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Canadian Intellectual Property Office

CA 3106549 A1 2019/02/14

(21) 3 106 549

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2018/08/10
(87) Date publication PCT/PCT Publication Date: 2019/02/14
(85) Entrée phase nationale/National Entry: 2021/01/12
(86) N° demande PCT/PCT Application No.: US 2018/046196
(87) N° publication PCT/PCT Publication No.: 2019/032952
(30) Priorités/Priorities: 2017/08/11 (US62/544,396); 2017/08/11 (US62/544,411)

(51) Cl.Int./Int.Cl. *A61K 38/17* (2006.01), *C12N 9/82* (2006.01)

(71) Demandeurs/Applicants:
THE BOARD OF TRUSTEES OF THE UNIVERSITY OF ILLINOIS, US;
THE UNITED STATES OF AMERICA AS REPRESENTED BY THE DEPARTMENT OF VETERANS AFFAIRS, US

(72) Inventeurs/Inventors:
LAVIE, ARNON, US;
NGUYEN, HIEN-ANH, US;
SCHALK, AMANDA, US

(74) Agent: RIDOUT & MAYBEE LLP

(54) Titre : VARIANTES TRONQUEES DE LA L-ASPARAGINASE DE COCHON D'INDE ET PROCEDES D'UTILISATION
(54) Title: TRUNCATED GUINEA PIG L-ASPARAGINASE VARIANTS AND METHODS OF USE

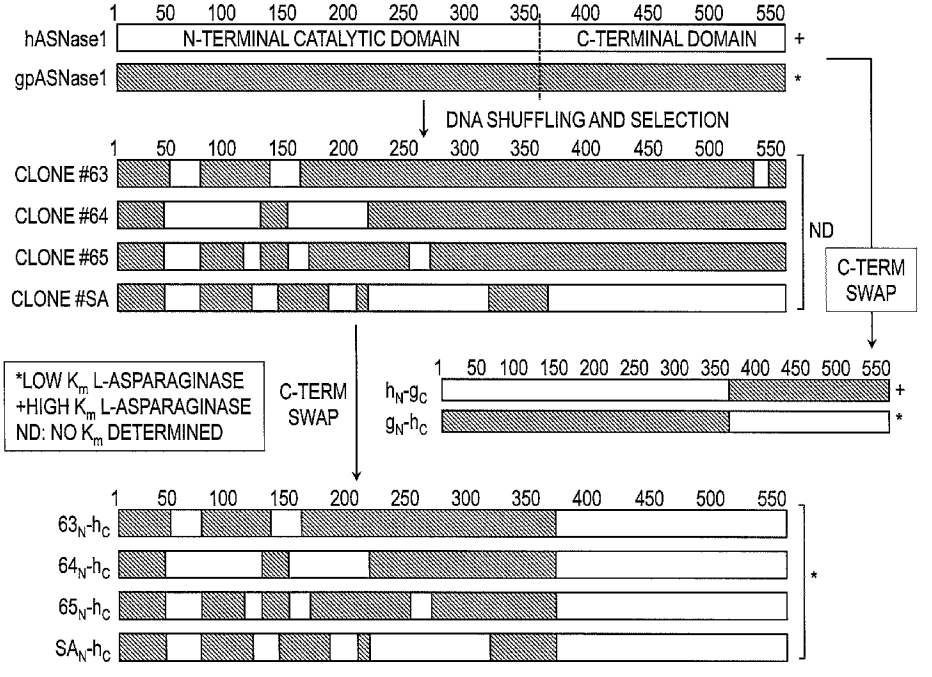

FIG. 1

(57) Abrégé/Abstract:
Variant guinea pig L-asparaginases which are truncated and humanized are described as are fusion proteins containing the L-asparaginase and use of the L- asparaginases in the treatment of cancers such as acute lymphoblastic leukemia and acute myeloid leukemia.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 February 2019 (14.02.2019)

WIPO | PCT

(10) International Publication Number
WO 2019/032952 A8

(51) International Patent Classification:
A61K 38/17 (2006.01) *C12N 9/82* (2006.01)

(21) International Application Number:
PCT/US2018/046196

(22) International Filing Date:
10 August 2018 (10.08.2018)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/544,411 11 August 2017 (11.08.2017) US
62/544,396 11 August 2017 (11.08.2017) US

(71) Applicant: **THE BOARD OF TRUSTEES OF THE UNIVERSITY OF ILLINOIS** [US/US]; 352 Henry Administration Building, 506 South Wright Street, Urbana, IL 61801 (US).

(72) Inventors: **LAVIE, Arnon**; 910 S. Michigan Avenue, Unit 1906, Chicago, IL 60605 (US). **NGUYEN, Hien-Anh**; 915 S. Lytle Street, Apartment 101, Chicago, IL 60607 (US). **SCHALK, Amanda**; 1368 W. Evergreen Avenue, Apartment 4F, Chicago, IL 60642 (US).

(74) Agent: **LICATA, Jane, Massey** et al.; Licata & Tyrrell P.C., 66 E. Main Street, Marlton, NJ 08053 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK,

(54) Title: TRUNCATED GUINEA PIG L-ASPARAGINASE VARIANTS AND METHODS OF USE

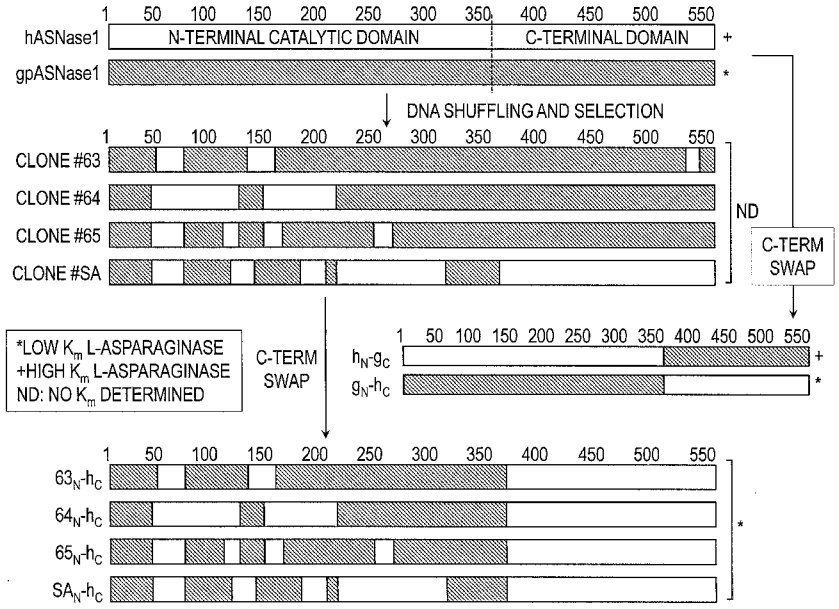

FIG. 1

(57) Abstract: Variant guinea pig L-asparaginases which are truncated and humanized are described as are fusion proteins containing the L-asparaginase and use of the L- asparaginases in the treatment of cancers such as acute lymphoblastic leukemia and acute myeloid leukemia.

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[Continued on next page]

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EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

(48) Date of publication of this corrected version:
25 July 2019 (25.07.2019)

(15) Information about Correction:
see Notice of 25 July 2019 (25.07.2019)

Truncated Guinea Pig L-Asparaginase Variants and Methods of Use

Introduction

[0001] This application claims the benefit of priority of U.S. Provisional Application Nos. 62/544,396, filed August 11, 2017, and 62/544,411, filed August 11, 2017, the contents of which are incorporated herein by reference in their entireties.

[0002] This invention was made with government support under Grant Number EB013685 awarded by the National Institutes of Health and Grant Number BX001919 awarded by the Department of Veteran Affairs. The government has certain rights in the invention.

Background

[0003] Certain cancers, such as acute lymphoblastic leukemia (ALL), are dependent upon scavenging Asn from blood, a factor most commonly attributed to the lack/low expression of asparagine synthetase in such cancers. Accordingly, L-asparaginases have been identified as critical components in the treatment of these cancers. All commercially available L-asparaginases have dual activities. The predominant one, the L-asparaginase activity, hydrolyzes the amino acid L-asparagine (Asn) into L-aspartic acid (Asp) and ammonia. The secondary activity is an L-glutaminase activity, which hydrolyze L-glutamine (Gln) to L-glutamic acid (Glu) and ammonia. For FDA-approved enzymes, *e.g.*, ELSPAR® (enzyme obtained from *Escherichia coli*) and ERWINAZE® (enzyme obtained from *Erwinia chrysanthemi*), the L-glutaminase activity ranges from 2 to 10% of the primary L-asparaginase activity. Whereas the importance of the L-asparaginase activity of

these drugs is accepted, there are conflicting reports as to the importance of L-glutaminase activity in killing leukemic cells. Moreover, the L-glutaminase activity has been associated with much of the clinical toxicity of L-asparaginases. In fact, toxic side effects of L-asparaginase treatment severely limit the use of this anti-cancer drug.

[0004] Another disadvantage to the use of bacterial enzymes as therapeutics is their immunogenicity, which can pose a direct threat to the patient due to hypersensitivity reactions, up to anaphylactic shock. Moreover, generated antibodies can inactivate and clear the enzyme drug, thus reducing or even eliminating its effectiveness. Methods have been developed to reduce these severe side effects, such as conjugation of the *E. coli* enzyme with polyethylene glycol (PEGylation) or deimmunization by mutation of residues 115, 118, 120, 123, 215, 219, 307 and 312 of the wild-type *E. coli* enzyme (WO 2012/075173 A2). However, alternative L-asparaginase preparations with reduced immunogenicity and reduced L-glutaminase activity are needed.

[0005] A guinea pig L-asparaginase has been purified and characterized from guinea pig serum (Zhang, et al. (1995) *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 112(4):607-12). The guinea pig enzyme, annotated as H0W0T5_CAVPO, exhibits antitumor activity, has a low K_m value for asparagine and lacks L-glutaminase activity (Schalk, et al. (2014) *J. Biol. Chem.* 289:33175-33186). See also EP 0726313 B1 and US 6,537,547.

[0006] Other approaches for treating cancer using L-asparaginase have included co-administration of the L-asparaginase with a TNF-related apoptosis-inducing ligand (TRAIL) agonist or TRAIL receptor agonist, *e.g.*, three

soluble TRAIL domains and an additional functional domain such as an antibody fragment. See US 2015/0337027; US 2009/0131317 and WO 2012/170640. In this respect, L-asparaginase has been shown to overcome resistance to both intrinsic apoptosis induced by the Bcl-2/Bcl-xL inhibitor, ABT263, and extrinsic apoptosis mediated by TRAIL in glioma cells that are resistant toward L-asparaginase single treatment (Karpel-Massler, et al. (2016) *Oncotarget* 7(23):33512-28).

Summary of the Invention

[0007] This invention provides a C-terminally truncated Guinea pig L-Asparaginase (GpA) variant sharing at least 85% sequence identity with residues 1 to 359 of SEQ ID NO:1. In some embodiments, the C-terminal truncation is at a position between 359 and 396 of SEQ ID NO:1. In certain embodiments, the C-terminal truncation is at position 369 of SEQ ID NO:1. In other embodiments, the truncated GpA variant further includes at least one amino acid substitution relative to SEQ ID NO:1, *e.g.*, at position 7, 10, 23, 25, 48, 49, 52, 53, 54, 57, 58, 59, 60, 62, 92, 98, 101, 102, 106, 108, 121, 122, 134, 147, 193, 198, 217, 233, 236, 250, 257, 281, 301, 311, 340, 344, 360, 362, 363, 364, 365, 366, 367, or 368, or a combination thereof. In particular embodiments, the at least one amino acid substitution relative to SEQ ID NO:1 is H10R, Q23R, K25E, K48E, Q52R, Q54R, P57S, D58E, H59D, A60T, A62V, D91A, D92E, K98Q, E101K, Q108H, S121F, G122A, H134Q, R147H, K193R, C198A, C198S, C198V, D217E, N233S, H236Q, S250A, Q288E, R301Q, E344D, L360P, T362S, A363V, D364E, L365E, H366R, Q367R, or S368P, or a combination thereof. In still other embodiments, the at least one amino acid substitution relative to SEQ ID NO:1 includes: (a) a cysteine residue at

position 49, 52, 225, 257, 281 or 340, or a combination thereof; or (b) a lysine residue at position 7, 53, 54, 57, 58, 98, 106, 233, 250, 257, 281, 311 or 340, or a combination thereof. Ideally, the variant has a catalytic activity equal to or greater than wild-type GpA. Optionally, the truncated GpA variant can further include a histidine tag, a SUMO tag, an albumin-binding domain, three tandem soluble domains of TRAIL (*e.g.*, residues 115-281 of human TRAIL), or a combination thereof.

[0008] Nucleic acid molecules, expression vectors, host cells and pharmaceutical compositions containing the truncated GpA variant or fusion proteins are also provided, as are methods of treating cancer by administering to a subject in need of treatment an effective amount of the truncated GpA variant or fusion proteins, optionally in combination with a stable form of TRAIL.

Brief Description of the Drawings

[0009] Fig. 1 is a schematic representation of the clones obtained from the DNA shuffling process and C-terminal domain swapping. hASNase1 sequences are open boxes, gpASNase1 is shaded boxes. Clones isolated from selection (#63, 64, 65 and SA) or generated by C-terminal swapping (63-hC, 64-hC, 65-hC and SA-hC) are a shuffle between hASNase1 (open boxes) and gpASNase1 (filled boxes) sequences.

[0010] Fig. 2A and 2B show an amino acid sequence alignment of hASNase1 (hASN), gpASNase1 (GpA), and select humanized clones. Underlined residues are residues derived from GpA.

Detailed Description of the Invention

[0011] L-asparaginase is a chemotherapy drug used to treat acute lymphoblastic leukemia (ALL). The main prerequisite

for clinical efficacy of L-asparaginases is micromolar K_m for asparagine to allow for complete depletion of this amino acid in the blood. Since currently approved L-asparaginases are of bacterial origin, immunogenicity is a challenge, which would be mitigated by a human enzyme. However, all human L-asparaginases have millimolar K_m for asparagine. A low K_m guinea pig L-asparaginase (gpASNase1) has been identified, which shares ~70% amino-acid identity with human L-asparaginase 1 (hASNase1). Like the human enzyme, gpASNase1 contains two domains; an N-terminal domain of ~360 residues where the L-asparaginase activity resides, and a C-terminal domain of ~200 residues of unknown function. To improve half-life, shorter yet stable versions of GpA were sought. Accordingly, GpA was C-terminally truncated to identify the shortest fragment of GpA that retained activity. This analysis indicated that the 359 amino acid residue N-terminal catalytic domain could be expressed with a SUMO tag and retained wild-type activity. However, upon removal of the SUMO tag, the protein became unstable. Extending the truncation to residue 369 provided both a stable and active enzyme.

[0012] To decrease the immunogenicity of the truncated GpA variant, two different approaches were taken to humanize the enzyme: DNA shuffling and structure-based mutation of surface residues. Humanization of GpA yielded variants sharing approximately 80% sequence identity with hASNase1, wild-type GpA activity, a low K_m value for asparagine, and no detectable L-glutaminase activity. Upon introduction of cysteine or lysine residues and conjugation to PEG, the truncated GpA variants maintained, and in some instanced, exhibited an increase in their L-asparaginase activity. In addition, modifications such as N- or C-terminal addition of a histidine tag, SUMO tag, and/or albumin binding domain

can increase *in vivo* circulation time and fusion of the truncated GpA variant to three tandem soluble domains of TRAIL can facilitate cell death by both providing the necessary signals for the intrinsic apoptotic cascade (L-asparaginase) and inducing the extrinsic apoptotic cascade (TRAIL).

[0013] Accordingly, this invention provides truncated GpA variants, and fusion proteins thereof, for use in the treatment of cancers such as lymphomas and leukemias, which are dependent upon the presence of an external supply of Asn. The improved safety of the instant L-asparaginases will provide benefit to current patient populations (*e.g.*, those with pediatric ALL), and extended use in other patient populations (*e.g.*, adult ALL, AML, and other cancers).

[0014] As is known in the art, L-asparaginases (L-asparagine aminohydrolase, E.C. 3.5.1.1) are amidases that hydrolyses the amide bond in Asn to Asp and ammonia (Kumar & Verma (2012) *Asian J. Biochem. Pharma Res.* 3:197-205). Wild-type guinea pig (*Cavia porcellus*) L-asparaginase, referred to herein as "gpASNase1" or "GpA," is a 565 amino acid residue protein available under Uniprot Accession No. H0W0T5_CAVPO and SEQ ID NO:1. GpA exhibits antitumor activity, has a low K_m value for asparagine and lacks L-glutaminase activity. However, wild-type GpA only shares approximately 70% sequence identity with hASNase1. Accordingly, this invention provides a truncated GpA, which in some embodiments is humanized to decrease immunogenicity. In particular, this invention provides a truncated GpA variant sharing at least 85% sequence identity with residues 1 to 359 of SEQ ID NO:1.

[0015] A "GpA variant" refers to a non-naturally occurring form of GpA that exhibits L-asparaginase activity, has a

low K_m for Asn, and lacks L-glutaminase activity. By comparison, a "wild-type" GpA refers to the typical form of the L-asparaginase when isolated from a naturally occurring source. A wild-type is that which is most frequently observed in a natural population and is thus arbitrarily designated the normal or wild-type form. Wild-type GpA has a reaction rate (k_{cat}) of approximately 39 s^{-1} and K_m for Asn of 58 μM (Schalk, et al. (2014) *J. Biol. Chem.* 289:33175-33186). A truncated GpA variant of this invention typically exhibits a k_{cat} of at least 75%, 80%, 85%, 90%, 95%, or 100% of wild-type *GpA* enzyme and exhibits a K_m for Asn of less than 250 μM, 200 μM, 150 μM, 100 μM, 80 μM or 60 μM.

[0016] At a minimum, a GpA variant is truncated. "Truncated GpA" refers to a GpA wherein all or a portion of the approximately 206 C-terminal amino acid residues have been removed. In certain embodiments, a truncated GpA protein retains the N-terminal 359 amino acid residue catalytic domain. Ideally, the full-length GpA (SEQ ID NO:1) is C-terminally truncated at a position between residue 359 and 396. In particular, the full-length GpA (SEQ ID NO:1) is C-terminally truncated at position 359, 367, 369, 374, 384, 392 or 396. In certain embodiments, a truncated GpA variant is C-terminally truncated at position 369. Exemplary truncated GpA variants are provided in SEQ ID NO:3 (GpA359), SEQ ID NO:4 (GpA367), SEQ ID NO:5 (GpA369), SEQ ID NO:6 (GpA374), SEQ ID NO:7 (GpA384), SEQ ID NO:8 (GpA392) and SEQ ID NO:9 (GpA396). In some embodiments, a C-terminally truncated GpA variant comprises or consists of SEQ ID NO:3. In certain embodiments, a C-terminally truncated GpA variant comprises or consists of SEQ ID NO:5.

[0017] According to other aspects of this invention, a GpA variant is truncated and includes at least one amino acid substitution or modification (*e.g.*, PEGylation or protein

fusion) that increases stability, increases sequence identity with hASNase1, and/or increases *in vivo* circulation time of the GpA as compared to a wild-type GpA enzyme. As demonstrated herein, amino acid residues located on the surface of GpA were mutated to either humanize GpA and/or provide a site suitable for PEGylation. Further, fusion of GpA to protein tags or trimeric TRAIL provides stability and/or enhanced tumor cell killing activity.

[0018] Accordingly, in some aspects, a GpA variant has at least one amino acid substitution relative to wild-type GpA (SEQ ID NO:1). In particular embodiments, the amino acid residue that is substituted is a surface residue. The term "surface residue" refers to a residue located on a surface of a protein. In contrast, a buried residue is a residue that is not located on the surface of a protein. A surface residue usually includes a hydrophilic side chain. Operationally, a surface residue can be identified computationally from a structural model of a protein as a residue that contacts a sphere of hydration rolled over the surface of the molecular structure. A surface residue also can be identified experimentally through the use of deuterium exchange studies, or accessibility to various labeling reagents such as, *e.g.*, hydrophilic alkylating agents. In particular embodiments, the amino acid substitution is not at one of the active site residues, *e.g.*, Thr19, Ser85, Ser86, Thr116, Asp117, Ala142, Lys188, Asn272, and Tyr308.

[0019] Surface residues of GpA that can be substituted to generate the GpA variant of this invention include, but are not limited to, amino acid residues at position 7, 10, 23, 25, 40, 48, 49, 52, 53, 54, 57, 58, 59, 60, 62, 92, 98, 101, 106, 108, 121, 122, 131, 132, 134, 147, 193, 198, 217, 221, 222, 223, 224, 225, 226, 233, 236, 250, 253, 257, 261,

281, 282, 283, 284, 301, 311, 340, 344, 345, 347, 352, 358, 359, 360, 362, 363, 364, 365, 366, 367, or 368 of SEQ ID NO:1. In some embodiments, a GpA variant includes at least one amino acid substitution. In other embodiments, a GpA variant includes about 1 to 50, 1 to 45, 1 to 40, 1 to 35, 1 to 30, 1 to 25, 1 to 20, 1 to 15, 1 to 10, 1 to 7, or 1 to 4 amino acid substitutions. In particular embodiments, a GpA variant includes an amino acid substitution at one or more of positions 7, 10, 23, 25, 48, 49, 52, 53, 54, 57, 58, 59, 60, 62, 92, 98, 101, 106, 108, 121, 122, 134, 147, 193, 198, 217, 233, 236, 250, 257, 281, 301, 311, 340, 344, 360, 362, 363, 364, 365, 366, 367 and 368 of SEQ ID NO:1.

[0020] As indicated herein, truncated GpA369 shares about 72% sequence identity with the N-terminal 371 amino acid residues of hASNase1. Ideally, the at least one amino acid substitution of GpA generates a GpA variant with increased amino acid sequence identity with hASNase1 compared to wild-type GpA. Accordingly, one aspect of this invention provides for the humanization of GpA. In accordance with this aspect of the invention, the truncated GpA variant preferably has an amino acid sequence identity with the N-terminal 371 amino acid residues of hASNase1 of at least 70%, 72%, 74%, 76%, 78%, 80%, 82% or 84%. Further, the truncated GpA variant shares at least 85%, 87%, 89%, 91%, 93%, 95%, 97% or 99% sequence identity with wild-type truncated GpA.

[0021] Preferably, humanization of truncated GpA is achieved by replacing one or more surface residues of wild-type GpA with the corresponding surface residues of hASNase1. In particular, humanization of truncated GpA is achieved by replacing wild-type GpA residue H10, Q23, K25, K48, Q52, Q54, D91, D92, K98, E101, Q108, S121, G122, H134, R147, K193, D217, N233, H236, S250, Q288, R301, E344, L360,

T362, A363, L365, H366, Q367, or S368, or any combination thereof, with the corresponding surface residue of hASNase1. Specifically, humanization of truncated GpA is achieved by making one or more of the following amino acid substitutions: H10R, Q23R, K25E, K48E, Q52R, Q54R, D91A, D92E, K98Q, E101K, Q108H, S121F, G122A, H134Q, R147H, K193R, D217E, N233S, H236Q, S250A, Q288E, R301Q, E344D, L360P, T362S, A363V, L365E, H366R, Q367R and/or S368P to wild-type GpA. Exemplary truncated and humanized GpA variants are provided in SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, SEQ ID NO:62, SEQ ID NO:64, SEQ ID NO:65 and SEQ ID NO:86.

[0022] PEGylation of L-asparaginases has been shown to increase in vivo circulation time. The term "PEGylated" or "PEGylation" refers to conjugation of an L-asparaginase with polyethylene glycol (PEG). "PEG" or "polyethylene glycol" refers to any water-soluble poly(ethylene glycol) or poly(ethylene oxide). The expression PEG thus comprises the structure $(CH_2CH_2O)_n$, wherein n is an integer from 2 to about 1000. A commonly used PEG is end-capped PEG, wherein one end of the PEG termini is end-capped with a relatively inactive group such as alkoxy, while the other end is a hydroxyl group that may be further modified by linker moieties. In one embodiment, the capping group is methoxy and the corresponding end-capped PEG is denoted mPEG. Hence, mPEG is $CH_3O(CH_2CH_2O)_n$, wherein n is an integer from 2 to about 1000. In another embodiment, the capping group is hydroxyl and the corresponding end-capped PEG is hydroxyPEG. "PEG" followed by a number (not being a subscript) indicates a PEG moiety with the approximate molecular weight equal the number multiplied by 1,000. Hence, "PEG40" or "PEG40K" is a PEG moiety having an

approximate molecular weight of 40 kDa. Examples of methods that may be used for determining PEG molecular weight include, without limitation, mass spectrometry, such as, for example, TOF-MS. PEG may be provided, for example, by NOF Corporation, Tokyo, Japan; Creative PEG-works, Winston Salem, NC; and Nanocs, Boston, MA.

[0023] In one embodiment, the PEG moiety may be attached by nucleophilic substitution (acylation) on N-terminal alpha-amino groups or on lysine residue(s) on the gamma-positions, *e.g.*, with OSu-activated esters. In another embodiment, the PEG moiety may be attached by reductive alkylation on amino groups present in the GpA protein using PEG-aldehyde reagents and a reducing agent, such as sodium cyanoborohydride. In another embodiment, the PEG moiety may be attached to the side-chain of an unpaired cysteine residue in a Michael addition reaction using for example PEG maleimide reagents. Other PEGylation methods include, but are not limited to, bridging PEGylation, transglutaminase PEGylation, glycoPEGylation, PEGylation using genetic engineering, releasable linkers PEGylation. For a review on PEGylation methods, see Pasut & Veronese (2012) *J. Contr. Rel.* 161:461-472; and Roberts, et al. (2012) *Adv. Drug Del. Rev.* 64:116-127. In one embodiment, the PEG moieties are attached to side chain(s) of lysine or cysteine residue(s).

[0024] "Linker" refers to a chemical moiety which connects an -HN- group of the GpA protein with the -O- group of a PEG moiety. In a preferred embodiment, the linker does not have any adverse influence on the activity of GpA. The linker is typically a derivative of a carboxylic acid, wherein the carboxylic acid functionality is used for attachment to the GpA protein via an amide bond. Examples of linkers include, but are not limited to, an acetic acid

moiety with the linking motif: CH_2CO, a propionic acid moiety with the linking motif: CH_2CH_2CO or $CHCH_3CO$, a butyric acid moiety with the linking motif: $CH_2CH_2CH_2CO$ or CH_2CHCH_3CO, a CO group, N-(aminocarbonyl)succinimide derivatives (such as, for example, N-(N-propylpropanamide) succinimide, N-(N-propylhexanamide)succinimide and N-(N-ethylpropanamide)succinimide), pentanoic acid ($(CH_2)_5CO$), α-methyl butanoic acid ($CH_2CH_2CH(CH_3)CO$), succinic acid ($CO(CH_2)_2CO$), glutaric acid ($CO(CH_2)_3CO$), succinamide derivatives (such as, for example, $(CH_2)_2NHCO(CH_2)_2CO$), glutaramide derivatives (such as, for example, $(CH_2)_3NHCO(CH_2)_3CO$ and $(CH_2)_2NHCO(CH_2)_3CO$). Exemplary PEG molecules of use in this invention include, but are not limited to, methoxy PEG succinimidyl carbonate (mPEG-SC), mPEG-succinimidyl carboxy methyl ester (mPEG-SCM) and mPEG-succinamide succinimidyl ester (mPEG-SAS).

[0025] Methods of PEGylation that can be used to PEGylate an L-asparaginase of the invention are provided, for example, in US 4,179,337, US 5,766,897, US 2002/0065397, and US 2009/0054590. Ideally, a PEGylated GpA variant is produced by reacting PEG with a truncated GpA variant in a ratio of PEG:GpA of about 20:1 to 100:1, or more particularly 20:1 to 50:1, or most particularly 20:1, depending on the PEG size. In some aspects, the GpA variant has at least one cysteine residue, the PEG is a maleimide PEG, the reaction is carried out in the absence of glycine or aspartic acid, and the reaction product is treated with beta-mercaptoethanol. In other aspects, the GpA variant has at least one lysine residue, the PEG is mPEG-SC, mPEG-SCM or mPEG-SAS, and the reaction is carried out in the absence of DTT and glycerol.

[0026] As disclosed herein, an L-asparaginase can be PEGylated by site-specific PEGylation at 1 to 6 cysteine or

10 to 30 lysine residues introduced into the amino acid sequence of the L-asparaginase by amino acid substitution. See Examples 4 and 5. In particular, truncated GpA, or a humanized and truncated GpA may be PEGylated by introduction of: (a) a cysteine residue at position 49, 52, 225, 257, 281 and/or 340; or (b) a lysine residue at position 7, 53, 54, 57, 58, 98, 106, 233, 250, 257, 281, 311 and/or 340. In addition to introduction of cysteine or lysine residues at specific locations, endogenous cysteine or lysine residues may be mutated (*i.e.*, substituted with another residue) to more evenly distribute the PEGylation sites over the length of the protein. In this respect, certain embodiments include replacing residue Cys198 with Ala, Val or Ser and/or replacing residue Lys223 with Asp. In one embodiment, Cys198 is replaced with Ala and the L-asparaginase derivative is PEGylated at Cys79. In other embodiments, Cys198 is replaced with Ala and one or more of the amino acid residues at positions 49, 225 and 340 are replaced with cysteine. Exemplary truncated GpA variants suitable for PEGylation are provided in SEQ ID NO:51 (GpA369-C198A), SEQ ID NO:52 (GpA369-C198S), SEQ ID NO:53 (GpA369-C198V), SEQ ID NO:54 (GpA369-C198A+K225C), SEQ ID NO:55 (GpA369-C198A+K225C+E340C), SEQ ID NO:56 (GpA369-C198A+K225C+E340C+E49C), SEQ ID NO:57 (GpA369-C198A+K225C+E340C+E49C+Q257C), SEQ ID NO:58 (GpA369(hum)-Group1+2+3-C198A), SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:64, SEQ ID NO:65 and SEQ ID NO:86.

[0027] To increase the *in vivo* circulation time of truncated GpA, this invention also provides the variants, in particular truncated GpA variants, having a histidine tag (His), a SUMO tag (SUMO), an albumin-binding domain (ABD), or a combination thereof. The truncated GpA variant

can include the histidine tag, SUMO tag and/or an albumin-binding domain at its C-terminus, N-terminus, and/or an internal location within the GpA sequence. Ideally, when a His tag, SUMO tag or ABD is inserted within the GpA sequence (*i.e.*, not at the N- or C-terminus), the insertion is in one or more flexible loops of the GpA protein. By way of illustration, the truncated GpA variant can have the N- to C-terminal structure of SUMO-Asparaginase-ABD, His-SUMO-ABD-Asparaginase, His-SUMO-Asparaginase-ABD, His-SUMO-Asparaginase, His-ABD-Asparaginase, ABD-Asparaginase, ABD-SUMO-Asparaginase, Asparaginase$^{1-225}$-ABD-Asparaginase$^{226-369}$, Asparaginase$^{1-340}$-ABD-Asparaginase$^{341-369}$, and the like. Nucleic acids encoding the His, SUMO or ABD can be inserted in-frame either 5′ or 3′ of the nucleic acids encoding the truncated GpA variant thereby creating a fusion protein.

[0028] Ideally, the inclusion of one or more of a histidine tag, a SUMO tag, an albumin-binding domain, or a combination thereof can significantly increase the *in vivo* circulation time of an L-asparaginase. Alternatively stated, the variant has a longer t½ than wild-type L-asparaginase administered at an equivalent protein dose. As used herein, the term "t½" or "half-life" refers to the time that would be required for the concentration of a truncated GpA variant or fusion protein thereof to fall by half *in vitro* or *in vivo*, for example, after injection in a mammal. Given that efficacy of L-asparaginases is related to the *in vivo* half-life of the drug, the truncated GpA variants of this invention are particularly useful in the treatment of cancers, such as leukemias and lymphomas.

[0029] As used herein, a "histidine tag" refers to an amino acid motif composed of at least one histidine (His) residue and preferably at least 6 His residues. The histidine tag includes a polyhistidine of 6 (hexa histidine tag, 6xHis

tag, or His_6 tag), 7, 8, 9, 10, or up to 20 histidine residues.

[0030] A "SUMO tag" refers to the fusion of a SUMO (small ubiquitin-related modifier) protein to a protein of interest to enhance the solubility/stability of the protein of interest. The inclusion of a SUMO tag can be achieved using known expression systems such as the CHAMPION pET SUMO expression system (Invitrogen), the EXPRESSO T7 SUMO cloning and expression system (Lucigen), or pET His6 SUMO TEV LIC cloning vector (Addgene). In addition to SUMO tag, it is contemplated that other Ubl proteins can be used including, but not limited to, Ub, Rub1, Hub1, ISG15, Ubi-L (MNSF), FAT10, Apg12, Apg8 and Urm1 (Larsen & Wang (2002) *J. Proteome Res.* 1(5):411-9). See also US 7,655,413, incorporated herein by reference in its entirety. Exemplary SUMO tags are set forth in SEQ ID NO:66, SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:69 and SEQ ID NO:70.

[0031] An "albumin-binding domain" or "ABD" refers to a polypeptide that binds albumin *in vivo* or *in vitro* and enhances the serum half-life and biodistribution of a therapeutic agent. Albumin may be derived from any animal species, for example human, monkey, or rodent. Albumin-binding domains are described, for example, in US 6,267,964, WO 1991/19741, WO 2005/097202, WO 2001/45746, WO 2013/043071 and US 2004/0001827. Further, US 9,156,887 discloses non-natural albumin-binding domains, which may be used in this invention. In some embodiments, the albumin-binding domain has the amino acid sequence set forth in SEQ ID NO:71, SEQ ID NO:72 or SEQ ID NO:73.

[0032] Enhanced cytotoxic activity of the truncated GpA variants disclosed herein can be achieved by conjugating or fusing the GpA to three tandem soluble domains of TRAIL or co-administering the GpA with a stable form of TRAIL. The

resulting fusion protein can provide at least a 20-, 25-, 30-, 35-, or 40-fold decrease in IC_{50} value compared to a truncated GpA variant lacking the three tandem soluble domains of TRAIL. Accordingly, a truncated GpA variant fused to three tandem soluble domains of TRAIL is particularly useful in the treatment of cancer, in particular, L-asparaginase-insensitive cancers.

[0033] A "fusion protein" refers to a chimeric protein containing proteins or protein fragments (*e.g.*, GpA variants) operably linked in a non-native way. In accordance with the fusion protein of this invention, three tandem soluble domains of TRAIL ($TRAIL_{trimer}$) are fused, in-frame, with a truncated GpA variant. Ideally, a glycine or serine residue is inserted between each TRAIL repeat to facilitate folding of the TRAIL trimer (see, *e.g.*, SEQ ID NO:87 and SEQ ID NO:88). Optionally, the TRAIL trimer and Asparaginase components can be separated by a linker as set forth in SEQ ID NO:81, SEQ ID NO:82, SEQ ID NO:83, or SEQ ID NO:84. The fusion protein can include the TRAIL trimer fused to the C-terminus (GpA-$TRAIL_{trimer}$; *e.g.*, SEQ ID NO:89 and SEQ ID NO:90) or N-terminus ($TRAIL_{trimer}$-GpA; *e.g.*, SEQ ID NO:91 and SEQ ID NO:92) of GpA, where the GpA component can be any the previously described variants. When further used in combination with a tag or modification, the fusion protein can have the structure of: SUMO-$TRAIL_{trimer}$-GpA-ABD, His-SUMO-ABD-$TRAIL_{trimer}$-GpA, His-SUMO-$TRAIL_{trimer}$-GpA-ABD, His-SUMO-GpA-$TRAIL_{trimer}$, His-ABD-$TRAIL_{trimer}$-GpA, ABD-GpA-$TRAIL_{trimer}$, ABD-SUMO-$TRAIL_{trimer}$-GpA, and the like. In addition to being inserted at the N- or C-terminus, the ABD peptide can be engrafted in one or more flexible loops of GpA variants. In one example, the flexible loop spans residues 215 to 228. In another example, the flexible loop spans residues 337 to 342 of GpA. See, *e.g.*, SEQ ID NO:94

and SEQ ID NO:95. Nucleic acids encoding TRAIL in particular $TRAIL_{trimer}$ can be inserted in-frame either 5′ or 3′ of the nucleic acids encoding the L-asparaginase thereby creating the fusion protein.

[0034] Preferably, the soluble domains of TRAIL are derived from a mammalian, particularly human TRAIL including allelic variants and/or derivatives thereof. The soluble domains include the extracellular portion of TRAIL including the receptor binding domain without membrane localized domains. Like other proteins of the TNF superfamily, TRAIL is anchored to the membrane via an N-terminal portion of 15-30 amino acids, the so-called stalk-region. The stalk region contributes to trimerization and provides a certain distance to the cell membrane. However, the stalk region is not part of the receptor binding domain (RBD). Accordingly, it is preferred that the soluble TRAIL domains include the receptor binding domain of the TRAIL lacking any amino acids from the stalk region (see US 2015/0337027).

[0035] The soluble TRAIL domain may be derived from human TRAIL. Preferably, the soluble TRAIL domains are derived from human TRAIL, particularly starting from amino acid residues 115-122, and include amino acid residues 115-281, 120-281, 121-281 or 122-281 of human TRAIL. In certain embodiments, each of the soluble domains of $TRAIL_{trimer}$ are composed of residues 115-281 of human TRAIL. Residues 115-281 of human TRAIL are set forth herein in SEQ ID NO:80. To facilitate correct folding, a glycine or serine residue may be inserted between each TRAIL repeat. See, *e.g.*, SEQ ID NO:87 and SEQ ID NO:88.

[0036] Derivatives and variants of the death receptor binding TRAIL domains are all contemplated and can be made by altering their amino acid sequences by substitutions,

additions, and/or deletions/truncations or by introducing chemical modifications that result in functionally equivalent polypeptides. It will be understood by one of ordinary skill in the art that certain amino acids in a sequence of any polypeptide may be substituted for other amino acids without adversely affecting the activity of the polypeptides.

[0037] The TRAIL domains disclosed herein include substitutions of one or more of the amino acids in the disclosed sequences. A skilled artisan will be able to determine using well-known techniques suitable sequence variants of the peptides set forth herein. In certain embodiments, one skilled in the art may identify suitable areas of the molecule that may be changed without destroying activity by targeting regions not believed to be important for activity. In other embodiments, the skilled artisan can identify residues and portions of the molecules that are conserved among similar polypeptides. In further embodiments, even amino acid residues important for biological activity or for structure may be subject to conservative amino acid substitutions without destroying the biological activity thereof or without adversely affecting the peptide structure.

[0038] The three soluble domains of TRAIL are preferably linked to one another via peptide linking group composed of one to eight amino acid residues. Likewise, it is preferable that $TRAIL_{trimer}$ is linked to the L-asparaginase via a peptide linking group composed of one to twenty amino acid residues. The term "peptide linking group" or "linker" is meant to refer to a peptide moiety that acts as a molecular bridge to operably link two different molecules together. Desirably, the linkers of this invention are composed of glycine or serine, or a combination thereof. In

particular embodiments, each of the soluble domains of $TRAIL_{trimer}$ are preferably linked to one another by a single glycine or single serine residue. An exemplary $TRAIL_{trimer}$ includes a single serine residue between the three soluble TRAIL domains (SEQ ID NO:88). Regarding the peptide linking group located between the $TRAIL_{trimer}$ and L-asparaginase, it is desirable that this linker is a flexible linker. The flexible linker preferably has a length of one to 20 amino acid residues, particularly a length of 6, 9, 12, 15 or 18 amino residues. The flexible linker is preferably a glycine/serine linker, *i.e.*, a peptide linker composed primarily of the amino acids glycine and serine. In a particular embodiment, the linker between the $TRAIL_{trimer}$ and L-asparaginase is a $(GGGS)_n$ linker (SEQ ID NO:81), wherein n is 1 to 4, or a permutation thereof including, *e.g.*, $GGGS(GGGGS)_n$ (SEQ ID NO:82), herein n is 1 to 4. In certain embodiments, the linker between the $TRAIL_{trimer}$ and L-asparaginase has the amino acid sequence set forth in SEQ ID NO:83. In other embodiments, the linker between the $TRAIL_{trimer}$ and L-asparaginase has the amino acid sequence set forth in SEQ ID NO:84. An example of a $TRAIL_{trimer}$-GpA variant fusion protein has an N-terminal $TRAIL_{trimer}$ including a single glycine or serine residue between the three soluble TRAIL domains and a glycine/serine linker between the $TRAIL_{trimer}$ and truncated GpA variant. See, *e.g.*, SEQ ID NO:89, SEQ ID NO:90, SEQ ID NO:91 and SEQ ID NO:92.

[0039] A stable form of TRAIL is intended to refer to a form of TRAIL that promotes trimerization. In particular, to promote trimerization of TRAIL, the FOLDON sequence (GYIPEAPRDGQAYVRKDGEWVLLSTFL; SEQ ID NO:85), a small trimerization domain, has been shown to maintain TRAIL stability and biological activity at 37°C for at least 48 hours (Kouno, et al. (2013) *J. Invest. Dermatol.*

133(9):2212-2220). Accordingly, the FOLDON peptide was inserted between a His-SUMO tag and the N-terminus of TRAIL to result in a His-SUMO-FOLDON-TRAIL fusion protein. The fusion protein was expressed and a yield of >10 mg/L of the fusion protein was obtained in the bacterial culture. The protein was very stable, showing that the inclusion of FOLDON results in a stable form of TRAIL. Accordingly, a stable form of TRAIL can be co-administered and/or combined in a pharmaceutical composition with a truncated GpA variant to enhance cytotoxic activity of the GpA.

[0040] The truncated GpA variants and fusion proteins disclosed herein can be readily prepared by conventional recombinant protein techniques, wherein recombinant host cells are transformed or transduced with an expression construct or vector harboring a nucleic acid molecule encoding the variant or fusion protein, the recombinant host cells are grown under suitable conditions to provide for expression of the variant or fusion protein, and the variant or fusion protein is subsequently isolated and optionally purified. Accordingly, this invention also provides a nucleic acid molecule encoding a truncated GpA variant or a fusion protein thereof, as well as an expression cassette and/or expression vector containing the same. Ideally, the expression cassette and expression vector contain the necessary regulatory sequences (*e.g.*, promoter, terminator, and the like) to facilitate expression in the host cell of interest. Host cells including a nucleic acid molecule encoding a truncated GpA variant or fusion protein are also included within the scope of this invention. Host cells can include eukaryotic cells (*e.g.*, mammalian, fungal or yeast cells) or prokaryotic cells (*e.g.*, *E. coli*).

[0041] Once produced and isolated/purified, the truncated GpA variant and/or fusion protein of the invention can be used as is or formulated in a pharmaceutical composition containing a pharmaceutically acceptable excipient. Pharmaceutical compositions provided herein can be specially formulated for intravenous administration in solid or liquid form or for intravenous injection. Optimal pharmaceutical compositions can be determined by one skilled in the art depending upon, for example, the intended route of administration, delivery format and desired dosage. See, for example, Remington's Pharmaceutical Sciences (19th edition, 1995).

[0042] The truncated GpA variant and/or fusion protein can be incorporated in a conventional systemic dosage form, such as an injectable formulation. The dosage forms may also include the necessary physiologically acceptable carrier material, excipient, lubricant, buffer, surfactant, antibacterial, bulking agent (such as mannitol), antioxidants (ascorbic acid or sodium bisulfite) or the like.

[0043] The primary carrier or excipient in a pharmaceutical composition may be either aqueous or non-aqueous in nature. For example, a suitable carrier or excipient may be water for injection, physiological saline solution or artificial cerebrospinal fluid, possibly supplemented with other materials common in compositions for parenteral administration. Neutral-buffered saline or saline mixed with serum albumin are further exemplary vehicles. Pharmaceutical compositions can include Tris buffer of about pH 7.0-8.5, or acetate buffer of about pH 4.0-5.5, which may further include sorbitol or a suitable substitute therefor. Pharmaceutical compositions of the invention may be prepared for storage by mixing the selected composition

having the desired degree of purity with optional formulation agents (Remington's Pharmaceutical Sciences, *Id.*) in the form of a lyophilized cake or an aqueous solution. Further, the truncated GpA variant or fusion protein of the invention may be formulated as a lyophilizate using appropriate excipients such as sucrose or glycine.

[0044] Administration routes for the truncated GpA variant, fusion protein, or pharmaceutical compositions of the invention include injection by intravenous, intraperitoneal, intracerebral (intra-parenchymal), intracerebroventricular, intramuscular, intra-ocular, intraarterial, intraportal, or intralesional routes; by sustained release systems or by implantation devices. Compositions may be administered by bolus injection or continuously by infusion, or by implantation device. Compositions also can be administered locally via implantation of a membrane, sponge or another appropriate material onto which the desired molecule has been absorbed or encapsulated. Where an implantation device is used, the device may be implanted into any suitable tissue or organ, and delivery of the desired molecule may be via diffusion, timed-release bolus, or continuous administration.

[0045] The compositions of the invention can be delivered parenterally. When parenteral administration is contemplated, the therapeutic compositions for use in this invention may be in the form of a pyrogen-free, parenterally acceptable aqueous solution comprising the desired compound identified in a screening method of the invention in a pharmaceutically acceptable vehicle. A particularly suitable vehicle for parenteral injection is sterile distilled water in which the compound identified in a screening method of the invention is formulated as a

sterile, isotonic solution, appropriately preserved. Preparation can involve the formulation of the desired molecule with an agent, such as injectable microspheres, bio-erodible particles, polymeric compounds (such as polylactic acid or polyglycolic acid), beads or liposomes, that may provide controlled or sustained release of the product which may then be delivered via a depot injection. Formulation with hyaluronic acid has the effect of promoting sustained duration in the circulation. Implantable drug delivery devices may be used to introduce the desired molecule.

[0046] The compositions may also be formulated for inhalation. In these embodiments, the truncated GpA variant or fusion protein is formulated as a dry powder for inhalation, or inhalation solutions may also be formulated with a propellant for aerosol delivery, such as by nebulization. Pulmonary administration is further described in WO 1994/020069, which describes pulmonary delivery of chemically modified proteins.

[0047] The compositions of the invention can be delivered through the digestive tract, such as orally. The preparation of such pharmaceutically acceptable compositions is within the skill of the art. The truncated GpA variant or fusion protein of the invention that is administered in this fashion may be formulated with or without those carriers customarily used in the compounding of solid dosage forms such as tablets and capsules. A capsule may be designed to release the active portion of the formulation at the point in the gastrointestinal tract when bioavailability is maximized and pre-systemic degradation is minimized. Additional agents can be included to facilitate absorption of the peptides of the invention disclosed herein. Diluents, flavorings, low melting point

waxes, vegetable oils, lubricants, suspending agents, tablet disintegrating agents, and binders may also be employed.

[0048] These compositions may also contain adjuvants such as preservative, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms can be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid and the like. It may also be desirable to include isotonic agents such as sugars, sodium chloride and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

[0049] The truncated GpA variant and/or fusion protein of the invention find particular use in the treatment of a disease or condition treatable by depletion of asparagine. Accordingly, this invention also provides methods for treating a disease, in particular cancer, by administering to a subject in need of treatment and effective amount of a truncated GpA variant or fusion protein. An "effective amount" is used herein to refer to an amount of an active ingredient sufficient to achieve the intended purpose of (a) reducing the severity of the disorder; (b) limiting or preventing development of symptoms characteristic of the disorder(s) being treated; (c) inhibiting worsening of symptoms characteristic of the disorder(s) being treated; (d) limiting or preventing recurrence of the disorder(s) in patients that have previously had the disorder(s); (e) limiting or preventing recurrence of symptoms in patients that were previously symptomatic for the disorder(s); (f) reduction of mortality after occurrence of a disease or a disorder; (g) healing; and (h) prophylaxis of a disease.

The effective amount in each individual case may be determined empirically by a skilled artisan according to established methods in the art. As used in the context of the invention, "administering" includes *in vivo* administration to an individual as well as administration directly to cells or tissue *in vitro* or *ex vivo*. An effective amount of a truncated GpA variant or fusion protein is generally that which can induce apoptosis and reduce circulating L-asparagine in cancer cells or a tumor of the subject. A clinician may titer the dosage or route of administration to obtain the optimal therapeutic effect.

[0050] In certain embodiments, the truncated GpA variant or fusion protein is useful in the treatment or the manufacture of a medicament for use in the treatment of cancers such as acute lymphoblastic Leukemia (ALL) in both adults and children, as well as other conditions where asparagine depletion is expected to have a useful effect. Such conditions include, but are not limited to, malignancies or cancers, including but not limited to hematalogic malignancies, non-Hodgkin's lymphoma, NK lymphoma, pancreatic cancer, ovarian cancer, Hodgkin's disease, acute myelocytic Leukemia, acute myelomonocytic Leukemia, chronic lymphocytic Leukemia, lymphosarcoma, reticulosarcoma, and melanosarcoma. Representative non-malignant hematologic diseases 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). Non-hematologic diseases associated with asparagine dependence include autoimmune diseases, for example rheumatoid arthritis, SLE, autoimmune, collagen vascular diseases, AIDS, etc. Other autoimmune diseases include osteo-arthritis, Issac's syndrome, 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), primary billiary cirrhosis, chronic active hepatitis, glomerulonephritis, myasthenia gravis, pemphigus vulgaris, and Graves' disease. In particular embodiments, the truncated GpA variant or fusion protein is used in the treatment of non-Hodgkin's lymphoma, leukemia, acute lymphoblastic leukemia, acute myeloid leukemia, B cell lymphoma, Burkitt's lymphoma, chronic myelocytic leukemia, chronic lymphocytic leukemia, and hairy cell leukemia.

[0051] Cells suspected of causing disease can be tested for asparagine dependence in any suitable *in vitro* or *in vivo* assay, *e.g.*, an *in vitro* assay wherein the growth medium lacks asparagine. Thus, in one aspect, the invention is directed to a method of treating a disease treatable in a patient, the method comprising administering to the patient an effective amount of truncated GpA variant or fusion protein of the invention. In a specific embodiment, the disease is ALL. In a particular embodiment, the truncated GpA variant or fusion protein used in the treatment of a disease treatable by asparagine depletion includes a truncated GpA variant from guinea pig.

[0052] The truncated GpA variant or fusion protein can be administered on a schedule ranging from about 3-times 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. As an example, patients with ALL will be

administered the truncated GpA variant or fusion protein of the invention as a component of multi-agent chemotherapy during 3 chemotherapy phases including induction, consolidation or intensification, and maintenance. In a specific example, the truncated GpA variant or fusion protein is not administered with an asparagine synthetase inhibitor (*e.g.*, see WO 2007/103290). In another specific example, the truncated GpA variant or fusion protein is not administered with an asparagine synthetase inhibitor but is administered with other chemotherapy drugs. The truncated GpA variant or fusion protein can be administered before, after, or simultaneously with other compounds as part of a multi-agent chemotherapy regimen.

[0053] In a specific embodiment, the method involves administering a truncated GpA variant or fusion protein of the invention at an amount of about 1 U/kg to about 1000 U/kg. In a more specific embodiment, the truncated GpA variant or fusion protein is administered at an amount selected from the group consisting of about 20, 50, 60, 70, 100, 200, 300, 400, 500 and 600 U/kg. In another specific embodiment, the truncated GpA variant or fusion protein is administered at a dose ranging from about 1000 IU/m^2 to about 20000 IU/m^2 (*e.g.*, 1000 IU/m^2, 2000 IU/m^2, 3000 IU/m^2, 4000 IU/m^2, 5000 IU/m^2, 6000 IU/m^2, 7000 IU/m^2, 8000 IU/m^2, 9000 IU/m^2, 10000 IU/m^2, 11000 IU/m^2, 12000 IU/m^2, 13000 IU/m^2, 14000 IU/m^2, 15000 IU/m^2, 16000 IU/m^2, 17000 IU/m^2, 18000 IU/m^2, 19000 IU/m^2, or 20000 IU/m^2). In another specific embodiment, the truncated GpA variant or fusion protein is administered at a dose that depletes Asn to undetectable levels for a period of about 3 days to about 10 days (*e.g.*, 3, 4, 5, 6, 7, 8, 9, or 10 days) for a single dose. In another embodiment, the method involves administering a truncated GpA variant or fusion protein of

the invention that has a longer *in vivo* circulating half-life after a single dose compared to the wild-type L-asparaginase.

[0054] The following non-limiting examples are provided to further illustrate the present invention.

Example 1: Generation of Active, C-Terminally Truncated GpA

[0055] Mammalian L-asparaginases such as human L-asparaginase (hASNase1; UNIPROT entry Q86U10; SEQ ID NO:2) and the guinea pig L-asparaginase (gpASNase1 or GpA; UNIPROT entry H0W0T5; SEQ ID NO:1) contain two domains; an N-terminal domain of ~360 residues where the L-asparaginase activity resides, and a C-terminal domain of ~200 residues of unknown function. By comparison, the clinically relevant bacterial L-asparaginases from *E. coli* and *Erwinia chrysanthemi* are about 350 amino acid residues in length and do not contain such a C-terminal domain.

[0056] One of the challenges of injectable biologics therapeutics comes from the short half-lives resulting in poor bio-availability. Clearance mostly results from proteolysis, renal filtration or from neutralization by the immune system. Larger molecules of foreign sequence have more chance to be detected by the immune system and get quickly cleared from the system. Identifying shorter yet stable versions of GpA was expected to provide a better therapeutic, especially in the context where the C-terminal domain of the full-length protein was of unknown function.

[0057] To assess the function of the C-terminal residues of GpA, C-terminally truncated GpA variants were constructed and analyzed for stability and full L-asparaginase activity. Sequence alignment of hASNase1, GpA, *E. coli* L-asparaginase, and *Erwinia* L-asparaginase enzymes and crystal structure analysis (indicated that catalytic domain

ends at residue 359. A construct composed of residues 1-359 of GpA (SEQ ID NO:3) was recombinantly expressed, but the purified C-truncated enzyme was unstable. Therefore, additional C-terminally truncated GpA constructs were prepared (Table 1) fused to a SUMO tag.

TABLE 1

GpA	Residues*	SEQ ID NO:
Full-length GpA	1-565	1
GpA359	1-359	3
GpA367	1-367	4
GpA369	1-369	5
GpA374	1-374	6
GpA384	1-384	7
GpA392	1-392	8
GpA396	1-396	9

*Relative to SEQ ID NO:1.

[0058] Recombinant overexpression of these C-terminally truncated GpA constructs indicated that all proteins were well overexpressed and purified to >95% of purity (measured by SDS-PAGE). While GpA367 precipitated quickly in a matter of minutes after the stability SUMO tag was cut off, the longer variants showed excellent stability and activity, which was comparable to the full-length protein. Notably, longer constructs carrying an extra Cys388 tended to form an unspecific disulfide bond, altering the tetrameric form necessary for L-asparaginase activity. While a C-terminally truncated GpA composed of at least residues 1-369 yields a stable GpA enzyme with L-asparaginase activity, as demonstrated herein the stability of a C-terminally truncated GpA composed of residues 1-359 or 1-367 can be stabilized by fusion with a heterologous peptide/protein (*e.g.*, a SUMO tag or albumin) or chemical modification (*e.g.*, PEGylation).

Example 2: Humanized GpA Variants Produced by Directed Evolution

[0059] *Overview.* Directed evolution, or the process to mimic natural evolutionary processes in the laboratory, is widely used to improve enzyme properties. See, *e.g.*, Dalby (2011) *Curr. Opin. Struct. Biol.* 21:473-480; Goldsmith & Tawfik (2012) *Curr. Opin. Struct. Biol.* 22:406-412; Labrou (2010) *Curr. Protein Pept. Sci.* 11:91-100; Wang & Zhao (2012) *Bioresour. Technol.* 115:117-125. Given the advances in library generation, screening techniques and foremost a better understanding of mechanisms of natural protein evolution, large improvements in the evolved catalytic activity (relative to the starting point, and in absolute k_{cat}/K_m values) have been obtained (Fasan, et al. (2008) *J. Mol. Biol.* 383:1069-80; Bar-Even, et al. (2011) *Biochemistry (Mosc.)* 50:4402-10).

[0060] To overcome immunogenicity, a human-like L-asparaginase with kinetic properties similar to that of the type II *E. coli* L-asparaginase was generated via a directed evolution approach. Using DNA family shuffling, several clones with high sequence identity to hASNase1 but with the low K_m properties of the gpASNase1 were identified.

[0061] *Strains.* Chromosomal gene deletions were performed using the λ-red recombinase system (Datsenko & Wanner (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97:6640-6645). The tyrosine aminotransferase gene *tyrB*, the aspartate aminotransferase gene *aspC* and the L-asparaginase genes *ansA, ansB, iaaA* were deleted from the chromosome of *E. coli* BW25113 F^-, DE(araD-araB)567, lacZ4787(del)::rrnB-3, LAM-, rph-1, DE(rhaD-rhaB)568, hsdR514 resulting in *E. coli* BW5Δ. Briefly, a primer pair was used to amplify the gene replaced by the kanamycin resistance cassette from the appropriate keio strain according to known methods

(Datsenko & Wanner (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97:6640-6645). Subsequently, the linear PCR product was used to replace the entire open reading frame of the targeted gene on the BW25113 chromosome. Colonies containing the correct gene deletions were transformed with the FLP recombinase plasmid pCP20 to remove the kanamycin resistance marker, and the pCP20 was then cured from the resulting strain. The strain *E. coli* BW2Δ was obtained following the same process and after the deletion of the genes *ansA* and *ansB*.

[0062] *Cloning of hASNase1 and gpASNase1 into BW2Δ and BW5Δ*. The gene encoding the codon-optimized sequence of the hASNase1 was amplified using the primers NdeI-hA_F1 and hA-BamHI_R573 and the codon-optimized sequence of gpASNase1 with the primers NdeI-gpA_F1 and gpA-BamHI_R565 (Table 2). After a *Bam*HI/*Nde*I digestion, the PCR products were inserted into the pBAD vector. The resulting vectors were subsequently used to transform the strains BW5Δ and BW2Δ, resulting in the strains BW5Δ pBAD_hASNase1, BW5Δ pBAD_gpASNase1, BW2Δ pBAD_hASNase1 and BW2Δ pBAD_gpASNase1. BW5Δ and BW2Δ were also transformed with the empty pBAD vector to serve as controls.

TABLE 2

Primer	Sequence (5'->3')	SEQ ID NO:
gpA-BamHI_R565	AACGTCGGATCCTTAAATAGCCGGCGGGACTTC	10
gpA_F	ATGGCACGCGCTTCGGGCTCGGAA	11
gpA_R	CCAGATTACGCAGCAGAGC	12
hA-F	GCTCGTGCTGTGGGTCCGGAA	13
hA_R	GACACCCGGCAGCACTTC	14
gpA360-367_R	CTGATGCAGGTCTGCCGTCGGCAGCGTCATTTCA CCGCGCAGGT	15
hA354-361_F	AAGGACCTGCGCGGTGAAATGACGCTGCCGACGG CAGACCTGC	16
hA362-369_R	GCGACGTTCTTCCACAGACGGCGGGGTCATTTCA CCACGCAGATC	17

gpA352-359_F	AAAGATCTGCGTGGTGAAATGACCCCGCCGTCTG TGGAAGAACG	18
Lib1 M22R23_F	TACACCGGCGGCACCATTGGCNNKNNKAGTGAGC TCGGCGTGCTTGTG	19
Lib1 M22R23_R	CACAAGCACGCCGAGCTCACTMNNMNNGCCAATG GTGCCGCCGGTGTA	20
Lib2 D84S86_F	CTGGAGTGCCAGCCCCTCTTCNNKTCCNNKGACA TGACCATCGCTGAGTGG	21
Lib2 D84S86 R	CCACTCAGCGATGGTCATGTCMNNGGAMNNGAAG AGGGGCTGGCACTCCAG	22
Lib3 H114G115_F	CAGTACCACGGCTTTGTGGTCATCNNKNNKACCG ACACCATGGCCTTTGCTGCC	23
Lib3 H114G115_R	GGCAGCAAAGGCCATGGTGTCGGTMNNMNNGATG ACCACAAAGCCGTGGTACTG	24
Lib4 A142Q143V144_F	AGAAGACTGTCATCCTCACTGGGNNKNNKNNKCC CATCCATGCCCTGTGGAGC	25
Lib4 A142Q143V144_R	GCTCCACAGGGCATGATGGGMNNMNNMNNCCCAG TGAGGATGACAGTCTTCT	26
Lib5 A191R192_F	GCAACCGGGCAACCAAGGTAGACNNKNNKAGGTT CGCAGCTTTCTGCTCCCC	27
Lib5 A191R192_R	GGGGAGCAGAAAGCTGCGAACCTMNNMNNGTCTA CCTTGGTTGCCCGGTTGC	28
Lib6 T118F121_F	TCATCCACGGCACCGACNNKATGGCCNNKGCTGC CTCGATGCTGTCC	29
Lib6 T118F121_R	GGACAGCATCGAGGCAGCMNNGGCCATMNNGTCG GTGCCGTGGATGA	30
Lib7 A91C95T99_F	TCCAGTGACATGACCATCNNKGAGTGGGTTNNKC TTGCCCAGNNKATCAAGAGGCACTACGAG	31
Lib7 A91C95T99_R	CTCGTAGTGCCTCTTGATMNNCTGGGCAAGMNNA ACCCACTCMNNGATGGTCATGTCACTGGA	32
Lib8 R23E25L26_F	GGCGGCACCATTGGCATGNNKAGTNNKNNKGGCG TGCTTGTGCCCGGG	33
Lib8 R23E25L26_R	CCCGGGCACAAGCACGCCMNNMNNACTMNNCATG CCAATGGTGCCGCC	34

[0063] *Media and Growth Experiments*. M9 complete medium was made from M9 minimum salt (Sigma) supplemented with 0.4% glycerol, 2 μM thiamine, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 100 μg/mL ampicillin. For complete M9 plates, 15 g/L of agar was added. For the experiments with the strain BW2Δ, M9 medium was made without NH_4Cl. When required, L-asparaginase was added to M9 medium at different concentrations. To induce the expression of hASNase1 and gpASNase1 cloned into

the pBAD vector, 0.02% arabinose was added to the M9 medium. For the growth experiments on complete M9 on agar plates, the strains were first grown in LB overnight at 37°C, spun down and washed in M9 medium. An appropriate dilution of this suspension was then spread on the M9 plates so as to get the same number of colonies for each strain. Plates were incubated at 37°C for 48 to 96 hours.

[0064] *DNA Shuffling*. DNA shuffling was performed as described (Meyer, et al. (2014) *Curr. Protoc. Mol. Biol.*, Ausubel (ed) 105:Unit-15.12) with slight adjustments. Briefly, an equimolar mixture of the hASNase1 and GpA genes were digested with 0.5 U of DNase (NEB) for 2 minutes and 30 seconds. Fragments between 100 bp and 200 bp were extracted using the QIAQUICK gel extraction kit (Qiagen), reassembled by PCR and then amplified using either the primers specific to the GpA gene or the one specific to the hASNase1 gene. The obtained shuffled fragments were cloned into a pBAD vector using the Megawhop method (Miyazaki (2003) *Methods Mol. Biol.* 231:23-28). Briefly, 100 to 300 ng of shuffled fragments were used as megaprimers to run a whole plasmid PCR using either pBAD_hASNase1 or pBAD_gpASNase1 as template. After digestion of the template with *Dpn*I, 20 to 40 ng of newly synthesized plasmids containing the shuffled sequences were used to transform electrocompetent BW5Δ cells. After the pulse, the cells were resuspended in 1 ml SOC medium and incubated with shaking at 37°C for one hour. The cells were then spun down at 4000 x g for 4 minutes and gently resuspended in 200 µL of M9 medium. One hundred µL were plated on M9 plate supplemented with 0.2 mM Asn or 2 mM Asn. After 4 days of incubation of the plates at 37°C, the colonies from the 2 mM Asn plate were isolated and pooled into 200 µL of fresh M9 medium. Two dilutions were successively carried out and

used to plate fresh 0.2 mM Asn M9 plates. Clones capable of growing on the 0.2 mM Asn plate after 4 days of incubation at 37°C and three additional days at room temperature were isolated and streaked on LB plates.

[0065] *C-Terminal Domain Swapping.* To build the clone h_N-g_C, the sequence corresponding to the N-terminal domain of hASNase1 (h_N, residue 1-361) was amplified using the primers NdeI-hA_F1 and gpA360-367_R and the sequence corresponding to the C-terminal domain of gpASNase1 (gC, residue 360-565) was amplified using the primers hA354-361_F and gpA-BamHI_R565. To build the clone g_N-h_C the sequence corresponding to the N-terminal domain of gpASNase1 (g_N, residue 1-359) was amplified using the primers NdeI-gpA_F1 and hA362-369_R and the sequence corresponding to the C-terminal domain of hASNase1 (h_C, residue 362-573) was amplified using the primers gpA352-359_F and hA_BamHI_R573. Primers are listed in Table 2. The chimera was then constructed by PCR fusion of fragment h_N and g_C or g_N and h_C using the appropriate primers and then subsequently cloned into the pBAD and pET vectors.

[0066] *Cloning and Expression of the Selected Clones.* The isolated clones were cultured; the plasmid was extracted and sequenced. The corresponding genes were transferred to a pET vector (modified pET14b to include a His-SUMO tag, using the same primers as the one used for cloning into the pBAD vector) to allow the expression of the His-tagged protein in C41 (DE3) cells. The culture was carried out in 1 L of 2YT medium supplemented with 100 µg/mL ampicillin. Expression was induced with 0.1 mM IPTG and cells were grown overnight at 18°C. Cells were harvested, lysed and purified as previously described for the wild type gpASNase1 (Schalk, et al. (2014) *J. Biol. Chem.* 289:33175-86). The protein was eluted in a 25 mM Tris-HCl pH 7.5, 200

mM KCl, 500 mM imidazole buffer and dialyzed against the same buffer containing no imidazole but 1 mM of DTT. Expression and purification of the *E. coli ansB* enzyme was described previously (Schalk, et al. (2014) *J. Biol. Chem.* 289:33175-86).

[0067] *Kinetic Assays.* The catalytic activity of the clones was determined using a spectroscopic NADH-dependent enzyme-coupled assay (Fernandez, et al. (2013) *Int. J. Clin. Exp. Med.* 6:478-487; Hejazi, et al. (2002) *Biochem. J.* 364:129-136), which measures the production of L-aspartic acid (Asp) through the 1:1 oxidation of reduced NADH. The conversion of NADH to NAD was measured spectrophotometrically as a decrease in absorbance at 340 nm at 37°C. All measurements were taken in triplicate in a buffer containing 100 mM Tris pH 7.5, 0.4 mM α-ketoglutarate and 0.4 mM NADH with 50 nM (hASNase1, hN-gC); 10 nM (gpASNase1, gN-hC, 63-hC, 64-hC, 65-hC, SA-hC) or 3 nM (*ansB*) enzyme. Glutamic-oxalacetic transaminase (Sigma) and malic dehydrogenase (Sigma) were helper enzymes for the coupled enzymatic reactions; 5 and 1 unit were used, respectively. Data were fit to the Michaelis-Menten equation using SigmaPlot (Systat Software Inc). Due to the cooperative nature of hASNase1, this enzyme was analyzed using the Hill equation.

[0068] *Cell Culture.* The LOUCY (Ben-Bassat, et al. (1990) *Cancer Genet. Cytogenet.* 49(2):241-8) and SUP-B15 (ATCC CRL-1929) cell lines are described in the art. All cell lines were analyzed by STR (Short Tandem Repeat) and confirmed to match 100% to corresponding STR profile data from the Global Bioresource Center ATCC. All cell lines were verified to be mycoplasma free. LOUCY and SUP-B15 lines were cultivated in a humid atmosphere (5% CO_2, 37°C) using RPMI 1640 media supplemented with 10% FBS (Hyclone)

and 1x penicillin-streptomycin solution (Invitrogen). L-Glutamine was added directly into cell cultures to a final concentration of 2 mM. Ninety µL aliquots of cell suspension ($5x10^5$ cells per mL) were cultured in triplicate in round-bottomed 96-well microtiter plates in the presence of 10 µL of either DPBS (Dulbecco's phosphate-buffered saline, Mediatech) or different L-asparaginases to a final concentration ranging from 0.00001 to 0.1 IU/mL. After incubating the plates for 4 days at 37°C in humidified air containing 5% CO_2, 11 µL of Alamar Blue (Invitrogen) was added to a final concentration of 10% (v/v) and the plates were incubated for an additional two hours, followed by reading of the fluorescence signal. The leukemic cell viability was calculated as percentage of fluorescence counts in the presence of L-asparaginase versus that in the DPBS control.

[0069] *Development of the Selection Systems.* To create a selection system for L-asparaginase activity, a bacterial strain whose growth is dependent on any product of the enzymatic reaction was required. Since L-asparaginase catalyzes the hydrolysis of Asn into Asp and ammonia, two selections systems were developed and tested: one employing a bacterial strain dependent on the reaction product ammonium as the sole nitrogen source and the other uses a strain auxotroph for Asp.

[0070] *Strain BW2Δ: Dependence on L-Asparaginase Reaction for Nitrogen Source.* All life forms require a nitrogen source. For *E. coli* grown in a minimal media such as M9, the nitrogen source is usually obtained in the form of NH_4Cl salt. However, in the absence of NH_4Cl, it was verified that the *E. coli* BW strain used in this study could grow using 2 mM Asn in the media. This demonstrates that *E. coli* can use Asn as a nitrogen source through the activity of its

endogenous L-asparaginases. To make the BW *E. coli* strain dependent on the NH_4Cl produced by exogenous L-asparaginase activity, two endogenous L-asparaginase genes (*ansA* and *ansB*) were deleted from the *E. coli* BW parental strain, which were then referred to as BW2Δ. Notably, the third endogenous L-asparaginase (*iiiA*) was not deleted, since it has a very high K_m value for Asn. Indeed, under the experimental conditions, even with 2 mM Asn in the media, growth of the BW2Δ strain was greatly impaired.

[0071] *Strain BW5Δ: Dependence on L-Asparaginase Reaction for Aspartate. E. coli* can generate Asp by hydrolyzing Asn (the L-asparaginase reaction) or by an aminotransferase reaction. Indeed, the parental BW strain grows well in M9 media independently and with no effect by Asp supplementation. To create an *E. coli* strain auxotrophic for Asp, all three endogenous L-asparaginase genes (*ansA*, *ansB*, and *iiiA*) were deleted, as were the two relevant aminotransferase genes (*aspC* and *tyrB*). The strain with these five genes deleted was referred to as strain BW5Δ. To ensure that the strain BW5Δ acquired Asp auxotrophy, growth was tested in minimal media M9 with and without Asp supplementation. It was observed that without supplementing the media with Asp, BW5Δ could not grow. However, growth was observed in the Asp supplemented conditions.

[0072] *Use of the BW2Δ and BW5Δ Strains to Select Clones Expressing L-Asparaginase.* To investigate whether these bacterial strains can be used as selection systems for L-asparaginase activities and foremost whether they would allow the differentiation between L-asparaginases based on their K_m property, the growth of the BW2Δ and BW5Δ strains expressing either an L-asparaginase with a low K_m or an L-asparaginase with a high K_m was analyzed. Human L-

asparaginase type I (hASNase1), the protein target to be evolved, is characterized by a K_m for Asn in the millimolar range (Karamitros & Konrad (2014) *J. Biol. Chem.* 289:12962-75; Schalk, et al. (2014) *J. Biol. Chem.* 289:33175-86) and was thus used as the high K_m L-asparaginase. The guinea pig L-asparaginase I (gpASNase1) was characterized as having a K_m for Asn in the micromolar range (Schalk, et al. (2014) *J. Biol. Chem.* 289:33175-86) and was thus used as the low K_m L-asparaginase. Both genes encoding the respective L-asparaginase were cloned into the pBAD vector in order to have well-controlled protein expression.

[0073] In the first screening system, which is based on the L-asparaginase reaction supplying the sole source of nitrogen, BW2Δ was transformed with pBAD (control vector), pBAD_hASNase1 (high K_m enzyme) or pBAD_gpASNase1 (low K_m enzyme). The transformed cells were grown in M9 medium lacking NH_4Cl but supplemented with an increasing concentration of Asn. The expectation was that a low Asn concentration would preferentially promote the growth of the bacteria carrying the pBAD_gpASNase1 plasmid, coding for the low K_M enzyme. Results show that growth of both BW2Δ pBAD_hASNase1 and pBAD_gpASNase1 did in fact depend on the concentration of Asn (*i.e.*, enhanced concentration of Asn leading to better growth). However, per Asn concentration, there was not a significant difference of growth between BW2Δ pBAD_hASNase1 and pBAD_gpASNase1, *e.g.*, at 0.2 mM Asn, BW2Δ bacteria that express the low K_m gpASNase1 enzyme did not form significantly more or bigger colonies than bacteria that express the high K_m hASNase1 enzyme. In sum, this screening system was found to be not suitable for discriminating between L-asparaginases that differ in their Asn K_m value.

[0074] In the second screening system, which is based on Asp auxotrophy, BW5Δ was similarly transformed with pBAD, pBAD_hASNase1 or pBAD_gpASNase1. The transformed cells were grown in complete M9 media supplemented with increasing concentration of Asn. Growth of BW5Δ pBAD_hASNase1 and pBAD_gpASNase1 were found to be dependent on the concentration of Asn. Interestingly, at 2 mM Asn, which represents a concentration at which the guinea pig enzyme is saturated with substrate (K_m = 50 μM) and the human enzyme is only partially saturated (K_m = 3,500 μM), hASNase1 and gpASNase1 transformed BW5Δ strains show similar growth. In other words, at this relatively high Asn concentration, the BW5Δ strain cannot be used to distinguish between those bacteria that express a low K_m L-asparaginase to those that express a high K_m enzyme. In contrast, at lower concentrations of the substrate Asn, only BW5Δ pBAD_gpASNase1 is capable of growing. Indeed, at 0.2 mM Asn, a concentration of Asn well below the K_m of hASNase1 but above the K_m of gpASNase1, colonies of BW5Δ pBAD_gpASNase1 developed whereas BW5Δ pBAD_hASNase1 could not grow. Noteworthy, this growth difference was found to be independent of enzyme expression level since no change in growth was noticed when arabinose (the inducer for protein expression) was used in the range 0.0002% - 0.2%. Taken together, the results suggest that the difference in growth seen between BW5Δ expressing hASNase1 and BW5Δ expressing gpASNase1 on minimal media plates directly reflects the K_m of the respectively expressed L-asparaginase.

[0075] The conclusion from this initial set of experiments is that the screen based on the L-asparaginase reaction supplying the nitrogen (using the BW2Δ strain) does not differentiate well enough between high and low K_m enzymes,

whereas the screen based on the L-asparaginase reaction supplying the amino acid Asp (using the BW5Δ strain) does indeed discriminate between enzymes with differing affinities to Asn - at low Asn concentration, only the bacteria expressing the low K_m L-asparaginase forms colonies. Hence, all further selection work aimed at discovering a human L-asparaginase variant that has acquired a low K_m was performed with the BW5Δ *E. coli* strain.

[0076] *DNA Family Shuffling*. DNA family shuffling is an alternative method for generating genetic diversity. The protein sequences of hASNase1 and gpASNase1 contain 573 and 565 amino acids, respectively, and are 69.8% identical at the amino acid level (differing by 170 amino acids). Working with synthetic codon-optimized versions of both hASNase1 (SEQ ID NO:35) and gpASNase1 (SEQ ID NO:36), the genes encoding hASNase1 and gpASNase1 show 75% identity at the DNA level. As mentioned herein, hASNase1 has a K_m of 3.5 mM for Asn, whereas the K_m of the guinea pig enzyme was determined to be 50 μM (Table 3). The DNA shuffling method was used to recombine the two L-asparaginases in order to obtain a chimera that displays the low K_m of the guinea pig but with as high as possible amino acid homology with the human enzyme. The shuffled fragments were then cloned into the pBAD vector. The resulting chimeric library was used to transform the BW5Δ strain. The presence of mutants possessing a low K_m was discovered using the selection protocol as described above. Four clones (#63, #64, #65 and #SA) were isolated from M9 plates at an Asn concentration of 0.2 mM. Sequence analyses of these clones revealed a shuffling pattern with recombination events occurring predominantly in the N-terminal (*i.e.*, catalytic) domain; one of the selected clones (#SA) carried a mutation that

introduced a premature stop codon (STOP) that was beyond the catalytic domain (Fig. 1).

[0077] *Swapping of the C-terminal domain.* To minimize immunogenicity against GpA, an enzyme was sought that was as identical as possible to the human L-asparaginase but that had the low K_m property of the guinea pig/*E. coli* type II enzymes. Shuffling experiments suggested that a chimera that had the guinea pig L-asparaginase domain followed by the human C-terminal domain would still retain the favorable kinetic properties of the gpASNase1 but would have an increased sequence identity with the human enzyme.

[0078] Accordingly, two chimeras were generated; one, which was referred to as h_N-g_C and included the human N-terminal domain fused to the guinea pig C-terminal domain, and the second, which was referred to as g_N-h_C and included the guinea pig N-terminal domain fused to the human C-terminal domain (Fig. 1, Table 3). *In vitro* kinetic characterization of these chimeras validated the prediction, with h_N-g_C displaying kinetic properties similar to hASNase1 and g_N-h_C displaying kinetic properties similar to gpASNase1 (Table 4).

[0079] This result demonstrates that the C-terminal domain of GpA does not influence the catalytic activity of the L-asparaginase and most importantly does not negatively affect the K_m. Since the goal was to identify a clone with gpASNase1 kinetic properties but with the highest sequence homology to hASNase1, the four clones, #63, #64, #65 and #SA, were engineered by replacing their shuffled C-terminal domain with the exact sequence of the human C-terminal domain (Fig. 1). The engineered clones, namely 63_N-h_C, 64_N-h_C, 65_N-h_C and SA_N-h_C, displayed respectively 85.7%, 91.1%, 87.1% and 91.6% identity with the wild-type hASNase1 sequence (Table 3 and Fig. 2A-2B).

TABLE 3

Enzyme Name	# of Residues	% Identity to hASNase1	SEQ ID NO:
hASNase1	573	100	2
ansB	326	26.9	-
gpASNase1	565	69.8	1
g_N-h_C	571	83.4	37
h_N-g_C	567	86.5	38
63_N-h_C	571	85.7	39
64_N-h_C	571	91.1	40
65_N-h_C	571	87.1	41
SA_N-h_C	571	91.6	42

[0080] *Catalytic Properties of the Variants.* For determining the precise kinetic properties of 63_N-h_C, 64_N-h_C, 65_N-h_C and SA_N-h_C, the genes encoding these enzymes were sub-cloned into a pET14b expression vector and expressed in C41 *E. coli* cells. The purified clones were tested for their L-asparaginase activity (Table 4). *E. coli* L-asparaginase ansB was also included in order to compare the clones to an L-asparaginase approved for cancer therapy. It was observed that the four clones selected for by directed evolution and carrying the C-terminal domain of hASNase1 displayed high sequence identity with hASNase1 (>85%) but kinetic properties similar to gpASNase1 with a K_m in the micromolar range. Clone 63_N-h_C (85.7% identity with the hASNase1 sequence) displayed the lowest K_m at 47 μM. Clone SA_N-h_C had the highest sequence identity to hASNase1 (91.6% identity), but this clone had a somewhat higher Asn K_m of 165 μM. The observed Asn hydrolysis rates of the enzymes at 50 μM Asn ($k_{obs@50\mu M}$) were compared as this is a relevant blood Asn concentration (Ollenschläger (1988) *Eur. J. Clin. Invest.* 18:512-6). The *E. coli* ansB $k_{obs@50\mu M}$ was found to be 41±0.3 sec^{-1} and that of wild-type gpASNase1 was 20 sec^{-1}. Importantly, the $k_{obs@50\mu M}$ values for the humanized clones were also in this range, being 17 sec^{-1}, 10 sec^{-1}, 17 sec^{-1}

and 6 sec^{-1} for clones 63_N-h_C, 64_N-h_C, 65_N-h_C and SA_N-h_C, respectively.

TABLE 4

Enzyme name	k_{cat} (sec^{-1})	K_m (μM)	k_{cat}/K_m ($sec^{-1}\mu M^{-1}$)	k_{obs} (sec^{-1}) @50 μM
hASNase1*	17±0.8	3500±300	0.005	ND
ansB	48±1	11±1	4.4	41±0.3
gpASNase1	41±2	50±7	0.8	20±1
g_N-h_C	24±1	35±4	0.7	14±1
h_N-g_C	14±0.5	3800±120	0.004	ND
63_N-h_C	32±0.6	47±3	0.7	17±0.5
64_N-h_C	60±2	202±17	0.3	10±1
65_N-h_C	40±1	74±5	0.5	17±1
SA_N-h_C	32±2	165±19	0.2	6±1

* Kinetic parameters using the Hill-equation (n=2.1)
ND: not determined

[0081] *Cell Culture Evaluation of the Humanized L-Asparaginase Clones.* To determine whether GpA enzyme exhibited anti-ALL activity, human T-ALL LOUCY and B-ALL SUP-B15 cell lines were exposed to increasing concentrations of gpASNase1. The results of this analysis indicated that gpASNase1 exhibited an IC_{50} of 0.00015 IU/ml and 0.00036 IU/ml for the LOUCY and SUP-B15 cell lines, respectively (Table 5). Notably, these IC_{50} values are comparable to those of the *E. coli* type II enzyme.

[0082] Chimeras of hASNase1 and gpASNase1 were also evaluated for their anti-ALL potency. Clones g_N-h_C, 63_N-hC and 65_N-hC were selected for these experiments since these clones have the lowest Asn K_m (35, 47 and 74 μM, respectively) and thereby have activities most similar to that of gpASNase1 (50 μM). Due to the higher K_m value of 65_N-hC, this clone had the highest IC_{50} value compared to the other enzymes but was still very effective in killing both the T-ALL and B-ALL cells (IC_{50} in the mIU/mL range;

Table 5). Clones g_N-h_C and 63_N-h_C proved to be very similar in their cell-killing power compared to gpASNase1. This is especially notable for clone 63_N-h_C, since this clone increased the percent identity to hASNase1 from 69.8% as present in gpASNase1 to 85.7%.

TABLE 5

Enzyme Name	IC_{50} value (IU/mL x 10^{-4})	
	LOUCY	SUB-B15
gpASNase1	1.5	3.6
g_N-h_C	2.9	5.7
63_N-h_C	3.8	6.0
65_N-h_C	85.3	13.0
E. coli type II	3.5	3.7

[0083] In summary, this analysis identified humanized variants of GpA that had the required low K_m property. Two of the identified clones, 63N-hC and 65N-hC, respectively shared 85.7% and 87.1% amino acid sequence identity with the hA-FL but had a K_m similar to full-length GpA. These clones possess 100- to 140-fold enhanced catalytic efficiency compared to full length hASNase1. Notably, these highly human-like L-asparaginases maintain their *in vitro* ALL killing potential.

Example 3: Humanized GpA Variants Produced via a Structured-Based Approach

[0084] As an alternative to DNA shuffling and domain swapping, a structure-based approach was taken to humanize the GpA enzyme and reduce the immunogenicity of the same. For this approach, the truncated GpA369 variant (SEQ ID NO:5) was modified. The crystal structure of GpA was examined and predictions were made as to which residues at the surface of the enzyme could be mutated to the corresponding amino acids in hASNase1, and whose presence would not be detrimental to the activity or stability of

the enzyme. The candidate surface residues were divided into three groups based upon the likelihood of having a possible impact on activity or stability of GpA (Group 1, no impact; Group 2, likely no impact; and Group 3, possible impact)(Table 6).

TABLE 6

Group 1		Group 2		Group 3	
GpA residue[1]	Human residue[2]	GpA residue[1]	Human residue[2]	GpA residue[1]	Human residue[2]
Q23	R23	H10	R10	R147	H147
K25	E25	D91	A91	K193	R193
K48	E48	D92	E92	D217	E217
Q52	R52	Q108	H108	R301	Q301
Q54	R54	H236	Q236		
K98	Q98	S250	A250		
E101	K101				
S121	F121				
G122	A122				
H134	Q134				
N233	S233				
Q288	E288				
E344	D346				
L360	P362				
T362	S364				
A363	V365				
D364	E366				
L365	E367				
H366	R368				
Q367	R369				
S368	P370				

[1]Reference sequence GpA369 (SEQ ID NO:5).
[2]Corresponding residue in reference sequence hASNase1 (SEQ ID NO:2).

[0085] A GpA variant containing all the Group 1 mutations, the combination of Groups 1 and 2 mutations, and the combination of Groups 1, 2 and 3 mutations were prepared (Table 7). The GpA variants were recombinantly expressed and found to retain 100% of the wild-type activity and stability.

[0086] *In vivo* stability data suggested a potential cleavage of one of the surface exposed loops present in the GpA enzyme. In particular, the data suggested different stabilities between proteins having the sequence of loop 1 as present in hASNase1 (loop1^{hum}, residues 57-62: SEDTLV (SEQ ID NO:43)) to proteins having the sequence as present in GpA (loop1^{gp}, residues 57-62: PDHALA (SEQ ID NO:44)). Accordingly, GpA variants containing combinations of Group 1, 2 and 3 mutations were further mutated to include the loop1^{hum} sequence (Table 7). Again, the Loop1 GpA variants were recombinantly expressed and found to retain 100% of the wild-type activity and stability.

TABLE 7

Variant Name	% Identity		SEQ ID NO:
	GpA359*	hASNase1^{+}	
GpA369	100%	72.2%	5
GpA369(hum)-Group 1	96.4%	77.9%	45
GpA369(hum)-Group 1+2	94.7%	79.5%	46
GpA369(hum)-Group 1+2+3	93.6%	80.6%	47
GpA369(hum)-Group1-loop1^{hum}	95.0%	79.2%	48
GpA369(hum)-Group1+2-loop1^{hum}	93.3%	80.9%	49
GpA369(hum)-Group1+2+3-loop1^{hum}	92.2%	81.9%	50

*Identity determined over the entire length of the catalytic domain of the variant (residues 1-359) as compared to GpA359 (SEQ ID NO:3).
$^{+}$Identity determined over the entire length of the variant as compared to residues 1-371 of hASNase1, since the human enzyme has a two-residue insertion at residue 311.

Example 4: Mutation of Surface Cysteine Residues for PEGylation

[0087] PEGylation is known to increase *in vivo* circulation time. PEG molecules can be readily conjugated to cysteine residues of a protein of interest by maleimide-based PEGylation chemistry. GpA369 and humanized GpA369 variants

contain five intrinsic cysteine residues, Cys79, Cys173, Cys198, Cys296 and Cys299. Based on crystal structure analysis, Cys173 and Cys296 are buried and thus predicted to not affect PEGylation. Cys299 was considered to be less accessible than Cys198 and contribute to tetramer stabilization. Attempts to exchange Cys299 to either Ala or Ser to avoid PEGylation at this site resulted in unstable proteins. Conversely, mutating the cysteine residue at position 198 to an alanine (GpA369-C198A, SEQ ID NO:51), serine (GpA369-C198S, SEQ ID NO:52) or valine (GpA369-C198V, SEQ ID NO:53) yielded equally stable and active proteins compared to the reference GpA369 enzyme. Moreover, maleimide-PEGylation of the GpA369-C198A, GpA369-C198S and GpA369-C198V proteins, now containing only a single reactive surface cysteine (Cys79), resulted in a homogenous product.

[0088] The degree of biologics protection by PEGylation depends on the structure and the complexity of the PEG agent, but in most cases, single-site PEGylation is not enough to cover the whole macromolecule. Therefore, additional GpA variants were generated, which contained between one and five surface cysteine residues. In this respect, the structure of GpA was analyzed in order to identify regions of residues that could be mutated to cysteines for multi-site PEGylated products. Useful regions must be on the surface, must be distant from oligomerization interfaces (GpA is a tetramer, so PEGylating a residue near an interface could be detrimental to the activity of the enzyme), and include residues that point outwards. Regions of GpA meeting these criteria are listed in Table 8.

TABLE 8

Region				
1	2	3	4	5
R40	E131	R253	E340	S358
K48	N132	Q257	E344	S359
E49	K221-D226	K261	R345	
Q52	N233	Q281-R284	Q347	
Q54		Q288	K352	
D58				

[0089] Amongst the possible residues that could be mutated (Table 8), E49 from Region 1, K225 from Region 2, Q257 from Region 3 and E340 from Region 4 were identified as good candidates for mutation to a cysteine. Variants of GpA369-C198 and GpA369(hum)-Group1+2+3-C198, which included one or more of K225C, E340C, E49C and Q257C mutations were generated (Table 9).

TABLE 9

Variant Name	% Identity		SEQ ID NO:
	GpA359*	hASNase1+	
GpA369-C198A	99.7%	72.0%	51
GpA369-C198A+K225C	99.4%	71.7%	54
GpA369-C198A+K225C+E340C	99.2%	71.7%	55
GpA369-C198A+K225C+E340C+E49C	98.9%	71.4%	56
GpA369-C198A+K225C+E340C+E49C+Q257C	98.6%	71.2%	57
GpA369(hum)-Group1+2+3-loop1^{hum}-C198A	91.9%	81.7%	58
GpA369(hum)-Group1+2+3-loop1^{hum}-C198A+R52C	91.9%	81.4%	59
GpA369(hum)-Group1+2+3-loop1^{hum}-C198A+R52C+K225C	91.6%	81.1%	60
GpA369(hum)-Group1+2+3-loop1^{hum}-C198A+R52C+K225C+E340C	91.4%	81.1%	61
GpA369(hum)-Group1+2+3-loop1^{hum}-C198A+R52C+K225C+E340C+Q281C	91.1%	80.9%	62

*Identity determined over the entire length of the catalytic domain of the variant (residues 1-359) as compared to GpA359 (SEQ ID NO:3).

[+]Identity determined over the entire length of the variant as compared to residues 1-371 of hASNase1, since the human enzyme has a two-residue insertion at residue 311.

[0090] Each of the variants listed in Table 9 were recombinantly expressed, purified and PEGylated with one or more of mPEG-10K, mPEG-20K and mPEG-40K (Table 10). As determined by SDS-PAGE analysis, the size of the PEGylated enzymes increased with an increase in the the number of active cysteines. For example, using maleimide-PEG10K, GpA369(hum)-C198A migrated at a higher molecular weight after PEGylation (due to the extra 10K Da supplied by the PEG), and GpA369(hum)-C198A+R52C migrated at an even higher apparent molecular weight since this variant can react with two PEG molecules (for a total of an extra 20K Da supplied by two 10K molecules linked to the enzyme). Of note, the GpA369-C198A-K225C-E340C-E49C-Q257C mutant was prepared, expressed, purified and PEGylated with mPEG-10K. However, PEGylation of this variant with mPEG-10K resulted in multiple-species product. Chaperone 60KDa was present with at a 1:1 ratio with this protein, suggesting that the GpA369-C198A-K225C-E340C-E49C-Q257C mutant was not well folded.

TABLE 10

Variants	k_{obs} at 2mM ASN (sec^{-1})	% vs GpA-FL
Full length GpA (GpA-FL)	41	100
GpA369	44	107.3
GpA369-C198A	43.3	105.6
GpA369-C198A+K225C	41.4	100.9
GpA369-C198A+K225C+E340C	42.4	103.4
GpA369-C198A+K225C+E340C+E49C	41.6	101.5
10K-PEGylated-GpA369-C198A	83.3	203.2
20K-PEGylated-GpA369-C198A	83.7	204.1
40K-PEGylated-GpA369-C198A	76.5	186.6
10K-PEGylated-GpA369-C198A+K225C	92.6	225.9

20K-PEGylated-GpA369-C198A+K225C	88.0	214.6
40K-PEGylated-GpA369-C198A+K225C	92.8	226.3
10K-PEGylated-GpA369-C198A+K225C+E340C	84.9	207.1
10K-PEGylated-GpA369-C198A+K225C+E340C+E49C	83.9	204.6

*PEGylation was with linear PEG.

[0091] To further assess the PEGylation of the variants, different PEG:protein ratios were used. For this analysis, 2 mg/mL of GpA369-C198A was used along with 2-, 10-, and 20-fold excess m-PEG10K linear. Further, analysis was carried out to determine the effect of adding 5 mM beta-mercaptoethanol after the PEGylation reactions were completed, and including additives such as 10 mM or 100 mM glycine or 10 mM aspartic acid during the PEGylation reaction. This analysis indicated that a molar ratio of 20:1 of maleimide PEG:protein was needed to ensure full PEGylation of GpA369 variants. Further, the addition of beta-mercaptoethanol was found to provide homogenous product, whereas glycine or aspartic acid should be excluded in the PEGylation reaction.

[0092] In addition to linear maleimide-PEG10K, the GpA369 variants with PEGylated with linear maleimide-PEG20K, linear maleimide-PEG40K two-branched maleimide-PEG20K, four-arm maleimide-PEG10K, and Y-shape maleimide-PEG40K. PEGylation with each of these different types of PEG was observed. Notably, the PEGylated variants showed a significant increase in L-asparaginase activity compared to the naked variants of GpA369 (Table 10).

Example 5: Mutation of Surface Lysine Residues for PEGylation

[0093] Most conventional biotherapeutics are PEGylated on lysine residues, where the epsilon amino group of the lysine side chain reacts with the PEG molecule. Lysine is a common amino acid present at the surface of proteins. Therefore, PEGylation using this strategy often results in a product of low homogeneity, with a variable number of PEG molecules linked to the protein.

[0094] To increase homogeneity, lysine resides of GpA that are in close proximity to another lysine residue were replaced with a residue that would not react with PEG. In addition, residues having a possible negative impact on the structural integrity and thus the enzymatic activity of the tetrameric L-asparaginase were replaced with surface lysine residues in order to have the whole enzyme's surface fully and evenly protected.

[0095] Using this strategy, truncated GpA variants having the mutations listed in Table 11 were considered to be of use in this invention.

TABLE 11

Variant Name	%Identity		SEQ ID NO:
	GpA359*	hASNase1+	
GpA369-Q54K+D91K+K223D+S311K	98.9%	72.5%	63
GpA369(hum)-Group1+2+3-C198A-R54K+A91K+K223D+S311K	92.8%	80.1%	64
GpA369(hum)-Group1+2+3-loop1^{hum}-C198A-R54K+A91K+K223D+S311K	91.4%	81.4%	65
GpA369(hum)-Group1+2+3-loop1^{hum}-C198A-S7K+A53K+E58K+Q98K+Q106K+S233K+Q257K+Q281K+S311K+E340K	90.3%	79.2%	86

*Identity determined over the entire length of the catalytic domain of the variant (residues 1-359) as compared to GpA359 (SEQ ID NO:3).

+Identity determined over the entire length of the variant as compared to residues 1-371 of hASNase1, since the human enzyme has a two-residue insertion at residue 311.

[0096] To demonstrate the activity, stability and PEGylation via lysine residues, the GpA369(hum)-Group1+2+3-C198A-R54K+A91K+K223D+S311K variant was generated, recombinantly expressed, purified, and PEGylated using different PEG:protein ratios. This analysis showed that a molar ratio of more than 20:1 of amine PEG versus protein was needed to ensure full PEGylation of the GpA369 variant. It was also observed that additives such as DTT and glycerol should be excluded in the PEGylation reaction. Different size and linker types were tested, including Methoxy PEG Succinimidyl Carbonate 10K (mPEG-SC-10K), mPEG-Succinimidyl Carboxy Methyl Ester 5K (mPEG-SCM-5K) and mPEG-Succinamide Succinimidyl Ester 5K (mPEG-SAS-5K). Notably, none of the PEGylated products exhibited any loss in L-asparaginase activity compared to the naked version (Table 12).

TABLE 12

Variants	k_{obs} at 2mM ASN (sec^{-1})	% vs GpA-FL
Full length GpA (Gpa-FL)	41	100
GpA369(hum)-Group1+2+3-C198A-R54K+A91K+K223D+S311K	69.5	169.5
mPEG-SC-10K-PEGylated-GpA369(hum)-Group1+2+3-C198A-R54K+A91K+K223D+S311K	69.3	169.1
mPEG-SCM-5K-PEGylated-GpA369(hum)-Group1+2+3-C198A-R54K+A91K+K223D+S311K	70.9	172.9
mPEG-SAS-5K-PEGylated-GpA369(hum)-Group1+2+3-C198A-R54K+A91K+K223D+S311K	66.9	163.2

Example 6: Tags for Increasing *In Vivo* Half-Life.

[0097] Efficacy of L-asparaginase is related to the *in vivo* half-life of the drug; the longer the half-life, the longer the enzyme acts to hydrolyze the blood asparagine. Accordingly, to increase the *in vivo* half-life of L-

asparaginase variants disclosed herein, tags are fused to the N-terminus of the L-asparaginase. Such tags include a histidine tag, a yeast SUMO tag; a human SUMO tag; a His_6-human SUMO tag where the SUMO tag can be one of the four homologous SUMO domains present in humans (SUMO-1, SUMO-2, SUMO-3 or SUMO-4); and an albumin-binding peptide tag. Each of the tags can increase the circulation time of the L-asparaginase enzyme. In addition, combinations of tags can be used. In particular, the SA21 and SUMO tags can be combined to obtain variants with an even further extended half-life.

[0098] *The Histidine Tag.* The DNA sequence specifying a string of six to nine histidine residues is frequently used in vectors for production of recombinant proteins. The result is expression of a recombinant protein with a 6xHis or poly-His tag fused to its N- or C-terminus.

[0099] Expressed His-tagged proteins can be purified and easily detected thereby providing a means of specifically purifying or detecting the recombinant protein without a protein-specific antibody or probe. Kits are commercially available to His-tag proteins.

[00100] *SUMO Modification.* It has been found that SUMO as an N-terminal fusion partner enhances functional protein production in prokaryotic and eukaryotic expression systems, based upon significantly improved protein stability and solubility.

[00101] Following the expression and purification of the fusion protein, the SUMO-tag can be cleaved by specific (SUMO) proteases via their endopeptidase activity *in vitro* to generate the desired N-terminus of the released protein partner. SUMO tag expression systems are commercially available. In some embodiments, the SUMO tag is a yeast SUMO tag (*e.g.*, Smt3 (SEQ ID NO:66)). In other embodiments,

the SUMO tag is a human SUMO tag (*e.g.*, SUMO-1 (SEQ ID NO:67), SUMO-2 (SEQ ID NO:68), SUMO-3 (SEQ ID NO:69) or SUMO-4 (SEQ ID NO:70)).

[00102] *His-SUMO Modification.* Combining a histidine (*e.g.*, 1x-6xHis) tag and SUMO modification provides for efficient purification, increased expression and solubility, as well as increased half-life of L-asparaginases. Expression systems for providing the His-SUMO modification to a protein of interest are commercially available. See, *e.g.*, the CHAMPION pET SUMO protein expression system (Invitrogen).

[00103] *Albumin Binding Domain.* Using phage display, a series of peptides that bind to serum albumin from multiple species have been identified (Dennis, at al. (2002) *J. Biol. Chem.* 277(38):35035-35043; US 2016/0185874; and US 2004/0001827). One of these peptides, called SA21, was found to have an extended serum half-life. Exemplary albumin binding peptides include, but are not limited to, SA20 (QRLIEDICLPRWGCLWEDDF; SEQ ID NO: 71), SA21 (RLIEDICLPRWGCLWEDD; SEQ ID NO: 72), and SA31 (RLIEDICLPRWGCLW; SEQ ID NO: 73). By fusing such domains to the L-asparaginase enzyme disclosed herein, pharmacokinetic improvements are expected via non-covalent association with albumin. See Dennis, et al. (2002) *J. Biol. Chem.* 277(38):35035-35043; US 2016/0185874; and US 2004/0001827.

[00104] Exemplary truncated GpA variants with various tags are provided in Table 13.

TABLE 13

GpA Variant	Tag	SEQ ID NO:
GpA369-C79K-C198A	N-terminal yeast SUMO + 1X-His	74
GpA369-C79K-C198A	N-terminal human SUMO-1 + 1X-His	75
GpA369-C198A	N-terminal SA21	76
GpA369-C198A	C-terminal SA21	77

GpA369-C79A-C198A	N-terminal yeast SUMO + 1X-His, C-terminal SA21	78
GpA369-C198A	N-terminal human SUMO-1 + 1X-His, C-terminal SA21	79
GpA(hum)$_{1-225}$-SA21-GpA(hum)$_{226-369}$	SA21 engrafted between residues 225 and 226 of GpA	93
GpA(hum)$_{1-340}$-SA21-GpA(hum)$_{341-369}$	SA21 engrafted between residues 340 and 341 of GpA	94

Example 7: TRAIL-GpA Fusion Protein

[00105] TRAIL (TNF-related apoptosis inducing ligand) is a protein that induces cell death by apoptosis. Accordingly, a TRAIL-Asparaginase fusion protein is created to combine the activity of these two proteins. Using this fusion protein, the L-asparaginase component signals the cell to undergo apoptosis, and the TRAIL component induces cell death. By way of illustration, three tandem soluble domains of TRAIL ($TRAIL_{trimer}$) are fused, in-frame, with a truncated and humanized guinea pig L-asparaginase such as GpA369(hum)-Group1+2+3 (SEQ ID NO:47) or GpA369(hum)-Group1+2+3-loop1^{hum} (SEQ ID NO:50) to generate $TRAIL_{trimer}$-GpA or GpA-$TRAIL_{trimer}$ fusion proteins.

[00106] Efficacy of a fusion protein is assessed by culturing human acute myeloid leukemia MV4;11 cells in the presence of the fusion protein to a final concentration ranging from 0.0001 to 2.5 IU/ml. After incubating the plates for 4 days at 37°C in humidified air containing 5% CO_2, Alamar Blue (Invitrogen) is added to a final concentration of 10% v/v, and the plates are incubated for an additional 4 hours followed by reading of the fluorescence signal. Leukemic cell viability is calculated as the percentage of fluorescence counts in the presence of L-asparaginase versus that in the DPBS control. This

analysis will demonstrate that the active fusion protein possesses remarkably better killing activity against the MV4;11 cell line compared to the truncated and humanized guinea pig L-asparaginase alone.

[00107] *In vivo* efficacy of a fusion protein for killing leukemic cells is assessed by injecting 4-10 non-obese diabetic/severe combined immune-deficient γ (NSG) mice (The Jackson Laboratory) at 6 weeks of age with 150 µL DPBS containing $5x10^6$ luciferase-positive MV4;11 cells. At regular time points, the bioluminescence is measured using the IVIS Lumina II imaging system (PerkinElmer). After engraftment (Day 0), the mice are treated every day for one week with an i.p. injection of 15 IU/mouse of the fusion protein. The bioluminescence signal is measured at day 0 and day 7. The results of this analysis will demonstrate a remarkable killing effect of the MV4;11 cells by the fusion protein.

[00108] Efficacy of the fusion protein against solid cancers, such as pancreatic and ovarian cancers, is also determined. Pancreatic cancer cell lines such as Panc-1 and MiaPaca2 and ovarian cancer cell lines such as OVCAR3 and OVCAR4 are treated with the fusion protein or GpA alone to a final concentration ranging from 0.0001 to 2.5 IU/ml, or $TRAIL_{trimer}$ at a concentration that corresponds to that used for the fusion protein. After incubating the plates for 4 days at 37°C in humidified air containing 5% CO_2, Alamar Blue (Invitrogen) is added to a final concentration of 10% v/v, and the plates are incubated for an additional 4 hours followed by reading of the fluorescence signal. Cancer cell viability is calculated as the percentage of fluorescence counts in the presence of the fusion protein versus that in the DPBS control. This analysis will demonstrate that the active fusion protein possesses remarkably better killing

activity against the cancer cell lines compared to $TRAIL_{trimer}$ or GpA alone.

What is claimed is:

1. A C-terminally truncated Guinea pig L-Asparaginase (GpA) variant sharing at least 85% sequence identity with residues 1 to 359 of SEQ ID NO:1.

2. The truncated GpA variant of claim 1, wherein the C-terminal truncation is at a position between 359 and 396 of SEQ ID NO:1.

3. The truncated GpA variant of any preceding claim, wherein the C-terminal truncation is at position 369 of SEQ ID NO:1.

4. The truncated GpA variant of any preceding claim, further comprising at least one amino acid substitution relative to SEQ ID NO:1.

5. The truncated GpA variant of claim 4, wherein the at least one amino acid substitution relative to SEQ ID NO:1 is at position 7, 10, 23, 25, 48, 49, 52, 53, 54, 57, 58, 59, 60, 62, 92, 98, 101, 106, 108, 121, 122, 134, 147, 193, 198, 217, 233, 236, 237, 250, 257, 281, 301, 311, 340, 344, 360, 362, 363, 364, 365, 366, 367, or 368, or a combination thereof.

6. The truncated GpA variant of claim 4 or 5, wherein the at least one amino acid substitution relative to SEQ ID NO:1 is H10R, Q23R, K25E, K48E, Q52R, Q54R, P57S, D58E, H59D, A60T, A62V, D91A, D92E, K98Q, E101K, Q108H, S121F, G122A, H134Q, R147H, K193R, C198A, C198S, C198V, D217E, N233S, H236Q, S250A, Q288E, R301Q, E344D, L360P, T362S,

A363V, D364E, L365E, H366R, Q367R, or S368P, or a combination thereof.

7. The truncated GpA variant of any preceding claim, wherein the at least one amino acid substitution relative to SEQ ID NO:1 comprises:

(a) a cysteine residue at position 49, 52, 225, 257, 281, or 340, or a combination thereof; or

(b) a lysine residue at position 7, 53, 54, 57, 58, 98, 106, 233, 250, 257, 281, 311 or 340, or a combination thereof.

8. The truncated GpA variant of any preceding claim, wherein the variant has a catalytic activity equal to or greater than wild-type GpA.

9. The truncated GpA variant of claim 1, further comprising a histidine tag, a SUMO tag, an albumin-binding domain, or a combination thereof.

10. The truncated GpA variant of claim 1, further comprising three tandem soluble domains of TRAIL.

11. The truncated GpA variant of claim 10, wherein the soluble domains of TRAIL comprise residues 115-281 of human TRAIL.

12. A nucleic acid molecule encoding the truncated GpA variant of claim 1.

13. An expression vector comprising the nucleic acid molecule of claim 12.

14. A host cell comprising the nucleic acid molecule of claim 12.

15. A pharmaceutical composition comprising the truncated GpA variant of claim 1 and a pharmaceutically acceptable excipient.

16. The pharmaceutical composition of claim 15, further comprising a stable form of TRAIL.

17. The pharmaceutical composition of claim 16, wherein the stable form of TRAIL comprises the FOLDON sequence GYIPEAPRDGQAYVRKDGEWVLLSTFL (SEQ ID NO:85).

18. A method of treating cancer comprising administering to a subject in need of treatment an effective amount of the truncated GpA variant of claim 1 thereby treating the subject's cancer.

19. The method of claim 18, wherein the cancer is selected from non-Hodgkin's lymphoma, leukemia, acute lymphoblastic leukemia, acute myeloid leukemia, B cell lymphoma, Burkitt's lymphoma, chronic myelocytic leukemia, chronic lymphocytic leukemia, and hairy cell leukemia.

20. The method of claim 18, further comprising administering a stable form of TRAIL.

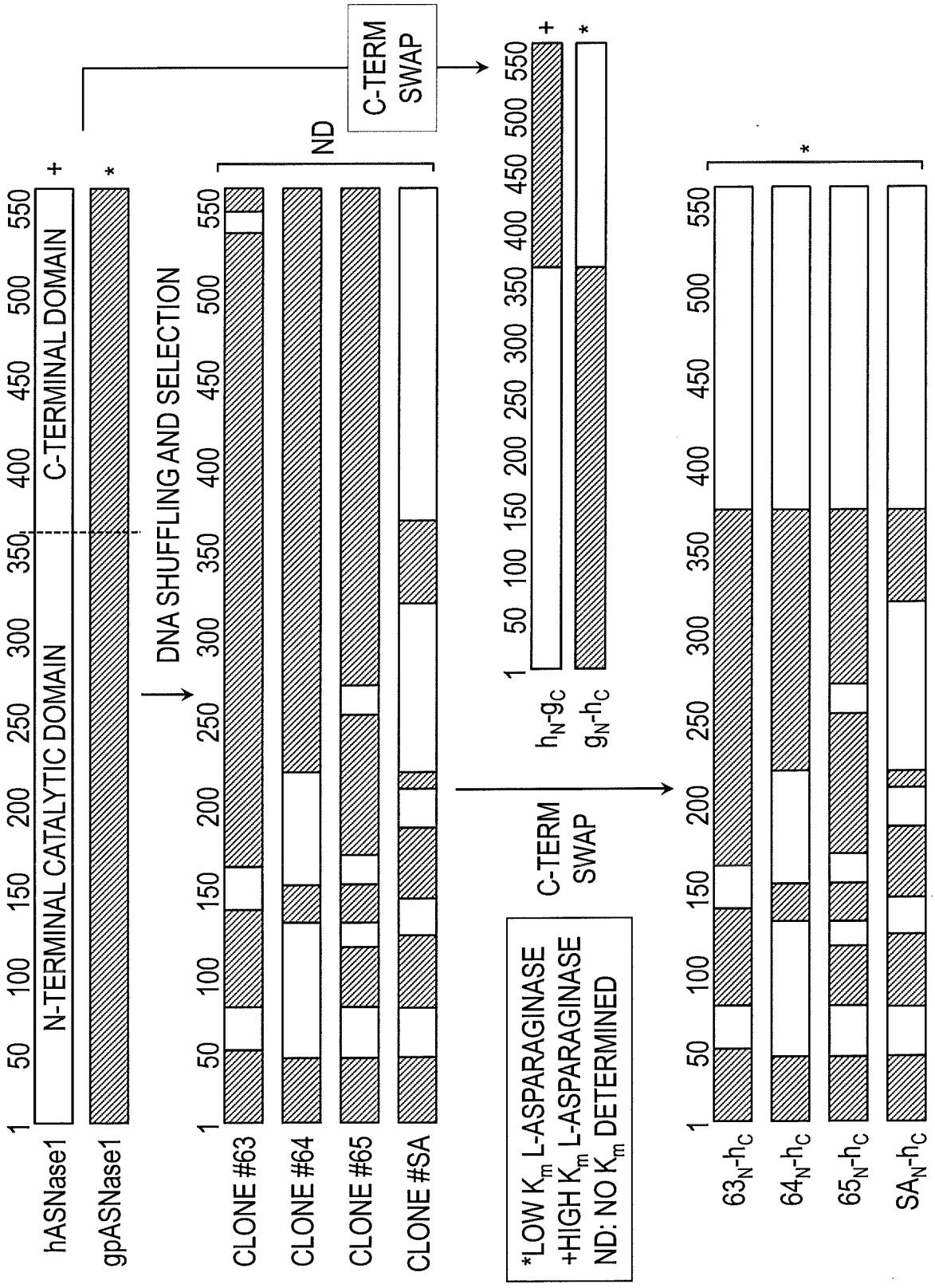
1
50
100
150
200
250
300
350
400
450
500
550
N-TERMINAL CATALYTIC DOMAIN
C-TERMINAL DOMAIN
+
*
hASNase1
gpASNase1
DNA SHUFFLING AND SELECTION
ND
C-TERM SWAP
CLONE #63
CLONE #64
CLONE #65
CLONE #SA
h_N-g_C
g_N-h_C
C-TERM SWAP
*LOW K_m L-ASPARAGINASE
+HIGH K_m L-ASPARAGINASE
ND: NO K_m DETERMINED
63_N-h_C
64_N-h_C
65_N-h_C
SA_N-h_C

FIG. 1

2/3

```
hASN      MARAVGPERRLLAVYTGGTIGMRSELGVLVPGTGLAAILRTLPMFHDEEH
GpA       MARASGSERHLLLIYTGGTLGMQSKGGVLVPGPGLVTLLRTLPMFHDKEF
hN-gC     MARAVGPERRLLAVYTGGTIGMRSELGVLVPGTGLAAILRTLPMFHDEEH
gN-hC     MARASGSERHLLLIYTGGTLGMQSKGGVLVPGPGLVTLLRTLPMFHDKEF
63-hC     MARASGSERHLLLIYTGGTLGMQSKGGVLVPGPGLVTLLRTLPMFHDKEF
64-hC     MARASGSERHLLLIYTGGTLGMQSKGGVLVPGPGLVTLLRTLPMFHDEEH
65-hC     MARASGSERHLLLIYTGGTLGMQSKGGVLVPGPGLVTLLRTLPMFHDEEH
SA-hC     MARASGSERHLLLIYTGGTLGMQSKGGVLVPGPGLVTLLRTLPMFHDEEH
          ****.*.**.**.:*****:**.*:.******.**.::**********:*.

hASN      ARARGLSEDTLVLPPASRNQRILYTVLECQPLFDSSDMTIAEWVCLAQTI
GpA       AQAQGLPDHALALPPASHGPRVLYTVLECQPLLDSSDMTIDDWIRIAKII
hN-gC     ARARGLSEDTLVLPPASRNQRILYTVLECQPLFDSSDMTIAEWVCLAQTI
gN-hC     AQAQGLPDHALALPPASHGPRVLYTVLECQPLLDSSDMTIDDWIRIAKII
63-hC     ARARGLSEDTLVLPPASRNQRILYTVLECQPLLDSSDMTIDDWIRIAKII
64-hC     ARARGLSEDTLVLPPASRNQRILYTVLECQPLLDSSDMTIDDWIRIAKII
65-hC     ARARGLSEDTLVLPPASRNQRILYTVLECQPLFDSSDMTIAEWVCLAQTI
SA-hC     ARARGLSEDTLVLPPASRNQRILYTVLECQPLLDSSDMTIDDWIRIAKII
          *.*.**.: :*.*****.. *:**********:*******.:*: :*: *

hASN      KRHYEQYHGFVVIHGTDTMAFAASMLSFMLENLQKTVILTGAQVPIHALW
GpA       ERHYEQYQGFVVIHGTDTMASGASMLSFMLENLHKPVILTGAQVPIRVLW
hN-gC     KRHYEQYHGFVVIHGTDTMAFAASMLSFMLENLQKTVILTGAQVPIHALW
gN-hC     ERHYEQYQGFVVIHGTDTMASGASMLSFMLENLHKPVILTGAQVPIRVLW
63-hC     ERHYEQYQGFVVIHGTDTMAFAASMLSFMLENLHKPVILTGAQVPIRVLW
64-hC     ERHYEQYQGFVVIHGTDTMAFAASMLSFMLENLHKPVILTGAQVPIHALW
65-hC     KRHYEQYHGFVVIHGTDTMAFAASMLSFMLENLHKPVILTGAQVPIHALW
SA-hC     ERHYEQYHGFVVIHGTDTMAFAASMLSFMLENLQKTVILTGAQVPIRVLW
          :******:************ .**********:*.**********..**

hASN      SDGRENLLGALLMAGQYVIPEVCLFFQNQLFRGNRATKVDARRFAAFCSP
GpA       NDARENLLGALLVAGQYIIPEVCLFMNSQLFRGNRVTKVDSQKFEAFCSP
hN-gC     SDGRENLLGALLMAGQYVIPEVCLFFQNQLFRGNRATKVDARRFAAFCSP
gN-hC     NDARENLLGALLVAGQYIIPEVCLFMNSQLFRGNRVTKVDSQKFEAFCSP
63-hC     NDARENLLGALLVAGQYIIPEVCLFMNSQLFRGNRVTKVDSQKFEAFCSP
64-hC     SDGRENLLGALLVAGQYIIPEVCLFMNSQLFRGNRVTKVDSQKFEAFCSP
65-hC     SDGRENLLGALLMAGQYVIPEVCLFFQNQLFRGNRATKVDARRFAAFCSP
SA-hC     NDARENLLGALLVAGQYIIPEVCLFMSNQLFRGNRATKVDARRFAAFCSP
          .*.*********:****:*******::.*******.****:..* *****

hASN      NLLPLATVGADITINRELVRKVDGKAGLVVHSSMEQDVGLLRLYPGIPAA
GpA       NLSPLATVGADVTIAWDLVRKVKWKDPLVVHSNMEHDVALLRLYPGIPAS
hN-gC     NLLPLATVGADITINRELVRKVDGKAGLVVHSSMEQDVGLLRLYPGIPAA
gN-hC     NLSPLATVGADVTIAWDLVRKVKWKDPLVVHSSMEQDVGLLRLYPGIPAS
63-hC     NLSPLATVGADVTIAWDLVRKVKWKDPLVVHSSMEQDVGLLRLYPGIPAS
64-hC     NLSPLATVGADVTIAWDLVRKVKWKDPLVVHSSMEQDVGLLRLYPGIPAS
65-hC     NLLPLATVGADVTIAWDLVRKVKWKDPLVVHSNMEHDVALLRLYPGIPAA
SA-hC     NLSPLATVGADITINRELVRKVDGKAGLVVHSSMEQDVGLLRLYPGIPAA
          **********:**  .:*****.  *  *****.**:**.**********:

hASN      LVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRVATERGLVIVNCTHCL
GpA       LVRAFLQPPLKGVVLETFGSGNGPSKPDLLQELRAAAQRGLIMVNCSQCL
hN-gC     LVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRVATERGLVIVNCTHCL
gN-hC     LVRAFLQPPLKGVVLETFGSGNGPSKPDLLQELRAAAERGLIMVNCSQCL
63-hC     LVRAFLQPPLKGVVLETFGSGNGPSKPDLLQELRAAAERGLIMVNCSQCL
64-hC     LVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRAAAERGLIMVNCSQCL
65-hC     LVRAFLQPPLKGVVLETFGSGNGPSKPDLLQELRAAAERGLIMVNCSQCL
SA-hC     LVRAFLQPPLKGVVMETFGSGNGPTKPDLLQELRVATERGLVIVNCTHCL
          **************:*********:*********.*::***::***::**
```

FIG. 2A

```
hASN      QGAVTTDYAAGMAMAGAGVISGFDMTSEAALAKLSYVLGQPGLSLDVRKE
GpA       RGSVTPGYAT--SLAGANIVSGLDMTSEAALAKLSYVLGLPELSLERRQE
hN-gC     QGAVTTDYAAGMAMAGAGVISGFDMTSEAALAKLSYVLGQPGLSLDVRKE
gN-hC     RGSVTPGYAT--SLAGANIVSGLDMTSEAALAKLSYVLGLPELSLERRQE
63-hC     RGSVTPGYAT--SLAGANIVSGLDMTSEAALAKLSYVLGLPELSLERRQE
64-hC     RGSVTPGYAT--SLAGANIVSGLDMTSEAALAKLSYVLGLPELSLERRQE
65-hC     RGSVTPGYAT--SLAGANIVSGLDMTSEAALAKLSYVLGLPELSLERRQE
SA-hC     QGAVTTGYAT--SLAGANIVSGLDMTSEAALAKLSYVLGLPELSLERRQE
          .*:**..**:  ::***.::**:****************.*.***: *:*

hASN      LLTKDLRGEMTPPSVEERRPSLQGNTLGGGVSWLLSLSGSQEADALRNAL
GpA       LLAKDLRGEMTLPTADLHQSSPPGSTLGQGVARLFSLFGCQEEDSVQDAV
hN-gC     LLTKDLRGEMTLPTADLHQSSPPGSTLGQGVARLFSLFGCQEEDSVQDAV
gN-hC     LLAKDLRGEMTPPSVEERRPSLQGNTLGGGVSWLLSLSGSQEADALRNAL
63-hC     LLAKDLRGEMTPPSVEERRPSLQGNTLGGGVSWLLSLSGSQEADALRNAL
64-hC     LLAKDLRGEMTPPSVEERRPSLQGNTLGGGVSWLLSLSGSQEADALRNAL
65-hC     LLAKDLRGEMTPPSVEERRPSLQGNTLGGGVSWLLSLSGSQEADALRNAL
SA-hC     LLAKDLRGEMTPPSVEERRPSLQGNTLGGGVSWLLSLSGSQEADALRNAL
          **:******** *:.: ...*  *.*** **:.*:** *.** *::.:*:

hASN      VPSLACAAAHAGDVEALQALVELGSDLGLVDFNGQTPLHAAARGGHTEAV
GpA       MPSLALALAHAGELEALQALMELGSDLRLKDSNGQTLLHVAARNGRDGVV
hN-gC     MPSLALALAHAGELEALQALMELGSDLRLKDSNGQTLLHVAARNGRDGVV
gN-hC     VPSLACAAAHAGDVEALQALVELGSDLGLVDFNGQTPLHAAARGGHTEAV
63-hC     VPSLACAAAHAGDVEALQALVELGSDLGLVDFNGQTPLHAAARGGHTEAV
64-hC     VPSLACAAAHAGDVEALQALVELGSDLGLVDFNGQTPLHAAARGGHTEAV
65-hC     VPSLACAAAHAGDVEALQALVELGSDLGLVDFNGQTPLHAAARGGHTEAV
SA-hC     VPSLACAAAHAGDVEALQALVELGSDLGLVDFNGQTPLHAAARGGHTEAV
          :**** * ****::******:****** * * **** **.***.*.  .*

hASN      TMLLQRGVDVNTRDTDGFSPLLLAVRGRHPGVIGLLREAGASLSTQELEE
GpA       TMLLHRGMDVNARDRDGLSPLLLAVQGRHRECIRLLRKAGACLSPQDLKD
hN-gC     TMLLHRGMDVNARDRDGLSPLLLAVQGRHRECIRLLRKAGACLSPQDLKD
gN-hC     TMLLQRGVDVNTRDTDGFSPLLLAVRGRHPGVIGLLREAGASLSTQELEE
63-hC     TMLLQRGVDVNTRDTDGFSPLLLAVRGRHPGVIGLLREAGASLSTQELEE
64-hC     TMLLQRGVDVNTRDTDGFSPLLLAVRGRHPGVIGLLREAGASLSTQELEE
65-hC     TMLLQRGVDVNTRDTDGFSPLLLAVRGRHPGVIGLLREAGASLSTQELEE
SA-hC     TMLLQRGVDVNTRDTDGFSPLLLAVRGRHPGVIGLLREAGASLSTQELEE
          ****:**:***:** **:*******.*** * ***:***.**.*:*::**

hASN      AGTELCRLAYRADLEGLQVWWQAGADLGQPGYDGHSALHVAEAAGNLAVV
GpA       AGTELCRLASRADMEGLQAWGQAGADLQQPGYDGRSALCVAEAAGNQEVL
hN-gC     AGTELCRLASRADMEGLQAWGQAGADLQQPGYDGRSALCVAEAAGNQEVL
gN-hC     AGTELCRLAYRADLEGLQVWWQAGADLGQPGYDGHSALHVAEAAGNLAVV
63-hC     AGTELCRLAYRADLEGLQVWWQAGADLGQPGYDGHSALHVAEAAGNLAVV
64-hC     AGTELCRLAYRADLE--------------------------------
65-hC     AGTELCRLAYRADLEGLQVWWQAGADLGQPGYDGHSALHVAEAAGNLAVV
SA-hC     AGTELCRLAYRADLEGLQVWWQAGADLGQPGYDGHSALHVAEAAGNLAVV
          *********:***:*
```

			Identity to hASN
hASN	AFLQSLEGAVGAQAPCPEVLPGV	(SEQ ID NO:2)	100%
GpA	ALLRNL-ALVG-----PEVPPAI	(SEQ ID NO:1)	69.8%
hN-gC	ALLRNL-ALVG-----PEVPPAI	(SEQ ID NO:38)	86.5%
gN-hC	AFLQSLEGAVGAQAPCPEVLPGV	(SEQ ID NO:37)	83.4%
63-hC	AFLQSLEGAVGAQAPCPEVLPGV	(SEQ ID NO:39)	85.7%
64-hC	----------	(SEQ ID NO:40)	87.1%
65-hC	AFLQSLEGAVGAQAPCPEVLPGV	(SEQ ID NO:41)	91.1%
SA-hC	AFLQSLEGAVGAQAPCPEVLPGV	(SEQ ID NO:42)	91.6%

FIG. 2B