFLQSLEGAVGAQAPCPEVLPGV (SEQ ID NO:
56)

An-72 h_c

MARAVGPERRLLAVYTGGTIGMRSELGVLVPGTGLAAILRTLPMFHDEEHARARGLSE
DTLVLPPASPNQRILYTVLECQPLFDSSDMTIAEWVRVAQTIERHYEQYHGFVVIHGTDT
MAFAASMLSFMLENLQKTVILTGAQVPIHALWSDGRENLLGALLMAGQYVIPEVCLFF
QNQLFRGNRTTKVDARRFAAFCSPNLPPLATVGADVTINRELVRKVGGKAGLVVHSSM
EQDVGLLRLYPGIPAALVRAFLQPPLKGMVMETFGSGNGPTKPDLLQELRVATERGLVI
VNCTHCLQGAVTTDYAAGMAMAGAGVISGFDMTSEAALAKLSYVLGQPGLSLDDRKE
LLTKDLRGEMTPPSVEERRPSLQGNTLGGGVSWLLSLSGSQEADALRNALVPSLACAAA
HAGDVEALQALVELGSDLGLVDFNGQTPLHAAARGGHTEAVTMLLQRGVDVNTRDTD
GFSPLLLAVRGRHPGVIGLLREAGASLSTQELEEAGTELCRLAYRADLEGLQVWWQAG
ADLGQPGYDGHSALHVAEAAGNLAVVAFLQSLEGAVGAQAPCPEVLPGV (SEQ ID NO:
57)

An-85 h_c

MARAVGPERRLLAVYTGGTIGMRSELGVLVPGTGLAAILRTLPMFHDEEHARARGLPE
DTLVLPPASPNQRILYTVLECQPLFDSSDMTIAEWVRVAQTIERHYEQYHGFVVIHGTDT
MAFAASVLSFMLENLQKTVILTGAQVPIHALWSDGRENLLGALLMAGQYVIPEVCLFFQ
NQLFRGNRTTKVDARRFAAFCSPNLPPLATVGADVTINRELVRKVGGKAGLVVHSSME
QDVGLLRLYPGIPAALVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRVATERGLVIV
NCTHCLQGAVTTDYAAGMAMAGAGVISGFDMTSEAALAKLSYVLGQPGLSLDDRKEL
LAKDLRGEMTPPSVEERRPSLQGNTLGGGVSWLLSLSGSQEADALRNALVPSLACAAA
HAGDVEALQALVELGSDLGLVDFNGQTPLHAAARGGHTEAVTMLLQRGVDVNTRDTD
GFSPLLLAVRGRHPGVIGLLREAGASLSTQELEEAGTELCRLAYRADLEGLQVWWQAG
ADLGQPGYDGHSALHVAEAAGNLAVVAFLQSLEGAVGAQAPCPEVLPGV (SEQ ID NO: 58)

An-88 h_c

MARAAGPERRLLLIYTGGTLGMRSERGVLVPGPGLAAVLRTLPMFHDEEHARAQGLPD
DTLVLPPASPGPRVIYTVLECQPLLDSSDMTITEWVQIAQTIERHYEQYHGFVVIHGTDT
MAFAASVLSFVLENLHKPVILTGAQVPIHALWNDSRENLLGALLMAGQYIIPEVCLFIQN
QLFRGNRVTKVDTQRFGAFCSPNLPPLATVGADVTIARELVRKASWKDALVVHSSMER
DVGLLRLYPGIPASLVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRAAAERGLIIVNC
SHCLRGPVTPGYASGLAMAGASIVSGFDMTSEAALAKLSYVLGQPGLSLDHRKELLAK
DLRGEMTLPTVEERRPSLQGNTLGGGVSWLLSLSGSQEADALRNALVPSLACAAAHAG
DVEALQALVELGSDLGLVDFNGQTPLHAAARGGHTEAVTMLLQRGVDVNTRDTDGFS
PLLLAVRGRHPGVIGLLREAGASLSTQELEEAGTELCRLAYRADLEGLQVWWQAGADL
GQPGYDGHSALHVAEAAGNLAVVAFLQSLEGAVGAQAPCPEVLPGV (SEQ ID NO: 59)

An-93 h_c

MARAAGPERRLLAIYTGGTIGMRSELGVLVPGRGLAAVLRTLPMFHDEEHARACGLPE
DTLVLPPASPDQRVIYTVLECQPLFDSSDMTITEWVQIAQTIERHYEQYHGFVVIHGTDT
MAFAASVLSFVLENLQKTVILTGAQVPIHALWSDGRENLLGALLMAGQYVIPEVCLFFQ
NQLFRGNRTTKVDARRFAAFCSPNLPPLATVGADVTINRELVRKVGGKEGLVVHSSME
RDVGLLRLYPGIPAALVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRAAAERGLVIV

NCTHCLQGAVTSDYAAGMAMAGAGIISGFDMTSEAALAKLSYVLGQPGLSLDDRKELL
AKDLRGEMTPPAVEERRPSLQGNTLGGGVSWLLSLSGSQEADALRNALVPSLACAAAH
AGDVEALQALVELGSDLGLVDFNGQTPLHAAARGGHTEAVTMLLQRGVDVNTRDTDG
FSPLLLAVRGRHPGVIGLLREAGASLSTQELEEAGTELCRLAYRADLEGLQVWWQAGA
DLGQPGYDGHSALHVAEAAGNLAVVAFLQSLEGAVGAQAPCPEVLPGV (SEQ ID NO: 60)

An-104 h_c

MARAAGPERRLLAIYTGGTIGMRSERGVLVPGRGLAAVLRTLPMFHDEEHARACGLPE
DTLVLPPASPDQRVIYTVLECQPLFDSSDMTITEWVQIAQTIERHYEQYHGFVVIHGTDT
MAFAASVLSFVLENLQKTVILTGAQVPIHALWNDGRENLLGALLMAGQYVIPEVCLFFQ
NQLFRGNRTTKVDARRFAAFCSPNLPPLATVGADVTINRELVRKVSGKDRLVVHSSME
RDVGLLRLYPGIPAALVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRAAAERGLVIV
NCTHCLQGAVTSDYAAGMAMAGAGIVSGFDMTSEAALAKLSYVLGQPGLSLDDRKEL
LAKDLRGEMTLPAVEERRPSLQGNTLGGGVSWLLSLSGSQEADALRNALVPSLACAAA
HAGDVEALQALVELGSDLGLVDFNGQTPLHAAARGGHTEAVTMLLQRGVDVNTRDTD
GFSPLLLAVRGRHPGVIGLLREAGASLSTQELEEAGTELCRLAYRADLEGLQVWWQAG
ADLGQPGYDGHSALHVAEAAGNLAVVAFLQSLEGAVGAQAPCPEVLPGV (SEQ ID NO: 61)

An-107 h_c

MARAAGPERRLLAIYTGGTIGMRSERGVLVPGRGLAAVLRTLPMFHDEEHARACGLPE
DTLVLPPASPDQRVIYTVLECQPLFDSSDMTITEWVQIAQTIERHYEQYHGFVVIHGTDT
MAFAASVLSFVLENLQKPVILTGAQVPIHALWNDGRENLLGALLMAGQYVIPEVCLFFQ
NQLFRGNRTTKVDARRFAAFCSPNLPPLATVGADVTINRELVRKASGKDRLVVHSSME
RDVGLLRLYPGIPAALVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRAAAERGLVIV
NCTHCLQGAVTSDYAAGMAMAGAGIVSGFDMTSEAALAKLSYVLGQPGLSLDDRKEL
LAKDLRGEMTLPAVEERRPSLQGNTLGGGVSWLLSLSGSQEADALRNALVPSLACAAA
HAGDVEALQALVELGSDLGLVDFNGQTPLHAAARGGHTEAVTMLLQRGVDVNTRDTD
GFSPLLLAVRGRHPGVIGLLREAGASLSTQELEEAGTELCRLAYRADLEGLQVWWQAG

ADLGQPGYDGHSALHVAEAAGNLAVVAFLQSLEGAVGAQAPCPEVLPGV (SEQ ID NO: 62)

An-108 h_c

MARAAGPERRLLAIYTGGTIGMRSERGVLVPGRGLAAVLRTLPMFHDEEHARACGLPE DTLVLPPASPDQRVIYTVLECQPLFDSSDMTITEWVQIAQTIERHYEQYHGFVVIHGTDT MAFAASVLSFVLENLQKPVILTGAQVPIHALWNDGRENLLGALLMAGQYIIPEVCLFFQ NQLFRGNRTTKVDARRFAAFCSPNLPPLATVGADVTINRELVRKASGKDRLVVHSSME RDVGLLRLYPGIPASLVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRAAAERGLVIV NCTHCLQGAVTSDYASGMAMAGAGIVSGFDMTSEAALAKLSYVLGQPGLSLDDRKEL LAKDLRGEMTLPAVEERRPSLQGNTLGGGVSWLLSLSGSQEADALRNALVPSLACAAA HAGDVEALQALVELGSDLGLVDFNGQTPLHAAARGGHTEAVTMLLQRGVDVNTRDTD GFSPLLLAVRGRHPGVIGLLREAGASLSTQELEEAGTELCRLAYRADLEGLQVWWQAG ADLGQPGYDGHSALHVAEAAGNLAVVAFLQSLEGAVGAQAPCPEVLPGV (SEQ ID NO: 63)

CLAIMS

1. A recombinant asparaginase comprising an amino acid sequence selected from SEQ ID NO: 1-53 or variant thereof having 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity.

2. The recombinant asparaginase of claim 1 having SEQ ID NO: 10 (An 104) or a variant having 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity.

3. The recombinant asparaginase of claim 1, wherein the recombinant asparaginase elicits a lower immunogenic response in a patient compared to E. coli L-asparaginase or Erwinia chrysanthemi L-asparaginase.

4. The recombinant asparaginase of claim 1, wherein the variant has 1 amino acid substitution.

5. The recombinant asparaginase of claim 1, wherein the variant has 2 or 3 amino acid substitutions.

6. The recombinant asparaginase of claim 1 conjugated to a biodegradable polymer.

7. The recombinant asparaginase of claim 6, wherein the biodegradable polymer comprises polyethylene glycol or monomethoxy polyethylene glycol.

8. The recombinant asparaginase of claim 6, wherein the biodegradable polymer comprises an amide linking group connecting polyethylene glycol or monomethoxy polyethylene glycol to the recombinant asparaginase through a lysine amino acid to the N-terminal amino acid.

9. A nucleic acid encoding a recombinant asparaginase of claim 1 in operable combination with a heterologous promoter.

10. A vector encoding a nucleic acid of claim 9.

11. A somatic cell comprising a nucleic acid of claim 9 or a vector of claim 10.

12. A method of treating diseases associated with asparagine dependence comprising administering a recombinant asparaginase of claim 1 to a subject in need thereof.

13. The method of claim 12, wherein the recombinant asparaginase elicits a lower immunogenic response in a patient compared to E. coli L-asparaginase or Erwinia chrysanthemi L-asparaginase.

14. A method of treating cancer comprising administering a recombinant asparaginase of claim 1 to a subject in need thereof.

15. The method of claim 14, wherein the recombinant asparaginase elicits a lower immunogenic response in a patient compared to E. coli L-asparaginase or Erwinia chrysanthemi L-asparaginase.

16. The method of claim 14, wherein the cancer is a hematological cancer selected from leukemia, lymphoma, acute lymphoblastic leukemia (ALL) lymphoblastic lymphoma (LBL), acute myelogenous leukemia (AML), chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma (SLL), chronic myelogenous leukemia, acute monocytic leukemia (AMOL), chronic myeloid leukemia (CML), B-cell acute lymphoblastic leukemia (B-ALL), myeloproliferative neoplasms (MPNs), and lymphomas, Hodgkin's lymphomas, and non-Hodgkin's lymphomas such as Burkitt lymphoma, B-cell lymphoma, or diffuse large B-cell lymphoma (DLBCL).

17. The method of claim 14, wherein the cancer is a solid cancer ovarian cancer, ovarian clear cell carcinoma, pancreatic cancer, pancreatic ductal adenocarcinoma (PDAC), colorectal cancer (CRC), breast cancer, metastatic breast cancer, hepatocellular carcinoma, or glioblastoma.

18. The method of claim 14, wherein the subject is an adult or pediatric patients 1 month or older.

19. The method of claim 14, wherein the recombinant asparaginase is administered in combination with another anticancer agent.

20. The method of claim 19, wherein the anticancer agent is prednisone, dexamethasone, vincristine, daunorubicin, doxorubicin, cyclophosphamide, methotrexate, cytarabine, 6-mercaptopurine, 6-thioguanine or nelarabine.

21. A pharmaceutical composition comprising a recombinant asparaginase of claim 1 and optionally a pharmaceutically acceptable excipient.

22. The pharmaceutical composition of claim 21, wherein the recombinant asparaginase elicits a lower immunogenic response in a patient compared to E. coli L-asparaginase or Erwinia chrysanthemi L-asparaginase.

23. The pharmaceutical composition of claim 21, wherein the recombinant asparaginase is in a vial in lyophilized form.

24. The pharmaceutical composition of claim 21, wherein the recombinant asparaginase is in an aqueous pH buffered isotonic solution.

25. A recombinant asparaginase comprising an amino acid sequence selected from SEQ ID NO: 54-63 or variant thereof having 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity.

26. The recombinant asparaginase of claim 25 having SEQ ID NO: 61 (An-104 h_c) or a variant having 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or greater sequence identity.

27. The recombinant asparaginase of claim 25, wherein the recombinant asparaginase elicits a lower immunogenic response in a patient compared to E. coli L-asparaginase or Erwinia chrysanthemi L-asparaginase.

28. The recombinant asparaginase of claim 25, wherein the variant has 1 amino acid substitution.

29. The recombinant asparaginase of claim 25, wherein the variant has 2 or 3 amino acid substitutions.

30. The recombinant asparaginase of claim 25 conjugated to a biodegradable polymer.

31. The recombinant asparaginase of claim 30, wherein the biodegradable polymer comprises polyethylene glycol or monomethoxy polyethylene glycol.

32. The recombinant asparaginase of claim 30, wherein the biodegradable polymer comprises an amide linking group connecting polyethylene glycol or monomethoxy polyethylene glycol to the recombinant asparaginase through a lysine amino acid to the N-terminal amino acid.

33. A nucleic acid encoding a recombinant asparaginase of claim 25 in operable combination with a heterologous promoter.

34. A vector encoding a nucleic acid of claim 33.

35. A somatic cell comprising a nucleic acid of claim 33 or a vector of claim 34.

36. A method of treating diseases associated with asparagine dependence comprising administering a recombinant asparaginase of claim 25 to a subject in need thereof.

37. The method of claim 36, wherein the recombinant asparaginase elicits a lower immunogenic response in a patient compared to E. coli L-asparaginase or Erwinia chrysanthemi L-asparaginase.

38. A method of treating cancer comprising administering a recombinant asparaginase of claim 25 to a subject in need thereof.

39. The method of claim 38, wherein the recombinant asparaginase elicits a lower immunogenic response in a patient compared to E. coli L-asparaginase or Erwinia chrysanthemi L-asparaginase.

40. The method of claim 38, wherein the cancer is a hematological cancer selected from leukemia, lymphoma, acute lymphoblastic leukemia (ALL) lymphoblastic lymphoma (LBL), acute myelogenous leukemia (AML), chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma (SLL), chronic myelogenous leukemia, acute monocytic leukemia (AMOL), chronic myeloid leukemia (CML), B-cell acute lymphoblastic leukemia (B-ALL), myeloproliferative neoplasms (MPNs), and lymphomas, Hodgkin's lymphomas, and non-Hodgkin's lymphomas such as Burkitt lymphoma, B-cell lymphoma, or diffuse large B-cell lymphoma (DLBCL).

41. The method of claim 38, wherein the cancer is a solid cancer ovarian cancer, ovarian clear cell carcinoma, pancreatic cancer, pancreatic ductal adenocarcinoma (PDAC), colorectal cancer (CRC), breast cancer, metastatic breast cancer, hepatocellular carcinoma, or glioblastoma.

42. The method of claim 38, wherein the subject is an adult or pediatric patients 1 month or older.

43. The method of claim 38, wherein the recombinant asparaginase is administered in combination with another anticancer agent.

44. The method of claim 43, wherein the anticancer agent is prednisone, dexamethasone, vincristine, daunorubicin, doxorubicin, cyclophosphamide, methotrexate, cytarabine, 6-mercaptopurine, 6-thioguanine or nelarabine.

45. A pharmaceutical composition comprising a recombinant asparaginase of claim 25 and optionally a pharmaceutically acceptable excipient.

46. The pharmaceutical composition of claim 45, wherein the recombinant asparaginase elicits a lower immunogenic response in a patient compared to E. coli L-asparaginase or Erwinia chrysanthemi L-asparaginase.

47. The pharmaceutical composition of claim 45, wherein the recombinant asparaginase is in a vial in lyophilized form.

48. The pharmaceutical composition of claim 45, wherein the recombinant asparaginase is in an aqueous pH buffered isotonic solution.

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Query  1    MARAVGPERRLLAVYTGGTIGMRSELGVLVPGTGLAAILRTLPMFHDEEHARARGLSEDT  60
            MARA GPERRLLA+YTGGTIGMRSE GVLVPG GLAA+LRTLPMFHDEEHARA GL EDT
Sbjct  1    MARAAGPERRLLAIYTGGTIGMRSERGVLVPGRGLAAVLRTLPMFHDEEHARACGLPEDT  60

Query  61   LVLPPASRNQRILYTVLECQPLFDSSDMTIAEWVCLAQTIKRHYEQYHGFVVIHGTDTMA  120
            LVLPPAS +QR++YTVLECQPLFDSSDMTI EWV +AQTI+RHYEQYHGFVVIHGTDTMA
Sbjct  61   LVLPPASPDQRVIYTVLECQPLFDSSDMTITEWVQIAQTIERHYEQYHGFVVIHGTDTMA  120

Query  121  FAASMLSFMLENLQKTVILTGAQVPIHALWSDGRENLLGALLMAGQYVIPEVCLFFQNQL  180
            FAAS+LSF+LENLQKTVILTGAQVPIHALW+DGRENLLGALLMAGQYVIPEVCLFFQNQL
Sbjct  121  FAASVLSFVLENLQKTVILTGAQVPIHALWNDGRENLLGALLMAGQYVIPEVCLFFQNQL  180

Query  181  FRGNRATKVDARRFAAFCSPNLLPLATVGADITINRELVRKVDGKAGLVVHSSMEQDVGL  240
            FRGNR TKVDARRFAAFCSPNL PLATVGAD+TINRELVRKV GK  LVVHSSME+DVGL
Sbjct  181  FRGNRTTKVDARRFAAFCSPNLPPLATVGADVTINRELVRKVSGKDRLVVHSSMERDVGL  240

Query  241  LRLYPGIPAALVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRVATERGLVIVNCTHCL  300
            LRLYPGIPAALVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELR A ERGLVIVNCTHCL
Sbjct  241  LRLYPGIPAALVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRAAAERGLVIVNCTHCL  300

Query  301  QGAVTTDYAAGMAMAGAGVISGFDMTSEAALAKLSYVLGQPGLSLDVRKELLTKDLRGEM  360
            QGAVT+DYAAGMAMAGAG++SGFDMTSEAALAKLSYVLGQPGLSLD RKELL KDLRGEM
Sbjct  301  QGAVTSDYAAGMAMAGAGIVSGFDMTSEAALAKLSYVLGQPGLSLDDRKELLAKDLRGEM  360

Query  361  TPPSVEERRPSLQGNTLGGGVSWLLSLSGSQEADALRNALVPSLACAAAHAGDVEALQAL  420
            T P+V+E RPSLQG+TLG GV+WLLSLSGSQEADA+R+AL+PSLACAAAHAGD+EALQAL
Sbjct  361  TLPAVDEHRPSLQGSTLGRGVAWLLSLSGSQEADAVRDALMPSLACAAAHAGDLEALQAL  420

Query  421  VELGSDLGLVDFNGQTFLHAAAARGGHTEAVTMLLQRGVDVNTRDTDGFSPLLLAVRGRHP  480
            VELGSDL L DFNGQTFLHAAAARGGH   VTMLLQRGVDVN RD DG SPLLLAVRGRH
Sbjct  421  VELGSDLSLEDFNGQTFLHAAAARGGHAGVVTMLLQRGVDVNARDQDGLSPLLLAVRGRHQ  480

Query  481  GVIGLLREAGASLSTQELEEAGTELCRLAYRADLEGLQVWWQAGADLGQPGYDGHSALHV  540
            GVIGLLR AGA LS QELE+AGTELCRLA RAD EGL+ WWQAGADLGQPGYDG SALHV
Sbjct  481  GVIGLLRAAGACLSPQELEDAGTELCRLASRADSEGLRAWWQAGADLGQPGYDGRSALHV  540

Query  541  AEAAGNLAVVAFLQSLEGAVGAQAPCPEVLPGV  573  SEQ ID NO: 64
            AEAAGNL VVA LQSLEG VGAQAP PEVLPGV       SEQ ID NO: 65
Sbjct  541  AEAAGNLEVVALLQSLEGGVGAQAPGPEVLPGV  573  SEQ ID NO: 10
```

FIG. 1

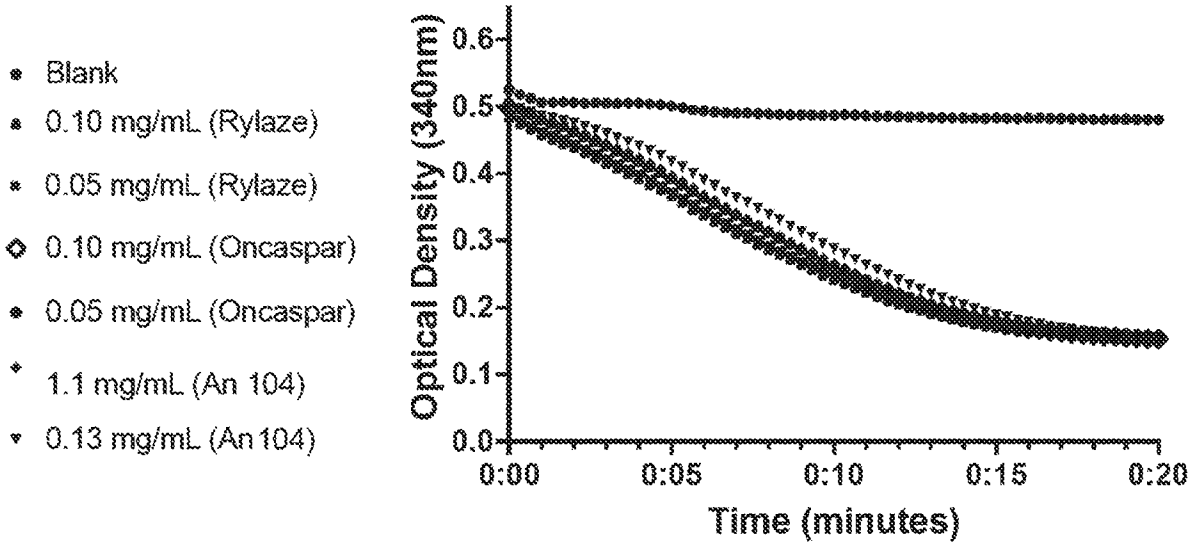
Blank
0.10 mg/mL (Rylaze)
0.05 mg/mL (Rylaze)
0.10 mg/mL (Oncaspar)
0.05 mg/mL (Oncaspar)
1.1 mg/mL (An 104)
0.13 mg/mL (An 104)
Optical Density (340nm)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0:00
0:05
0:10
0:15
0:20
Time (minutes)

FIG. 2A

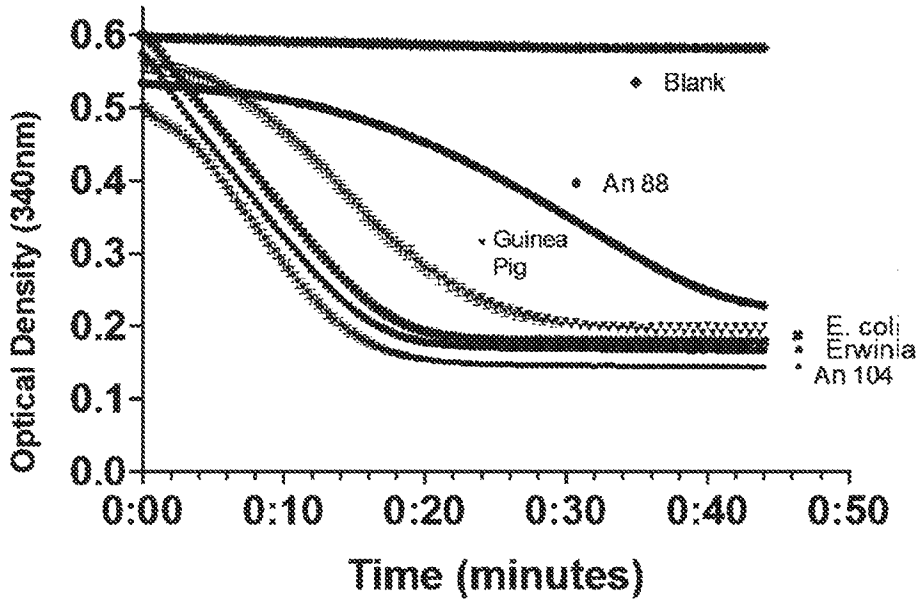
Optical Density (340nm)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0:00
0:10
0:20
0:30
0:40
0:50
Time (minutes)
Blank
An 88
Guinea Pig
E. coli
Erwinia
An 104

FIG. 2B

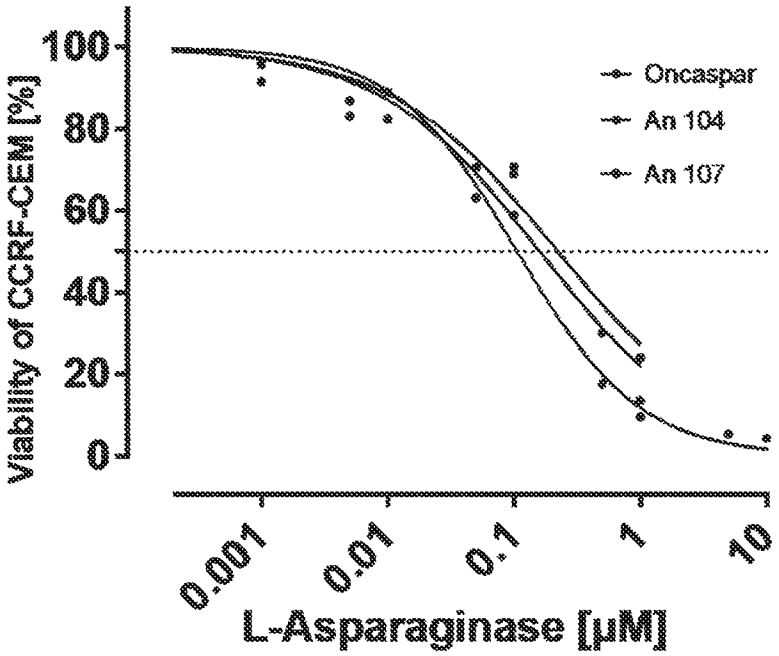
Viability of CCRF-CEM [%]
100
80
60
40
20
0
Oncaspar
An 104
An 107
0.001
0.01
0.1
1
10
L-Asparaginase [µM]

FIG. 3A

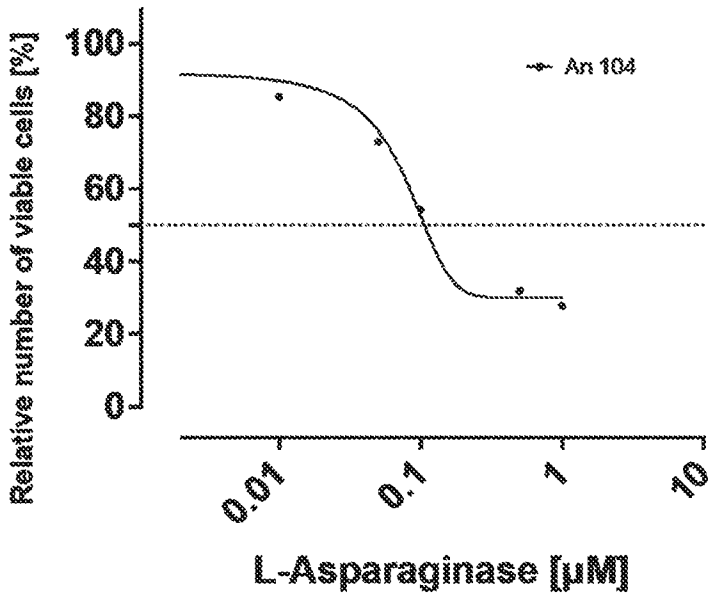
Relative number of viable cells [%]
100
80
60
40
20
0
An 104
0.01
0.1
1
10
L-Asparaginase [µM]

FIG. 3B

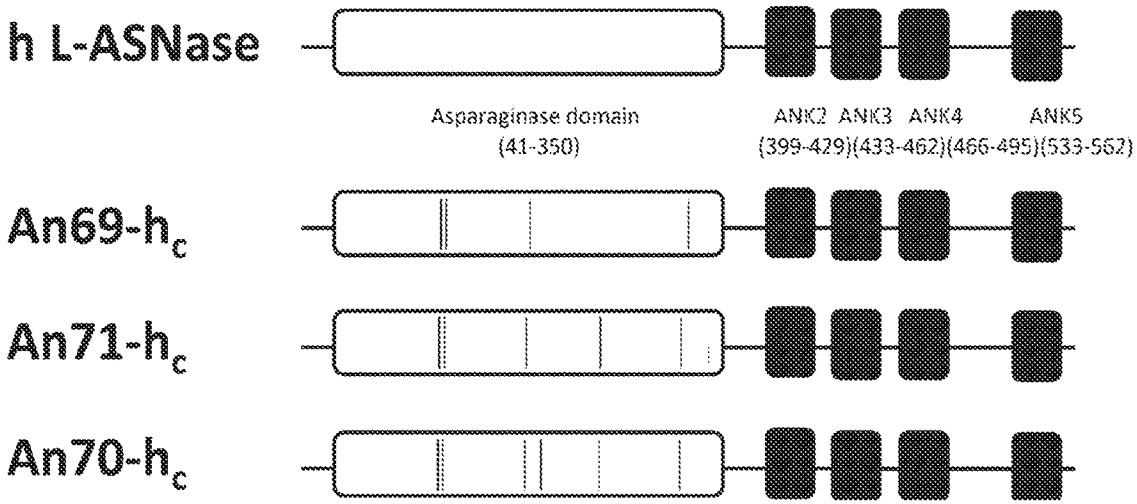

FIG. 4A

L-Asparaginase	Identity to Human L-Asparaginase (%)	Amino-Acid Replacements
Human	100	0
An-69-h_c	99.13	5
An-71-h_c	98.95	6
An-70-h_c	98.78	7
An-72-h_c	98.95	10
An-85-h_c	97.91	12
An-88-h_c	89.70	59
An-93-h_c	94.94	29
An-104-h_c	94.07	34
An-107-h_c	93.72	36
An-108-h_c	93.19	39

FIG. 4B

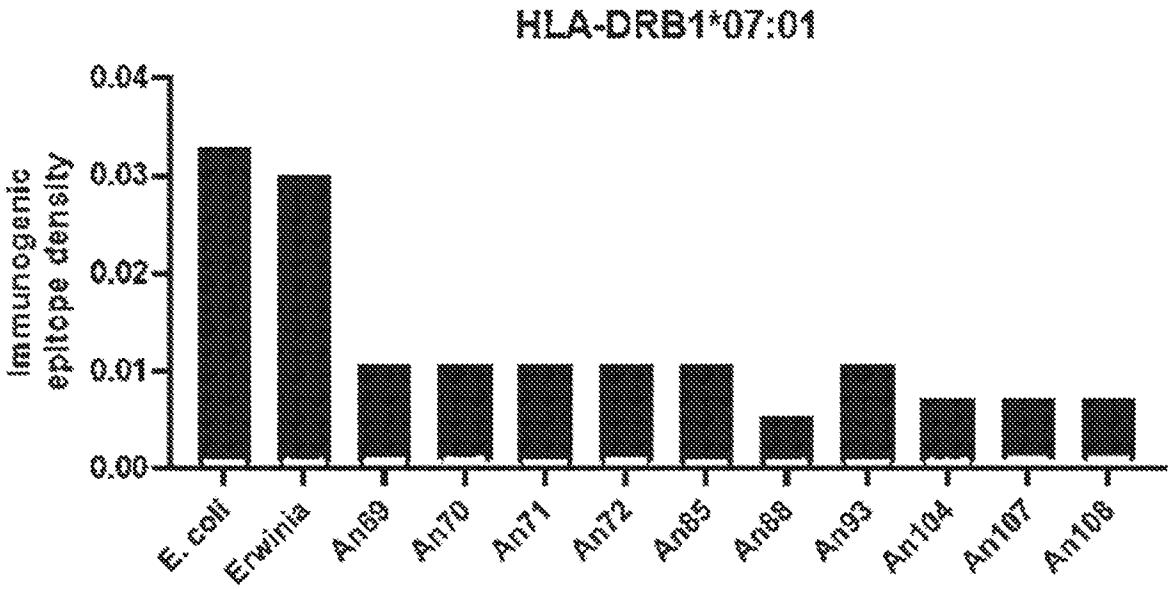
HLA-DRB1*07:01
Immunogenic epitope density
0.04
0.03
0.02
0.01
0.00
E. coli
Erwinia
An69
An70
An71
An72
An85
An89
An93
An104
An107
An108

FIG. 5