

## METHODS FOR PRODUCTION OF HUMAN RECOMBINANT ARGINASE 1 AND USES THEREOF

### TECHNICAL FIELD

**[0001]** The present disclosure generally relates to enzyme replacement therapy and treatment of Arginase 1 deficiency or hyperargininemia. The disclosure also encompasses methods for producing human recombinant Arginase 1. The Arginase 1 can also be used in the treatment of cancer.

### BACKGROUND

**[0002]** Arginase 1 deficiency or hyperargininemia is a rare disorder of amino acid metabolism caused by a deficiency in the enzyme Arginase 1. Arginase 1 is one of the 6 enzymes critical to the normal function of the urea cycle; it catalyzes the conversion of L-arginine to urea and ornithine in the last step of the cycle. The ornithine then reenters the mitochondrion to continue the cycle.

**[0003]** Arginase 1 is found predominantly in red blood cells (RBCs) and the liver. ARG1 is the only gene in which mutations are currently known to cause Arginase 1 deficiency. Clinically, Arginase 1 deficiency is characterized by a slow deterioration of the cerebral cortex and pyramidal tracts that leads to progressive dementia, psychomotor retardation, spastic diplegia, seizures, and growth failure. If left untreated the disease progresses to severe spasticity, loss of ambulation, loss of bowel and bladder control, and severe intellectual disability. Patients with Arginase 1 deficiency typically have elevated blood arginine levels (3 to 4 times the upper limit of normal [ULN]), mild hyperammonemia, and a mild increase in urinary orotic acid. Most patients have no detectable Arginase 1 enzyme activity in RBCs (< 1% of normal).

**[0004]** The current treatment of Arginase 1 deficiency is focused on the maintenance of plasma arginine concentrations at a level as near to normal as possible through lifelong dietary protein restriction. Protein intake is limited to the minimum required to maintain protein biosynthesis and growth. Half or more of dietary protein is given in the form of an arginine free essential amino acid mixture. Such dietary modification can reduce plasma arginine levels in most patients, but the diet is unpalatable, expensive, and difficult to maintain and manage especially in growing children.

[0005] The paucity of treatment options for Arginase 1 deficient patients highlights the significant unmet need for a therapy that will lower arginine levels to within the normal range and promote the lifelong maintenance of normal arginine levels. The development of such a therapeutic could be useful in the attempt to minimize exposure to the neurotoxic effects of arginine and its metabolites and offer the potential for normal neurocognitive development in these patients.

[0006] In addition to treatment of Arginase 1 deficiency or hyperargininemia, the Arginase produced by these methods can be used to treat other diseases. Arginase 1 has been used in clinical trials investigating its use in cancer treatment and in combination with immune-oncology agents such as pembrolizumab.

## SUMMARY

### Production of Arginase

[0007] One aspect of the present invention relates to methods for producing and/or purifying recombinant human Arginase proteins. In one or more embodiments, the recombinant human Arginase protein is recombinant human Arginase 1 (rhARG1) (SEQ ID NO: 1; shown Figure 1(a)). In other embodiments, the recombinant human Arginase protein is recombinant human Arginase 2 (rhARG2) (SEQ ID NO: 3; shown Figure 1(c)). Although specific reference is made herein to rhARG1, the methods, formulations and uses described herein can also be applied to rhARG2.

[0008] Human Arginase 1 and 2 proteins that are subjected to the methods of the invention have two  $Mn^{2+}$  sites; either or both sites can be substituted to generate a modified Arginase 1 or 2 protein with a non-native metal cofactor. In some embodiments, the protein displays a  $k_{cat}/K_M$  greater than  $200 \text{ mM}^{-1} \text{ s}^{-1}$  at pH 7.4. In a particular embodiment, the protein displays a  $k_{cat}/K_M$  in the range of about  $200 \text{ mM}^{-1} \text{ s}^{-1}$  to about  $4,000 \text{ mM}^{-1} \text{ s}^{-1}$  at pH 7.4. In another embodiment, the protein displays a  $k_{cat}/K_M$  in the range of about  $400 \text{ mM}^{-1} \text{ s}^{-1}$  to about  $2,500 \text{ mM}^{-1} \text{ s}^{-1}$  at pH 7.4 at  $37^\circ \text{ C}$ . In a particular embodiment, the present invention contemplates a protein comprising an amino acid sequence of human Arginase 1 or 2 and a non-native metal cofactor, wherein said protein exhibits a  $k_{cat}/K_M$  greater than  $400 \text{ mM}^{-1} \text{ s}^{-1}$  at  $37^\circ \text{ C}$ , pH 7.4. Exemplary  $k_{cat}/K_M$  values include about 200, about 250, about 300, about 350, about 400, about 450, about 500, about 550, about 600, about 650, about 700, about 800, about

900, about 1,000, about 1,100, about 1,200, about 1,500, about 2,000, about 2,500, about 3,000, about 3,500 and about 4,000  $\text{mM}^{-1} \text{s}^{-1}$  at pH 7.4 at 37° C.

**[0009]** In one or more embodiments, provided is a method for producing recombinant cobalt-substituted human Arginase protein (Co-rhARG). In one or more embodiments, the method comprises the following: strain fermentation of *E. coli* cells expressing rhARG1, substitution of cobalt for manganese in rhARG1 to provide a Co-Arginase 1 intermediate (Co-rhARG1), purification of this Co-Arginase 1 intermediate and PEGylation of Co-Arginase 1 intermediate to form the drug substance (Co-rhARG1-PEG). In one or more embodiments, the Co-rhARG1-PEG comprises pegzilarginase.

**[0010]** In one or more embodiments, the method comprises several steps: culturing *E. coli* cells in a bioreactor that produce the recombinant human Arginase (rhARG), lysing the *E. coli* cells, removing cell debris from the lysate, loading the cell lysate on a cation exchange column, eluting the recombinant human Arginase protein (rhARG) with a high salt solution, incubating the eluted recombinant human Arginase protein (rhARG) with a cobalt salt to form the cobalt-substitute recombinant human Arginase protein (Co-rhARG), applying the cobalt-substitute recombinant human Arginase protein (Co-rhARG) to an anion exchange column and collecting the flow-through, adding the flow-through onto a third chromatography column, and eluting the cobalt-substitute recombinant human Arginase protein (Co-rhARG) from the third chromatography column with a high salt concentration.

**[0011]** In one or more embodiments, the methods for producing recombinant cobalt-substitute human Arginase protein (Co-rhARG) comprises loading up to 60 grams of the recombinant human Arginase protein (rhARG) onto the cation exchange column per liter of cation exchange resin.

**[0012]** In one or more embodiments, the methods for producing recombinant cobalt-substitute human Arginase protein (Co-rhARG) comprises eluting the recombinant human Arginase protein (rhARG) from the cation exchange column using a high salt solution of up to about 0.5 M salt concentration. In some embodiments, the recombinant human Arginase protein (rhARG) is eluted from the cation exchange column using a high salt solution of 0.1 M concentration. In some embodiments, the recombinant human Arginase protein (rhARG) is eluted from the cation exchange column using a gradient of about 0.0 to about 0.5 M salt concentration. In some embodiments, the recombinant human Arginase protein (rhARG) is

eluted from the cation exchange column using a gradient of about 0.0 to about 0.2 M salt concentration.

[0013] In one or more embodiments, the methods for producing recombinant cobalt-substitute human Arginase protein (Co-rhARG) comprises incubating the recombinant human Arginase protein (rhARG) that was eluted from the cation exchange column with the cobalt salt comprising  $\text{Co}^{2+}$ . In some embodiments, the cobalt salt comprises  $\text{CoCl}_2$ .

[0014] In one or more embodiments, the methods for producing recombinant cobalt-substitute human Arginase protein (Co-rhARG) comprises adding the recombinant cobalt-substitute human Arginase protein (Co-rhARG) flow-through onto the third chromatography column comprising a multimodal chromatography (MMC) column.

[0015] In one or more embodiments, the methods for producing recombinant cobalt-substitute human Arginase protein (Co-rhARG) comprises reacting the recombinant cobalt-substitute human Arginase protein (Co-rhARG) or the recombinant cobalt-substitute human Arginase protein (Co-rhARG) with a PEGylation reactant to provide a PEGylated protein. In some embodiments, the PEGylated protein comprises one or more of PEGylated amino acid residues at K16, K32, K38, K40, K47, K67, K74, K82, L87, K88, K152, K154, K171, K222, K223, K312 and K321. In some embodiments, the PEGylated protein comprises one or more of about 15% to about 60% of K16, about 35% to about 80% of K32, about 20% to about 85% of K38, about 10% to about 60% of K40, about 10% to about 60% of K47, about 40% to about 90% of K67, about 30% to about 95% of K74, about 30% to about 98% of K82, about 15% to about 65% of K87, about 25% to about 70% of K88, about 25% to about 85% of K152, about 15% to about 65% of K154, about 20% to about 75% of K171, 0% to about 30% of K222, 0% to about 35% of K223, 0% to about 45% of K312, and 0% to about 45% of K321 is PEGylated. In some embodiments, the PEGylated protein comprises PEGylated amino acid residues at least at K16, K32, K38, K40, K47, K67, K74, K82, L87, K88, K152, K154, K171, K312 and K321. In some embodiments, the PEGylated protein does not have PEGylated amino acid residues at K3, K149, K190, K195, K29, K265 and K283.

[0016] One or more embodiments of Co-rhARG1-PEG relate to a cobalt-substituted, PEGylated human recombinant Arginase 1 enzyme expressed in *E. coli* that is formulated for intravenous (IV) or subcutaneous (SC) administration. The replacement of the native manganese ( $\text{Mn}^{2+}$ ) with cobalt ( $\text{Co}^{2+}$ ) in the active site of Arginase 1 enhances the stability and

catalytic activity at physiological pH. PEGylation also extends the circulating half-life ( $t_{1/2}$ ) of recombinant Arginase 1.

[0017] In various embodiments, the methods comprise culturing *E. coli* cells in a bioreactor to produce recombinant human Arginase 1, lysing the *E. coli* cells and purifying the recombinant human Arginase 1 (See Figures 2 and 3). Purification of Co-Arginase 1 intermediate can be performed by a purification procedure that includes one or more of the following steps: cell disruption by high pressure homogenization, homogenate clarification, SP Sepharose FF cation exchange capture chromatography, cobalt exchange, ultrafiltration/diafiltration, Q Sepharose FF anion exchange flowthrough chromatography, Capto MMC multimodal chromatography, and ultrafiltration/diafiltration. The purified Co-Arginase 1 intermediate may be processed to form PEGylated drug substance or frozen and stored for later conversion to drug substance.

[0018] In preferred embodiments of the methods, an *E. coli* lysate containing rhARG1 is loaded onto a cation exchange (CEX) chromatography column (also called "Column 1") to capture the rhARG1, and which is then eluted with a high salt solution to provide a first protein product ("First Protein Product").

[0019] In one or more embodiments, the method further comprises loading the First Protein Product onto an anion exchange (AEX) chromatography column (also called "Column 2") and collecting the flow-through to provide a second protein product ("Second Protein Product"). In another aspect of the methods, the method further comprises loading the Second Protein Product onto a multimodal chromatography (MMC) column which captures the Arginase 1 and is then eluted to provide a third protein product ("Third Protein Product"). In some embodiments, this third chromatography column (also called "Column 3") may be a size exclusion chromatography (SEC) column.

[0020] Various embodiments comprise changing the native manganese co-enzyme of Arginase for a cobalt co-enzyme. Cobalt substitution (also called cobalt loading) can be performed at any step in the manufacturing process. For example, Arginase 1 cobalt loading can be performed on either the *E. coli* lysate, the First Protein Product, the Second Protein Product, the Third Protein Product, or at any step on a PEGylated Arginase 1. In other embodiments the cobalt loading can be performed on Arginase 1 eluted from Column 1, or Arginase 1 eluted from Column 2, or Arginase 1 eluted from Column 3. Cobalt loading can be performed on an Arginase 1 that has been eluted from a CEX column, Arginase 1 eluted from

an AEX column, Arginase 1 eluted from an MMC column, or Arginase 1 eluted from an SEC column.

[0021] Cobalt loading of Arginase 1 can be done with a variety of solutions containing cobalt and at a variety of temperatures. In one or more embodiments, the cobalt salt comprises a  $\text{Co}^{2+}$  such as  $\text{CoCl}_2$ . In a preferred embodiment, cobalt loading of Arginase 1 is performed with  $\text{CoCl}_2$  at or about room temperature, such as about 15 to about 25°C or about 20 to about 25°C. The rate of cobalt loading can be manipulated by increasing or decreasing the temperature of the reaction. Cobalt loading can also be performed at a range of pH values.

[0022] One aspect of the present disclosure pertains to varying the conditions related to CEX chromatography (Column 1). The amount of protein loaded onto Column 1 can be increased or decreased to select for different Arginase 1 charge variants. Load factors can be manipulated to cause a shift towards more desirable CEX charge species profiles. Load factors (amount of protein in grams/volume of CEX column resin in liters) up to about 60 g/L can produce Arginase 1 with high specific activity. In various embodiments, the load factor is up to about 10 g/L, about 20 g/L, about 30 g/L, about 40 g/L, about 50 g/L or about 60 g/L.

[0023] In a preferred embodiment, Arginase 1 is first captured on Column 1 followed by sequential purification on Column 2, then Column 3. In an alternative embodiment, the *E. coli* lysate can be loaded onto an AEX column (for example Column 2) and the flow through applied to a CEX column to capture the Arginase 1. In another embodiment, cobalt loading of Arginase 1 can occur after the PEGylation reaction. Also, other chromatography columns can be used to replace the MMC column, such as an SEC column.

[0024] One aspect of the present invention relates to methods for producing recombinant cobalt-substitute human Arginase protein (Co-rhARG). In one or more embodiments, the recombinant human Arginase protein (rhARG) comprises an amino acid sequence that is at least 98% identical to SEQ ID NO:1. The method comprises several steps: culturing *E. coli* cells in a bioreactor that produce the recombinant human Arginase (rhARG), lysing the *E. coli* cells, removing cell debris from the lysate, loading the cell lysate on a cation exchange column, eluting the recombinant human Arginase protein (rhARG) with a high salt solution, incubating the eluted recombinant human Arginase protein (rhARG) with a cobalt salt to form the cobalt-substitute recombinant human Arginase protein (Co-rhARG), applying the cobalt-substitute recombinant human Arginase protein (Co-rhARG) to an anion exchange column and collecting the flow-through, adding the flow-through onto a third chromatography

column, eluting the cobalt-substitute recombinant human Arginase protein (Co-rhARG) from the MMC column with a high salt solution, reacting a molar excess of methoxy PEG succinimidyl carboxymethyl ester and removing excess PEG.

## 5 **Recombinant Human Arginase 1, Pharmaceutical Compositions and Formulations**

[0025] Another aspect of the present invention relates to rhARG1, Co-rhARG1 and/or Co-rhARG1-PEG produced by the methods described herein, or a composition comprising the same.

[0026] In one or more embodiments, the protein is covalently linked to a polyethylene glycol at one or more of K16, K32, K38, K40, K47, K67, K74, K82, L87, K88, K152, K154, K171, K222, K223, K312 and K321.

[0027] Another aspect of the present invention relates to a composition comprising a recombinant human Arginase (rhARG) protein, wherein the protein comprises an amino acid sequence that is at least 98% identical to SEQ ID NO: 1, wherein the protein is in a complex with a non-native metal cofactor, wherein the non-native metal cofactor is cobalt, and wherein the protein is covalently linked to a polyethylene glycol at one or more of K16, K32, K38, K40, K47, K67, K74, K82, L87, K88, K152, K154, K171, K222, K223, K312 and K321.

[0028] In one or more embodiments, the protein comprises an amino acid substitution at a position selected from the group consisting of: H100, D123, H125, D127, D231, D233, W121, D180, S229, C302, and E255.

[0029] In one or more embodiments, the protein comprises at least one amino acid substitution selected from the group consisting of: D180S, S229C, S229G, C302F, C302I, E255Q, D180E, and S229A.

[0030] In one or more embodiments, the at least one amino acid substitution is C302.

[0031] In one or more embodiments, the protein comprises at least two amino acid substitutions.

[0032] In one or more embodiments, the protein is a truncated arginase I protein.

[0033] In one or more embodiments, the protein further comprises an exogenous protein fragment.

[0034] In one or more embodiments, the exogenous protein fragment comprising the Fc region of an immunoglobulin or a portion of the Fc region of an immunoglobulin.

[0035] In one or more embodiments, the specific activity of Co-rhARG-PEG is in the range of about 400 U/mg to about 700 U/mg.

[0036] In one or more embodiments, the protein displays a  $k_{cat}/K_m$  for the hydrolysis of arginine in the range of about  $200 \text{ mM}^{-1} \text{ s}^{-1}$  to about  $4,000 \text{ mM}^{-1} \text{ s}^{-1}$  at pH 7.4 when assayed *in vitro*.

[0037] In one or more embodiments, the protein displays a  $k_{cat}/K_M$  for the hydrolysis of arginine in the range of about  $400 \text{ mM}^{-1} \text{ s}^{-1}$  to about  $2,500 \text{ mM}^{-1} \text{ s}^{-1}$  at pH 7.4 when assayed *in vitro*.

[0038] In one or more embodiments, the molar ratio of PEG:Co-rhARG is in the range of about 7 moles/mole to about 15 moles/mole.

[0039] In one or more embodiments, the free PEG concentration is less than or equal to  $100 \text{ }\mu\text{g/mL}$ .

[0040] In one or more embodiments, the total cobalt content of the composition is in the range of about  $9 \text{ }\mu\text{g/mL}$  to about  $15 \text{ }\mu\text{g/mL}$ .

[0041] In one or more embodiments, the composition produces at least 9 peaks when loaded on imaging capillary isoelectric focusing (iCIEF), wherein peak 1 is less than 20%, peak 2 is less than 30%, Peak 3+4 is in the range of 10-30%, peak 5 is in the range of 15-30%, peak 6 is in the range of 10-25%, peak 7 is less than 25%, peak 8 is less than 15%, and peak 9 is less than 8%.

[0042] In one or more embodiments, the composition produces at least 9 peaks when loaded on icIEF, wherein peak 1 is in the range of 5-7%, peak 2 is in the range of 8-11%, Peak 3+4 in the range of 16-20%, peak 5 in the range of 21-24%, peak 6 in the range of 21-22%, peak 7 in the range of 14-15%, peak 8 in the range of 5-8%, and peak 9 in the range of 2-3%.

[0043] Another aspect of the present invention relates to pharmaceutical compositions comprising the rhARG1, Co-rhARG1 and/or Co-rhARG1-PEG, and a pharmaceutically acceptable carrier. In one or more embodiments, the composition is formulated for intravenous or subcutaneous administration. In one or more embodiments, the composition comprises potassium phosphate, sodium chloride and glycerol. In one or more embodiments, the composition comprises about 50 mM NaCl, about 1 mM  $\text{K}_2\text{HPO}_4$ , about 4 mM  $\text{KH}_2\text{PO}_4$ , and about 1.5% w/v glycerol.

**Method of Treating Arginase 1 deficiency**

[0044] Another aspect of the present invention relates to the administration of a recombinant human Arginase 1 such as Co-rhARG1-PEG. Such administration can be by any suitable method, including IV or SC administration. In one or more embodiments of this aspect, the dose of Co-rhARG1-PEG is determined by a particular algorithm:

[0045] In one or more embodiments of this algorithm, the patient initiates therapy at 0.10 mg/kg. Plasma arginine levels are monitored. If the plasma arginine level is  $>150 \mu\text{M}$ , the dose will be increased to 0.20 mg/kg. If the plasma arginine level is  $<50 \mu\text{M}$ , the dose is decreased to 0.05 mg/kg. Otherwise, the patient remains on the dose at 0.10 mg/kg.

[0046] In one or more embodiments of this algorithm, dose modifications are as follows:

- If the plasma arginine level is  $>150 \mu\text{M}$ , a single 168-hour sample will be used to increase the dose by 2 dose levels in the table below (not to exceed 0.20 mg/kg) if the 2 doses prior to this sample were a) the same dose level in mg/kg, and b) consecutive (with no missed doses).
- If the plasma arginine levels from 2 sequential 168-hour samples (regardless of missed doses) are both  $<50 \mu\text{M}$ , the dose is decreased by 1 dose level in the table below, not to decrease below 0.05 mg/kg.

| Dose Level <sup>(a)</sup> | Dose       |
|---------------------------|------------|
| 1 (Minimum Possible Dose) | 0.05 mg/kg |
| 2 (Starting Dose)         | 0.10 mg/kg |
| 3                         | 0.15 mg/kg |
| 4 (Maximum Possible Dose) | 0.20 mg/kg |

(a) Pegzilarginase dosing starts at level 2, 0.10 mg/kg. Dose increases, when required, are by 2 dose levels. Dose decreases are by 1 dose level.

**BRIEF DESCRIPTION OF THE DRAWINGS**

[0047] Further features of the present invention will become apparent from the following written description and the accompanying figures, in which:

[0048] Figure 1 shows the amino acid and DNA sequence of Arginase 1, as well as the amino acid sequence of Arginase 2. Figure 1(a) shows the amino acid sequence of recombinant human Arginase 1 that was expressed in *E. coli*. (SEQ ID NO: 1); and Figure 1(b) shows the codon optimized DNA sequence (SEQ ID NO: 2) of recombinant human Arginase 1. The

expressed monomer of Arginase 1 is missing the N-terminal methionine found in native human Arginase 1 monomer. Figure 1(c) shows the amino acid sequence of Arginase 2 that is missing the N-terminal methionine found in native human Arginase 2 monomer.

5 [0049] Figure 2 is a schematic diagram of an exemplary process for the fermentation of *E. coli* and expression of Arginase 1.

10 [0050] Figure 3 is a schematic diagram of an exemplary process for purification of recombinant human Arginase 1, cobalt-substituted recombinant human Arginase 1 and PEGylated cobalt-substituted recombinant human Arginase 1. Figure 3(a) shows an exemplary process including a cation exchange column (Column 1), an anion exchange column (Column 2) and a Capto multimodal column (Column 3), as well as a cobalt loading step. Figure 3(b) shows PEGylation of the Co-Arginase 1 intermediate, followed by final filtering and formulation to provide the drug substance.

15 [0051] Figure 4 shows column chromatography purification of Arginase 1. Figure 4(a) shows loading the *E. coli* cell lysate onto a cation exchange column (Column 1), washing of the column, then elution with a high salt solution (to provide a First Protein Product). Protein loading and elution was assessed by measuring UV absorbance at 280 nm. Approximately 3 liters (L) of cell lysate was applied to the column, then the column washed with approx. 1.5 L of buffer, then elution was performed with less than about 1 L. Figure 4(b) shows loading of the Column 1 eluted Arginase 1 (the First Protein Product) onto an anion exchange column (Column 2), the protein concentration was measured using absorbance at 280 nm. Arginase 1 was collected in the flow through from this Column 2 to provide a Second Protein Product. Figure 4(c) shows capture of Arginase 1 onto the Capto Multimodal Cation Exchange column (Column 3) and elution with a high salt solution to provide a Third Protein Product.

25 [0052] Figure 5 shows the results of an analytical cation exchange HPLC method used to determine the charge heterogeneity profile of the Co-Arginase 1 intermediate sample eluted from Column 1 (also called First Protein Product). A 1 mg/ml sample of Arginase 1 was loaded onto a cation exchange column with a mobile phase of 20 mM MES, pH 6.0 buffer, with a flow rate of 1.0 mL/minute. A gradient of 0-500 mM NaCl was introduced over 40 minutes and the amount of protein eluted from this column was estimated by absorbance at 280 nm. Figure 5(a) shows a representative chromatogram of the charge heterogeneity species of Arginase 1. The Arginase 1 charge variants were eluted from this analytical HPLC column after 10-20 minutes. Figure 5(b) shows the same chromatogram as Figure 5(a) with greater

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magnification of the peaks. Figure 5(c) shows the assignment of Peak Numbers to the Arginase 1 cation exchange charge variants. Figure 5(d) shows a typical charge heterogeneity profile of drug substance resolved by an imaging capillary isoelectric focusing (iCIEF) method.

[0053] Figure 6 shows the results of a LC/MS method to determine Arginase 1 gluconylation variants produced by expression of rhARG in *E. coli*. The LC/MS analysis identifies unmodified Arginase 1 (monomer), gluconylated Arginase1, phosphogluconylated Arginase 1, and 2 times (2X) gluconylated Arginase 1. The trace is from two separate production runs of drug intermediate. The overlay of mass spectra was summed over 33-35 minutes on a RP LCMS at 35°C; Spectra are normalized to the peak intensities of the signals due to unmodified Arginase 1. Peak intensities of variants are proportional to relative abundance.

[0054] Figure 7 show the results of a gradient of 0.0 - 0.2 M NaCl applied to Column 1. Fractions were collected every 0.25 CV (Column Volume) through the gradient. The data represent two column 1 runs using two different lots of harvested cell slurry as feed material. The load factor used for the evaluations was 30 g/L. The gradient successfully separated different gluconoylated species while maintaining product recovery.

[0055] Figure 8 shows the enzyme activity of Co-Arginase 1 intermediate and Co-rhARG1-PEG drug substance. Figure 8(a) shows a representative enzyme kinetic analysis of Co-Arginase 1 intermediate (conversion of arginine to ornithine with substrate concentrations over a range of 0 - 2 mM at 37°C). Figure 8(b) shows a representative enzyme kinetic analysis for Co-rhARG1-PEG drug substance.

[0056] Figure 9 shows a pharmacokinetic analysis of Co-rhARG1-PEG drug substance. Figures 9(a) and (b) show mean ( $\pm$ SD) Arginase 1 concentration versus time profiles in patients following a single IV dose administration of Co-rhARG1-PEG: Part 1. Linear (a) and semi-log (b) plots are shown. Note that first mean BQL concentration is plotted at half of the LLOQ (0.125  $\mu$ g/mL). Mean circulating drug concentrations in all patients increased with escalating doses of Co-rhARG1-PEG. Figures 9(c) - 9 (f) show mean ( $\pm$ SD) Co-rhARG1-PEG concentration versus time profiles in patients following QW (weekly) IV dose administration of Co-rhARG1-PEG: Part 2. Linear plots for week 1 (c) and week 8 (d); semi-log plots for week 1 (e) and week 8 (f).

[0057] Figure 10 shows three representative integrated plots (a, b, c) for pharmacokinetics (PK) and pharmacodynamics (PD) in the Phase 1/2, open-label study to

evaluate administration of Co-rhARG1-PEG to patients with Arginase 1 deficiency. Using the escalation stop criteria, the doses settled upon in Part 2 were 0.09 mg/kg for Patient 1, 0.12 mg/kg for Patient 3, and 0.04 mg/kg (for the period of Part 2 shown). By applying the dose escalation stopping criteria, other patients in the trial settled upon a variety of Part 2 dosing levels. These same criteria can be applied to adjust (increase or decrease) the dose of any patient that is already on Co-rhARG1-PEG in response to arginine levels that move outside preferred (healthy) ranges.

[0058] Figure 11 shows a comparison of IV and subcutaneous administration of Co-rhARG1-PEG. The preferred plasma arginine concentrations for a patient are between 40  $\mu$ M and 115  $\mu$ M (dotted lines). Subcutaneous administration of Co-rhARG1-PEG results in arginine concentrations within this preferred range longer than via IV administration. Figure 11 (a) includes data from the first week subsequent to end of Part 2 and Figure 11 (b) excludes IV data from this week 1 extension. Plots are shown as an average of patient values and the data are drawn from the dose that the stopping criteria determined for each patient.

[0059] Figure 12 shows plasma arginine and plasma guanidino compound levels after administration of Co-rhARG1-PEG. Figure 12(a) shows plasma arginine levels at baseline, after dose 1, after dose 8 and during the open label extension (OLE). Figure 12(b) shows plasma levels for guanidinoacetic acid (GAA), N- $\alpha$ -acetyl-L-arginine (NAA),  $\alpha$ -keto- $\delta$ -guanidinovaleric acid (GVA) and argininic acid (ARGA) at baseline and during the OLE.

[0060] Figure 13 shows the baseline deficit and responses for clinical outcomes. Figure 13(a) shows the baseline deficit for Arginase 1 deficiency patients for the 6-Minute Walk Test (6MWT), Gross Motor Function Measure (GMFM) Parts D and E, and Adaptive Behaviour Assessment System (ABAS). Figure 13(b) shows the clinical response for the 6MWT, GMFM-D and GMFM-E.

[0061] Figure 14 shows the time-dependent improvements in 6MWT, GMFM-D and GMFM-E. Figure 14(a) shows the percentage of clinical responders for 6MWT across all patients and those with baseline deficits in 6MWT at dose 8 and dose 20. Figure 14(b) shows the percentage of clinical responders for GMFM-D across all patients and those with baseline deficits in 6MWT at dose 8 and dose 20. Figure 14(c) shows the percentage of clinical responders for GMFM-E across all patients and those with baseline deficits in 6MWT at dose 8 and dose 20.

[0062] Figure 15 shows a site-specific PEGylation analysis of different batches of Co-rhARG1-PEG.

## DETAILED DESCRIPTION

### Recombinant Human Arginase 1

5 [0063] Human Arginase 1, identified as hArg1, is a binuclear manganese metalloenzyme that catalyzes the hydrolysis of L-arginine (L-Arg) to yield L-ornithine and urea. Arginase 1 is a trimer of three non-covalently bound identical monomer units. Monomeric Arginase 1 is enzymatically active but less stable. The substitution of the native manganese ( $Mn^{2+}$ ) with cobalt ( $Co^{2+}$ ) in the active site of Arginase 1 enhances catalytic  
10 activity at physiological pH. The methods of producing cobalt-substituted Arginase 1 enzyme described herein provide an enzyme that is highly pure and highly active. The methods can also provide Co-Arginase 1 (Co-rhARG1) as an isolated intermediate in the manufacture of the drug substance. In one or more embodiments, the drug substance is PEGylated Co-Arginase 1 (Co-rhARG1-PEG). PEGylation of Co-Arginase 1 extends the circulating half-life  
15 significantly. Again, although specific reference is made herein to rhARG1, the methods, formulations and uses described herein can also be applied to rhARG2.

[0064] As used herein, the term “rhARG1” refers to a recombinant human Arginase 1 enzyme, such as a recombinant enzyme having at least 98% sequence identity to SEQ ID NO: 1.

20 [0065] As used herein, the terms “Co-rhARG1”, “Co-Arginase 1 intermediate” and the like refer to a rhARG1 that has at least some of the native manganese cofactor replaced with cobalt. In one or more embodiments, the Co-rhARG1 is an isolatable intermediate in a production and/or purification process for Co-rhARG1-PEG.

[0066] As used herein, the terms “Co-rhARG1-PEG”, “PEGylated Co-Arginase 1” and  
25 the like refers to a Co-rhARG1 that has one or more PEG units covalently linked to the enzyme, such as at free amine(s) at the N-terminal amino acid and/or at one or more lysine residues.

[0067] The quantity of Co-rhARG1-PEG drug substance can be expressed as the mass amount of un-PEGylated enzyme. In one embodiment of the method, each mg (enzyme basis)  
30 of Co-rhARG1-PEG drug substance also contains approximately 1-2 mg of PEG, such as about 1.4 mg of PEG.

[0068] Figure 1(a) shows the amino acid sequence that was expressed in *E. coli*. The hArg1 protein sequence was obtained from the NCBI database (UniProtKB: locus ARG1\_HUMAN, accession P05089). Overlapping oligonucleotides were used in a PCR reaction to generate Arginase 1 DNA that was codon optimized for expression in *E. coli* (Figure 1(b)). The 321 amino acid *E. coli* expressed monomer of Arginase 1 lacks the N-terminal methionine found in native human Arginase 1 monomer. The calculated molecular weight of Co-Arginase 1 is 34721.6 Daltons (Table 1). The calculated molecular weight of homotrimeric Co-Arginase 1 is 104164.8 Daltons. Arginase 1 does not have any disulfide bonds.

10 [0069] Table 1: Structural Information of an Exemplary Co-Arginase 1 Intermediate

|                                   |                                                                              |
|-----------------------------------|------------------------------------------------------------------------------|
| Molecular weight of Co-Arginase 1 | 34721.6 Daltons (calculated value with cobalt)                               |
| Homotrimeric Co-Arginase 1        | 104164.8 Daltons (calculated value with cobalt)                              |
| Length of the monomer             | 321 amino acid residues                                                      |
| Disulfide bonds                   | None                                                                         |
| Amino acid sequence               | The primary sequence of recombinant human Arginase 1 is provided in Figure 1 |

[0070] In one or more embodiments, the calculated molecular weight of monomeric Co-rhARG1-PEG is about 75-115 kDa. In one or more embodiments, the calculated molecular weight of homotrimeric Co-rhARG1-PEG is about 224-344 kDa. In one or more embodiments, the average number of PEG is about 8 to about 25 moles of PEG/mole Co-Arginase 1 monomer, such as about 8 to about 16 moles of PEG/mole Co-Arginase 1 monomer. Exemplary amounts of PEG include about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15 and about 16 moles of PEG/mole Co-Arginase 1 monomer. In one or more embodiments, each PEG has an average molecular weight of about 1,000 to about 10,000 Daltons, such as about 1,000, about 2,000, about 3,000, about 4,000, about 5,000, about 6,000, about 7,000, about 8,000, about 9,000 or about 10,000 Daltons. In a particular embodiment, the average MW of the PEG is about 5,000 Daltons.

[0071] In one or more embodiments, the Co-rhARG1-PEG comprises pegzilarginase. Pegzilarginase has the following two chemical names:

- 25 a. poly(oxy-1,2-ethanediyl),  $\alpha$ -(carboxymethyl)- $\omega$ -methoxy-, amide with arginase 1 [cobalt cofactor] (synthetic human) (1:10), trimer

b. Des-Met<sup>1</sup>-arginase-1 (liver-type arginase, EC 3.5.3.1) (*Homo sapiens*) from which manganese has been replaced with cobalt, an average of 10 primary amines (of N-terminal serines and N<sup>6</sup>-lysines) are amidified with [methoxypoly(ethyleneoxy)]acetyl, non-covalent homotrimer, produced in

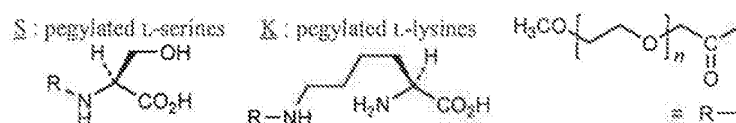
5 [0072] *Escherichia coli* The molecular formula for pegzilarginase is C<sub>1554</sub>H<sub>2492</sub>N<sub>416</sub>O<sub>453</sub>S<sub>6</sub> [C<sub>3</sub>H<sub>4</sub>O<sub>2</sub> (C<sub>2</sub>H<sub>4</sub>O)<sub>n</sub>]<sub>a</sub> monomer. The average molecular weight for pegzilarginase is 284 kDa for the trimer. The CAS registry number for pegzilarginase is 1659310-95-8.

[0073] Potential PEGylation sites of pegzilarginase are shown below:

Monomer sequence

|             |            |            |            |             |     |
|-------------|------------|------------|------------|-------------|-----|
| SAKSRITIGII | GAPFSKGQPR | GGVEEGPIVL | RKAGLLEKLE | EQECDVKDYG  | 50  |
| DLFFADIPND  | SFFQIVKNPR | SVGKASEQLA | QKVAEVKKNQ | RISLVLGQDH  | 100 |
| SLAIGSISCH  | ARVHPDLGVI | WVDAMTDINT | PLTTTSCNLI | GQPVSPILLKE | 150 |
| LKGKLPDVPG  | FSWVTPCISA | KDIVYIGLED | VDPGENYILK | TLGIKYFSMT  | 200 |
| EVDRLGIGKV  | MEETLSYLLG | RKKRPINLSF | DVDGLDPSPT | PATGTPVVG   | 250 |
| LTYREGLYIT  | EEIYKTGLLE | GLDIMEVNPS | LGKTPREVTR | TVNTAVAITL  | 300 |
| ACFGLAREGN  | HKPIDYLNPP | K          |            |             | 321 |

Potential modified residues



10

[0074] Typically, PEGylation reaction is performed on the Co-rhARG1. In some embodiments, PEGylation reaction may be performed on the rhARG1. In one or more embodiments, amount of reactants, time, temperature and solution and reactant handling (such as mixing, addition rate, PEG handling) are important to produce consistent PEGylated product. Typically, PEGylation reaction on Co-rhARG1 is performed in a reaction buffer at pH 8.4. In one or more embodiments, the Co-rhARG1 is PEGylated in 0.1M sodium phosphate buffer at pH 8.4. In one or more embodiments, the PEGylation reaction comprises reactant ratio, PEG (g) to Co-rhARG1 (g), is in the range of 4:1 to 1:1. In one or more embodiments, the PEGylation reaction comprises reactant ratio, PEG (g) to Co-rhARG1 (g), is about 2.77:1.

15

20 In one or more embodiments, the PEGylation reaction is performed by misting PEG and Co-rhARG1 from about 5 minutes to about 300 minutes, about 10 minutes to about 300 minutes, about 20 minutes to about 300 minutes, about 30 minutes to about 300 minutes, about 5 minutes to about 280 minutes, about 10 minutes to about 280 minutes, about 20 minutes to about 280 minutes, about 30 minutes to about 280 minutes, about 5 minutes to about 260

minutes, about 10 minutes to about 260 minutes, about 20 minutes to about 260 minutes, about 30 minutes to about 260 minutes, about 5 minutes to about 240 minutes, about 10 minutes to about 240 minutes, about 20 minutes to about 240 minutes, about 30 minutes to about 240 minutes. In one or more embodiments, the PEGylation reaction is stopped by removing excess  
5 PEG and reducing pH of the reaction buffer. In some embodiments, the excess PEG is removed by filtration technique. In one or more embodiments, the pH is reduced by exchanging the reaction buffer with a storage buffer. In some embodiments, the storage buffer comprises 5mM potassium phosphate, 50 mM NaCl, 1.5% w/v Glycerol and pH 7.4.

[0075] In one or more embodiments, the Co-rhARG1-PEG is PEGylated at one or  
10 more of K16, K32, K38, K40, K47, K67, K74, K82, L87, K88, K152, K154, K171, K222, K223, K312 and K321 amino acid residues. In some embodiments, the Co-rhARG1-PEG is PEGylated at least at K16, K32, K38, K40, K47, K67, K74, K82, L87, K88, K152, K154, K171, K312 and K321 amino acid residues. In some embodiments, the Co-rhARG1-PEG is PEGylated at K222 and/or K223 amino acid residues. In some embodiments, the Co-rhARG1-  
15 PEG is not PEGylated at K222 and/or K223 amino acid residues. In some embodiments, the Co-rhARG1-PEG is not PEGylated at one or more of K3, K149, K190, K195, K29, K265 and K283 amino acid residues. In some embodiments, the Co-rhARG1-PEG is not PEGylated at K3, K149, K190, K195, K29, K265 and K283 amino acid residues.

[0076] In one or more embodiments of the Co-rhARG1-PEG, K16 is PEGylated in the  
20 range of about 15% to about 60%. In one or more embodiments of the Co-rhARG1-PEG, K32 is PEGylated in the range of about 35% to about 80%. In one or more embodiments of the Co-rhARG1-PEG, K38 is PEGylated in the range of about 20% to about 85%. In one or more embodiments of the Co-rhARG1-PEG, K40 is PEGylated in the range of about 10% to about 60%. In one or more embodiments of the Co-rhARG1-PEG, K47 is PEGylated in the range of  
25 about 10% to about 60%. In one or more embodiments of the Co-rhARG1-PEG, K67 is PEGylated in the range of about 40% to about 90%. In one or more embodiments of the Co-rhARG1-PEG, K74 is PEGylated in the range of about 30% to about 95%. In one or more embodiments of the Co-rhARG1-PEG, K82 is PEGylated in the range of about 30% to about 98%. In one or more embodiments of the Co-rhARG1-PEG, K87 is PEGylated in the range of  
30 about 15% to about 65%. In one or more embodiments of the Co-rhARG1-PEG, K88 is PEGylated in the range of about 25% to about 70%. In one or more embodiments of the Co-rhARG1-PEG, K152 is PEGylated in the range of about 25% to about 85%. In one or more

embodiments of the Co-rhARG1-PEG, K154 is PEGylated in the range of about 15% to about 65%. In one or more embodiments of the Co-rhARG1-PEG, K171 is PEGylated in the range of about 20% to about 75%. In one or more embodiments of the Co-rhARG1-PEG, K222 is PEGylated in the range of 0% to about 30%. In one or more embodiments of the Co-rhARG1-PEG, K223 is PEGylated in the range of 0% to about 35%. In one or more embodiments of the Co-rhARG1-PEG, K312 is PEGylated in the range of 0% to about 45%. In one or more embodiments of the Co-rhARG1-PEG, K321 is PEGylated in the range of 0% to about 45%.

[0077] PEG-Protein Molar ratio is an attribute indicative of the extent of PEGylation. In one or more embodiments, about 1 to about 20 moles of PEG has PEGylated one mole of Co-rhARG1. Exemplary range of molar ratio for PEG:Co-rhARG1 include 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, 15:1, 16:1, 17:1, 18:1, 19:1 and 20:1. In some embodiments, the molar ratio of PEG:Co-rhARG is in the range of about 7 moles/mole to about 15 moles/mole.

[0078] The free PEG is measured to demonstrate PEG clearance and stability. In some embodiments, free PEG concentration ( $\mu\text{g}$ ) in PEGylated Co-rhARG1 (mL) is less than or equal to 500  $\mu\text{g/mL}$ , less than or equal to 400  $\mu\text{g/mL}$ , less than or equal to 300  $\mu\text{g/mL}$ , less than or equal to 200  $\mu\text{g/mL}$ , less than or equal to 100  $\mu\text{g/mL}$ , and less than or equal to 50  $\mu\text{g/mL}$ .

[0079] Human Arginase 1 catalyzes the fifth and final step in the urea cycle which is the conversion of L-arginine into L-ornithine and urea. The PEGylated drug substance, Co-rhARG1-PEG, catalyzes the same reaction. The assay to assess enzyme activity measures the conversion of L-arginine to L-ornithine during a fixed reaction time at pH 7.4 and 37°C. The amount of conversion of product is converted to a reaction rate and fit to the Michaelis-Menten equation to determine  $K_m$  and  $k_{cat}$ .

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

[0080]  $V_{max}$  is the maximum reaction rate achieved at saturating substrate concentration;  $K_m$  is the Michaelis-Menten binding constant to measure the substrate concentration yielding a velocity at the half of  $V_{max}$ . The enzymatic turnover number,  $k_{cat}$  is calculated by  $V_{max}/[E]$ .

[0081] Specific activity is determined by dividing the reaction velocity at 2 mM arginine expressed in  $\mu\text{moles/minute}$  by the enzyme concentration in mg.

[0082] The values for Co-rhARG1-PEG drug substance for  $K_M$  and  $k_{cat}$  as measured in the enzyme activity assay typically range from 0.15-0.22 mM and approximately 200-300/sec respectively. Upon PEGylation of the Co-Arginase 1 intermediate to form the drug substance, compared to the unPEGylated intermediate, the enzyme activity is not significantly changed.

5 However, PEGylation significantly increases the circulating half-life of the Co-rhARG1-PEG drug product compared to the Co-Arginase 1 intermediate.

[0083] In one or more embodiments, the protein (e.g. Co-rhARG1 or Co-rhARG1-PEG) displays a  $k_{cat}/K_M$  greater than  $200 \text{ mM}^{-1} \text{ s}^{-1}$  at pH 7.4. In a particular embodiment, the protein displays a  $k_{cat}/K_M$  in the range of about  $200 \text{ mM}^{-1} \text{ s}^{-1}$  to about  $4,000 \text{ mM}^{-1} \text{ s}^{-1}$  at pH 7.4.

10 In another embodiment, the protein displays a  $k_{cat}/K_M$  in the range of about  $400 \text{ mM}^{-1} \text{ s}^{-1}$  to about  $2,500 \text{ mM}^{-1} \text{ s}^{-1}$  at pH 7.4 at  $37^\circ \text{C}$ . In a particular embodiment, the present invention contemplates a protein comprising an amino acid sequence of human Arginase 1 and a non-native metal cofactor, wherein said protein exhibits a  $k_{cat}/K_M$  greater than  $400 \text{ mM}^{-1} \text{ s}^{-1}$  at  $37^\circ \text{C}$ , pH 7.4. Exemplary  $k_{cat}/K_M$  values include about 200, about 250, about 300, about 350,

15 about 400, about 450, about 500, about 550, about 600, about 650, about 700, about 800, about 900, about 1,000, about 1,100, about 1,200, about 1,500, about 2,000, about 2,500, about 3,000, about 3,500 and about  $4,000 \text{ mM}^{-1} \text{ s}^{-1}$  at pH 7.4 at  $37^\circ \text{C}$ , or any range in between these values.

[0084] The specific activity is an indication of potency of the protein (e.g. Co-rhARG1 or Co-rhARG1-PEG). In one or more embodiments, the specific activity of Co-rhARG-PEG is in the range of about 200 U/mg to about 1000 U/mg. Exemplary ranges of the specific activity include about 200 U/mg to about 1000 U/mg, about 300 U/mg to about 1000 U/mg, about 400 U/mg to about 1000 U/mg, about 200 U/mg to about 900 U/mg, about 300 U/mg to about 900 U/mg, about 400 U/mg to about 900 U/mg, about 200 U/mg to about 800 U/mg, about 300

25 U/mg to about 800 U/mg, about 400 U/mg to about 800 U/mg, about 200 U/mg to about 700 U/mg, about 300 U/mg to about 700 U/mg, and about 400 U/mg to about 700 U/mg.

[0085] In one or more embodiments, the rhARG1, Co-rhARG1 or Co-rhARG1-PEG can have at least 98%, 98.5%, 99% or 99.5% identity to SEQ ID NO: 1. In one or more embodiments, rhARG1, Co-rhARG1 or Co-rhARG1-PEG can have at 1, 2, 3, 4, 5, 6, 7, 8, 9,

30 10, 11, 12, 13, 14, 15 or more deletions, substitutions and/or insertions to the amino acid sequence described by SEQ ID NO: 1. Various alignment algorithms and/or programs may be used to calculate the identity between two sequences, including FASTA, or BLAST which are

available at the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/>).

**[0086]** In one or more embodiments, the rhARG1, Co-rhARG1 or Co-rhARG1-PEG has at least one amino acid substitution at a position selected from H100, D123, H125, D127, D231, D233, D180, S229, and C302. In some embodiments, rhARG1, Co-rhARG1 or Co-rhARG1-PEG comprises at least one amino acid substitution selected from the group consisting of: D180S, S229C, S229G, C302F, C302I, E255Q, D180E, and S229A. In one or more embodiments, rhARG1, Co-rhARG1 or Co-rhARG1-PEG comprises at least an amino acid substitution at C302.

**[0087]** Using the methods described herein, it is possible to replace almost all the manganese cofactor in Arginase 1 with cobalt. The change to cobalt cofactor results in a change in the  $K_m$  for arginine from 2.8 mM to about 0.18 mM at pH 7.4. In one or more embodiments, the Co-rhARG1-PEG comprises about 0.1 to about 2  $\mu\text{g}$  Co/mg protein. Exemplary cobalt loadings include about 0.1, about 0.2, about 0.3, about 0.4, about 0.5, about 0.6, about 0.7, about 0.8, about 0.9, about 1, about 1.1, about 1.2, about 1.3, about 1.4, about 1.5, about 1.6, about 1.7, about 1.8, about 1.9 and about 2  $\mu\text{g}$  Co/mg protein.

**[0088]** The free cobalt is measure to demonstrate cobalt clearance and stability. In some embodiments, the free cobalt is less than or equal to 0.10  $\mu\text{g/mL}$ , less than or equal to 0.09  $\mu\text{g/mL}$ , less than or equal to 0.08  $\mu\text{g/mL}$ , less than or equal to 0.07  $\mu\text{g/mL}$ , less than or equal to 0.06  $\mu\text{g/mL}$ , less than or equal to 0.05  $\mu\text{g/mL}$ , and less than or equal to 0.04  $\mu\text{g/mL}$ .

**[0089]** The total cobalt impacts potency of the protein and is an indicator of bound cobalt as the amount of free cobalt is relatively small. In some embodiments, the total cobalt concentration is in the range of about 5  $\mu\text{g/mL}$  to about 20  $\mu\text{g/mL}$ , about 6  $\mu\text{g/mL}$  to about 20  $\mu\text{g/mL}$ , about 7  $\mu\text{g/mL}$  to about 20  $\mu\text{g/mL}$ , about 8  $\mu\text{g/mL}$  to about 20  $\mu\text{g/mL}$ , about 9  $\mu\text{g/mL}$  to about 20  $\mu\text{g/mL}$ , about 5  $\mu\text{g/mL}$  to about 19  $\mu\text{g/mL}$ , about 6  $\mu\text{g/mL}$  to about 19  $\mu\text{g/mL}$ , about 7  $\mu\text{g/mL}$  to about 19  $\mu\text{g/mL}$ , about 8  $\mu\text{g/mL}$  to about 19  $\mu\text{g/mL}$ , about 9  $\mu\text{g/mL}$  to about 19  $\mu\text{g/mL}$ , about 5  $\mu\text{g/mL}$  to about 18  $\mu\text{g/mL}$ , about 6  $\mu\text{g/mL}$  to about 18  $\mu\text{g/mL}$ , about 7  $\mu\text{g/mL}$  to about 18  $\mu\text{g/mL}$ , about 8  $\mu\text{g/mL}$  to about 18  $\mu\text{g/mL}$ , about 9  $\mu\text{g/mL}$  to about 18  $\mu\text{g/mL}$ , about 5  $\mu\text{g/mL}$  to about 17  $\mu\text{g/mL}$ , about 6  $\mu\text{g/mL}$  to about 17  $\mu\text{g/mL}$ , about 7  $\mu\text{g/mL}$  to about 17  $\mu\text{g/mL}$ , about 8  $\mu\text{g/mL}$  to about 17  $\mu\text{g/mL}$ , about 9  $\mu\text{g/mL}$  to about 17  $\mu\text{g/mL}$ , about 5  $\mu\text{g/mL}$  to about 16  $\mu\text{g/mL}$ , about 6  $\mu\text{g/mL}$  to about 16  $\mu\text{g/mL}$ , about 7  $\mu\text{g/mL}$  to about 16  $\mu\text{g/mL}$ , about 8  $\mu\text{g/mL}$  to about 16  $\mu\text{g/mL}$ , about 9  $\mu\text{g/mL}$  to about 16  $\mu\text{g/mL}$ , about 5

μg/mL to about 15 μg/mL, about 6 μg/mL to about 15 μg/mL, about 7 μg/mL to about 15 μg/mL, about 8 μg/mL to about 15 μg/mL, and about 9 μg/mL to about 15 μg/mL.

[0090] In various embodiments, the Co-rhARG1-PEG comprises less than about 1 μg Mn/mg protein, such as less than about 1, about 0.9, about 0.8, about 0.7, about 0.6, about 0.5, about 0.4, about 0.3, about 0.2, about 0.15, about 0.1, about 0.09, about 0.08, about 0.07, about 0.06, about 0.05, about 0.04, about 0.03, about 0.02 or about 0.01 μg Mn/mg protein. In a particular embodiment, Co-rhARG1-PEG drug substance contains about 2 μg Co/mg protein and about 0.05 μg Mn/mg protein.

[0091] In various embodiments, the Co-rhARG1-PEG comprises less than about 1 μg Fe/mg protein, such as less than about 1, about 0.9, about 0.8, about 0.7, about 0.6, about 0.5, about 0.4, about 0.3, about 0.2, about 0.15, about 0.1, about 0.09, about 0.08, about 0.07, about 0.06, about 0.05, about 0.04, about 0.03, about 0.02 or about 0.01 μg Fe/mg protein.

#### **Production and Purification of rhARG1, Co-rhARG1 and PEGrhARG1**

[0092] An overview of exemplary upstream and downstream production methods can be seen in Figures 2 and 3.

#### **Shake Flask Expansion**

[0093] The purpose of the shake flask expansion/fermentation is to generate an inoculum to seed the production fermenter. Shake flask expansion creates cell mass for inoculation of the production reactor as well as extra for analytical purposes. A representative overview of the Arginase 1 fermentation processes can be seen in Figure 2.

[0094] An aliquot of the inoculum medium is introduced into one 500 mL flask (a Primary flask) and six 3 L disposable flasks (Secondary flasks). The flasks are autoclaved, and post-sterile additions are transferred to each flask. Prior to inoculation, the primary medium is pre-warmed to the processing temperature of 37°C. Before secondary inoculation, the secondary flasks are pre-warmed to the processing temperature of 37°C.

[0095] One vial of an Arginase 1-expressing *E. coli* working cell bank (WCB) is removed from cold storage and thawed. A target volume of thawed cells (approximately 1.1 mL) is added aseptically to the primary flask, and the flask is incubated at 37°C with agitation. Samples are removed from the flask hourly starting several hours post-inoculation to follow cell growth by optical density at 600 nm (OD600). Once the target OD600 of  $\geq 1.0$  is reached

in the primary flask, a target volume (15 mL) of primary culture is aseptically transferred into each secondary flask. The secondary flasks are incubated at 37°C with agitation. Samples are removed from one secondary flask hourly starting at 4 h post-inoculation, increasing to every 30 min once the OD600 has reached  $\geq 1.5$ . When the measurement has met the specified density of  $\geq 2.0$  OD600, the remaining secondary flasks are sampled. If the average OD600 of all secondary flasks meets a specified transfer criterion, the flasks are pooled, and the inoculum is transferred to the production fermenter. A representative overview of the fermentation process for Arginase 1 can be seen in Figure 2.

### Production Fermentation

10 [0096] The purpose of the Production Fermentation is to expand the shake flask culture and induce production of Arginase 1. Production fermentation can create large scale quantities of Arginase 1. Following a shake flask expansion phase to build cell mass, the fermentation process produces Arginase 1 (in *E. coli*) as a soluble protein. In one embodiment a 1500 L fermenter contains the initial batch medium including sterile additions before inoculation. 15 After inoculation, inputs to the fermenter include nutrient feed, antifoam solution, addition of acid or base to maintain culture pH. A secondary vessel holds the nutrient feed medium. An automated control strategy maintains important parameters for consistent cell growth including dissolved oxygen, sparge rate, agitation rate, pH, pressure, and temperature. Arginase 1 expression is induced by addition of IPTG (isopropyl beta-D-1-thiogalactopyranoside), with 20 harvest occurring approximately 18 hours later. The performance of the fermenter is assessed at the end of production by monitoring cell density, percent solids, and the proportion of soluble Arginase 1.

[0097] In a preferred embodiment, the fermentation medium is prepared directly in the production fermenter. Purified water is added to the fermentation medium to the required 25 weight before in-place sterilization (SIP). Post-sterilization additions of kanamycin, glucose, and potassium phosphate are filter-sterilized into the production fermenter once the medium has cooled. If necessary, the sterile medium is brought to a designated pre-inoculation weight with purified water using a 0.2  $\mu\text{m}$  sterile filter. The fermentation medium is titrated with base (ammonium hydroxide) to a controlled pH value.

30 [0098] The production fermenter at 37°C is inoculated aseptically using a pooled inoculum via a pressure-assisted transfer. Fermentation broth samples are collected at a regular frequency and measured for OD600 analysis from the time of inoculation until fermentation

cool down. Glucose samples are taken at a regular interval beginning at 3 h post-inoculation and increasing in frequency after 9 h post-inoculation. Antifoam solution is added as needed during the fermentation process to avoid excessive foaming of the culture. Dissolved oxygen is controlled by an agitation cascade with oxygen sparge on demand. Culture pH is maintained using acid and base inputs. Growth medium is preferably maintained at 36-38 °C and at a pH of 7.0 – 7.4 with agitation and aeration.

[0099] The nutrient feed consists of yeast extract, Martone B-1, L-cysteine HCl, and glycerol. The feed starts when the glucose concentration is less than 10 g/L (12 –14 h post-inoculation) and continues at a fixed rate until the end of production. Expression is triggered with addition of IPTG. Induction continues for 18 hours. Completion of the fermentation process is followed by a cool down in preparation for harvest operations. The production fermenter can generate titers of soluble Arginase 1 of approximately 6 g/L. An overview of the Production Fermentation can be seen in Figure 3.

## 15 Harvest Operations

[00100] Harvest operations capture cells containing soluble Arginase 1, break open the cells/lyse the cells, and clear the lysate of cell debris by using centrifugation and/or filtration. The recovered cell slurry can be frozen or kept at a low temperature for long-term storage. Harvest operations may collect the cells by centrifugation, lysed with two passes through a homogenizer or cell disruption under pressure (French press), centrifuged a second time, and membrane filtered prior to the first chromatography step.

[00101] In a preferred embodiment, whole cells are separated from fermentation medium using a disc stack centrifuge. The resulting cell slurry resuspended in 25 mM HEPES, pH 7.6, followed by two passes through a homogenizer. The pH of the 25 mM HEPES can also be used in the range of pH 7.2-7.6. The lysed material is clarified using a centrifuge to remove cell debris, then membrane filtered through 0.2µm grade filters. In a preferred embodiment, harvest steps are performed at a target temperature of  $\leq 15^{\circ}\text{C}$ .

[00102] In an alternative embodiment, cell disruption is performed using high pressure. Cell slurry is transferred to a homogenizer at a controlled rate and the homogenized outflow is passed through a heat exchanger to reduce the temperature increase seen during pressure homogenization. The chilled cells undergo two homogenization passes. The first pass lysis

pool is transferred from the collection vessel back to the feed vessel. The hold duration between passes is minimized to reduce potential microbial growth.

[00103] The post-lysis material is clarified by centrifugation to remove cellular debris from the soluble components of the lysate. The lysate is transferred at a controlled rate to a disk-stack, intermittent discharge, centrifuge. The clarified lysate is collected for further processing.

[00104] The clarified lysate is filtered, such as with an about 0.2  $\mu\text{m}$  filter. Process transition filters can also be used for microbial control during process operations. For this purpose, filters can be either 0.5  $\mu\text{m}$  or 0.2  $\mu\text{m}$  filters. This step also removes small particulates from clarified material that may not have separated during clarification operations. Prior to use, the filters are flushed extensively with purified water and equilibrated with 25 mM HEPES, pH 7.6 buffer. Each downstream process step can be preceded by a pre-filter to mitigate the potential for bioburden load.

#### 15 **Purification of rhARG1, Co-rhARG1 and Co-rhARG1-PEG**

[00105] Regardless of the methods used to culture cells that express the rhARG1 (e.g. the fermentation processes described above), the purification methods described herein can be used to capture rhARG1 and further purify the enzyme. The purification methods can include optional steps such as loading with cobalt to produce Co-rhARG1 and/or reacting with a PEGylation reactant to provide Co-rhARG1-PEG.

[00106] Various embodiments of the purification process relate to the use of a cation exchange (CEX) column to capture rhARG1. In one or more embodiments, the CEX column is the first column ("Column 1") in system with multiple chromatography columns. The protein product eluted from this Column 1 is "First Protein Product".

[00107] In one or more embodiments, Column 1 uses cation exchange chromatography to bind rhARG1 at a pH in the range of about 7 to about 8, such as a pH of about 7.6. In one or more embodiments, the rhARG1 is bound in the absence of salt or at low salt concentrations. In one or more embodiments, the rhARG1 is eluted with a buffer having a high salt (e.g. NaCl) concentration, such as up to about 0.5 M NaCl. Exemplary salt concentrations include about 0.01, about 0.02, about 0.03, about 0.04, about 0.05, 0.1, about 0.2, about 0.3, about 0.4 and about 0.5 M NaCl.

[00108] In various embodiments, a salt gradient is used to separate different charge variants of rhARG1. Exemplary salt gradients are from about 0 to about 0.5 M NaCl, about 0 to about 0.4 M NaCl, about 0 to about 0.3 M NaCl, about 0 to about 0.2 M NaCl or about 0 to about 0.1 M NaCl.

5 [00109] In one or more embodiments, the method further comprises loading the First Protein Product (optionally after cobalt substitution) onto an anion exchange (AEX) chromatography column ("Column 2") and collecting the flow-through to provide a second protein product ("Second Protein Product"). In another aspect of the methods, the method further comprises loading the Second Protein Product onto third column which captures the  
10 Arginase 1 and is then eluted to provide a third protein product ("Third Protein Product"). In some embodiments, this third chromatography column ("Column 3") may be a size exclusion chromatography (SEC) column or a multimodal chromatography (MMC) column.

[00110] Various embodiments provide that the rhARG1 is loaded with Co to replace the Mn cofactor. In one or more embodiments, the Co loading is performed using a  $\text{Co}^{2+}$  salt such as  $\text{CoCl}_2$ . Incubation times are temperature dependent, such that lower cobalt substitution  
15 temperatures require longer incubation times and higher cobalt substitution temperatures do not require as long incubation times. The cobalt loading temperature may be as low as 1 °C or greater than 50 °C and corresponding incubation times can be as long as over 8 hours or less than 10 minutes.

20 [00111] Various embodiments provide that the rhARG1 or Co-rhARG1 is reacted with a PEGylation reactant such as methoxy PEG succinimidyl carboxymethyl ester (MW 5000). The PEGylation reactant is typically provided in molar excess of 10-40 compared to the enzyme. Incubation times can be in the range of 0.5 to 4 hours. The pH during PEGylation can be about 8 to about 9, such as a pH of about 8.4.

25 [00112] In one or more embodiments, the purified PEGylated proteins, rhARG1 or Co-rhARG1, comprises Arginase I monomer, Gluconoylated Arginase I, Phosphogluconoylated Arginase I, 2 x Gluconoylated Arginase I, Gluconoylated + phosphogluconoylated Arginase I and 2x Phosphogluconoylated Arginase I.

[00113] In some embodiments, the purified PEGylated proteins, rhARG1 or Co-rhARG1, comprises at least 70% of Arginase I monomer. Exemplary amounts include at least  
30 70%, at least 75, at least 80%, at least 85%, or at least 90% Arginase I monomer. In some embodiments, the purified PEGylated proteins, rhARG1 or Co-rhARG1, comprises less than

10% of Gluconoylated Arginase I. Exemplary amounts include about 1%, about 2%, about 3%, about 4%, about 5%, about 6%, about 7%, about 8% or about 9% Gluconoylated Arginase I, or any range in between these values. In some embodiments, the purified PEGylated proteins, rhARG1 or Co-rhARG1, comprises less than 10% of Phosphogluconoylated Arginase I. Exemplary amounts include about 1%, about 2%, about 3%, about 4%, about 5%, about 6%, about 7%, about 8% or about 9% Phosphogluconoylated Arginase I. In some embodiments, the purified PEGylated proteins, rhARG1 or Co-rhARG1, comprises at least 70% of Arginase I monomer, less than 10% of Gluconoylated Arginase I, and less than 10% of Phosphogluconoylated Arginase I.

10 **[00114]** Imaged capillary isoelectric focusing (iCIEF) provides a measurement of consistency for pegylated protein due to the level of heterogeneity in the PEGylated trimer. In one or more embodiments, the iCIEF analysis of purified PEGylated protein, rhARG1 or Co-rhARG1, comprises nine distinctively peaks, peak-1, peak-2, peak-3, peak-4, peak-5, peak-6, peak-7, peak-8 and peak-9, each corresponding to nine distinctively charged species, species-1, species-2, species-3, species-4, species-5, species-6, species-7, species-8 and species-9. The area under curve for each peak corresponds to the proportion of that particular species. In one or more embodiments, certain peaks may be combined together for related species such as peaks-1+2 or peaks-3+4.

20 **[00115]** In some embodiments, the purified PEGylated protein, rhARG1 or Co-rhARG1, comprises the area under curve for peak-1 in the proportion of less than about 30%, less than about 25%, less than about 20%, less than about 15%, and less than about 10%.

**[00116]** In some embodiments, the purified PEGylated protein, rhARG1 or Co-rhARG1, comprises the area under curve for peak-2 in the proportion of less than about 40%, less than about 35%, less than about 30%, less than about 25%, less than about 20%, less than about 15%, and less than about 10%.

25 **[00117]** In some embodiments, the purified PEGylated protein, rhARG1 or Co-rhARG1, comprises the area under curve for combined peaks-1+2 in the proportion of less than about 50%, less than about 45%, less than about 40%, less than about 35%, less than about 30%, less than about 25%, less than about 20%, less than about 15%, and less than about 10%.

30 **[00118]** In some embodiments, the purified PEGylated protein, rhARG1 or Co-rhARG1, comprises the area under curve for peak-3+4 in the proportion range of about 2% to about 40%, about 2% to about 35%, about 2% to about 30%, about 2% to about 25%, about 4% to

about 40%, about 4% to about 35%, about 4% to about 30%, about 4% to about 25%, about 6% to about 40%, about 6% to about 35%, about 6% to about 30%, about 6% to about 25%, about 8% to about 40%, about 8% to about 35%, about 8% to about 30%, about 8% to about 25%, about 10% to about 40%, about 10% to about 35%, about 10% to about 30%, and about 10% to about 25%.

[00119] In some embodiments, the purified PEGylated protein, rhARG1 or Co-rhARG1, comprises the area under curve for peak-5 in the proportion range of about 5% to about 40%, about 5% to about 35%, about 5% to about 30%, about 5% to about 25%, about 10% to about 40%, about 10% to about 35%, about 10% to about 30%, about 10% to about 25%, about 15% to about 40%, about 15% to about 35%, about 15% to about 30%, and about 15% to about 25%.

[00120] In some embodiments, the purified PEGylated protein, rhARG1 or Co-rhARG1, comprises the area under curve for peak-6 in the proportion range of about 2% to about 35%, about 2% to about 30%, about 2% to about 25%, about 2% to about 20%, about 4% to about 35%, about 4% to about 30%, about 4% to about 25%, about 4% to about 20%, about 6% to about 35%, about 6% to about 30%, about 6% to about 25%, about 6% to about 20%, about 8% to about 35%, about 8% to about 30%, about 8% to about 25%, about 8% to about 20%, about 10% to about 35%, about 10% to about 30%, about 10% to about 25%, and about 10% to about 20%.

[00121] In some embodiments, the purified PEGylated protein, rhARG1 or Co-rhARG1, comprises the area under curve for peak-7 in the proportion of less than about 35%, less than about 30%, less than about 25%, less than about 20%, less than about 15% and less than about 10%.

[00122] In some embodiments, the purified PEGylated protein, rhARG1 or Co-rhARG1, comprises the area under curve for peak-8 in the proportion of less than about less than about 25%, less than about 20%, less than about 15%, less than about 10% and less than about 5%.

[00123] In some embodiments, the purified PEGylated protein, rhARG1 or Co-rhARG1, comprises the area under curve for peak-9 in the proportion of less than about 18%, less than about 16%, less than about 14%, less than about 12%, less than about 10%, less than about 8%, less than about 6%, and less than about 4%.

#### Administration of rhARG1, Co-rhARG1 and Co-rhARG1-PEG

[00124] The rhARG1, Co-rhARG1 and Co-rhARG1-PEG as described herein (and compositions comprising them) can be administered via any appropriate route, including intravenously, intrathecally, subcutaneously, intramuscularly, intratumorally, and/or intraperitoneally. In one or more embodiments, the rhARG1, Co-rhARG1 and Co-rhARG1-PEG (or compositions comprising them) are administered intravenously (IV) or subcutaneously (SC).

[00125] Compositions containing rhARG1, Co-rhARG1 and Co-rhARG1-PEG thereof can be provided in formulations together with physiologically tolerable liquid, gel or solid carriers, diluents, and excipients. Such compositions are typically prepared as liquid solutions or suspensions, as injectables. Suitable diluents and excipients are, for example, water, saline, dextrose, glycerol, or the like, and combinations thereof. In addition, if desired the compositions may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, stabilizing or pH buffering agents.

[00126] Exemplary methods and instructions regarding the administration of rhARG1, Co-rhARG1 and Co-rhARG1-PEG (e.g. pegzilarginase) are provided below. Although the following description is specific to pegzilarginase, the methods and instructions are also applicable to other recombinant Arginase 1 and 2 enzymes.

#### **Recommended Intravenous Dosage Regimen:**

[00127] Obtain a baseline plasma arginine concentration before initiating treatment. The initial recommended dosage of pegzilarginase for ARG1-D patients administered once weekly as a single intravenous infusion is 0.10 mg/kg. If the initial dose of 0.10 mg/kg does not reduce plasma arginine to levels  $\leq 150$  micromol/L, then the dose may be adjusted up to a maximum of 0.20 mg/kg once weekly. If during treatment, plasma arginine levels fall below 50 micromol/L, then consider reducing the dosage. Upon administration of 5 or more IV doses of pegzilarginase, consider using subcutaneous administration of pegzilarginase for ARG1-D patients and continue to periodically monitor plasma arginine levels.

#### **Recommended Subcutaneous Dosage Regimen:**

[00128] When transitioning from pegzilarginase intravenous therapy to subcutaneous administration, administer the first subcutaneous dose instead of the next scheduled intravenous dose. The initial subcutaneous dose should be the same mg/kg dose as the last IV

dose administered. The subcutaneous dose may be modified as clinically indicated to ensure plasma arginine levels remain in the range of 50 – 150 micromol/L.

#### **Blood Arginine Monitoring:**

- 5 [00129] After initiating treatment with pegzilarginase, plasma arginine monitoring should be performed until the patient's plasma arginine level is inside the target range of 50 to 150  $\mu\text{mol/L}$ . Thereafter, periodic plasma arginine monitoring is recommended to assess blood arginine control. Upon changing to subcutaneous dose administration or when making changes to diet, additional plasma arginine monitoring may be required.

10

#### **Preparation and Administration Instructions**

- [00130] Pegzilarginase is supplied as a frozen liquid formulation in 10 mL single-use glass vials that contain 5 mL of pegzilarginase at a concentration of either 1 mg/mL or 5 mg/mL. Each single-use glass vial of pegzilarginase is intended for use as a single intravenous  
15 injection or as a subcutaneous injection. Inspect pegzilarginase visually for particulate matter and discoloration prior to administration. Pegzilarginase is a colorless to slightly yellow or slightly pink solution. Discard if discolored, cloudy or if particulate matter is present in the vial. Remove the flip-top from the vial. Wipe the rubber stopper of the vial with alcohol swabs to disinfect. Use a sterile syringe with an 18G needle to remove the appropriate volume of drug  
20 from the vial. If more than one vial is required, please use a separate needle to draw the solution from each vial. Calculate the solution to be withdrawn from the vial for use in the syringe pump. Once the appropriate volume of drug has been drawn into the syringe, draw normal saline using a separate needle to achieve a total volume of 40 mL. Calculate the required amount of drug to be used as follows:

25

Amount of 1.0 mg/mL pegzilarginase = Patient Weight (kg) x dose level (mg/kg)

Amount of 5.0 mg/mL pegzilarginase =  $\frac{\text{Patient Weight (kg)} \times \text{dose level (mg/kg)}}{5}$

- 30 [00131] Administer pegzilarginase via intravenous infusion over 30 minutes using a syringe pump.

[00132] Table 2: Weight-Based Dosing for Administration of 0.1 mg/kg Once Weekly

| Body Weight (kg)* | Volume to Inject<br>(mL) | Vial Configuration |
|-------------------|--------------------------|--------------------|
| 3                 | 0.30                     | 1 mg/mL            |
| 4                 | 0.40                     | 1 mg/mL            |
| 5                 | 0.50                     | 1 mg/mL            |
| 6                 | 0.60                     | 1 mg/mL            |
| 7                 | 0.70                     | 1 mg/mL            |
| 8                 | 0.80                     | 1 mg/mL            |
| 9                 | 0.90                     | 1 mg/mL            |
| 10                | 1.0                      | 1 mg/mL            |
| 15                | 1.5                      | 1 mg/mL            |
| 20                | 2.0                      | 1 mg/mL            |
| 25                | 2.5                      | 1 mg/mL            |
| 30                | 3.0                      | 1 mg/mL            |
| 35                | 3.5                      | 1 mg/mL            |
| 40                | 4.0                      | 1 mg/mL            |
| 50                | 5.0                      | 1 mg/mL            |
| 60                | 1.20                     | 5 mg/mL            |
| 70                | 1.40                     | 5 mg/mL            |
| 80                | 1.60                     | 5 mg/mL            |

[00133] In one or more embodiments, the volume for a subcutaneous injection has a maximum volume, such as a maximum of 2 mL/injection for adult patients and/or a maximum volume of 1 mL/injection for pediatric patients. If the calculated volume for subcutaneous administration is greater than a maximum volume, then a higher vial concentration may be used (e.g. 5 mg/mL instead of 1 mg/mL) and/or the volume may be split into multiple smaller injections (e.g. a 4 mL injection is split into 2 injections of 2 mL each).

#### Dosage Forms and Strengths

[00134] pegzilarginase injection is a colorless to slightly yellow or slightly pink solution available as follows in 10mL vials:

- a. Solution for Injection: 5 mL of 1.0 mg/mL

b. Solution for Injection: 5 mL of 5.0 mg/mL

### **Warnings and Precautions**

[00135] Hypersensitivity reactions may occur with administration of pegzilarginase. Monitor all patients for signs and symptoms of acute allergic reactions (e.g. urticaria, pruritus, erythema, hypotension, tachycardia) during and following pegzilarginase infusion. In case of severe hypersensitivity reactions, slow or discontinue the administration of pegzilarginase immediately and administer appropriate medical care. Consider premedication of patients with a non-sedating antihistamine prior to dosing. In the event that corticosteroids are required, they should be used with caution due to their potential to cause hyperammonemia.

### **Pregnancy: Pregnancy Category B**

[00136] Reproduction studies have been performed in mice and rats at doses up to 100 mg/kg. There was no evidence of harm to the fetus due to pegzilarginase. There are, however, no adequate and well-controlled studies in pregnant women. Because animal reproduction studies are not always predictive of human response, pegzilarginase should be used during pregnancy only if clearly needed.

### **Nursing Mothers**

[00137] It is not known if pegzilarginase is present in human milk. The developmental and health benefits of breastfeeding should be considered along with the mother's clinical need for pegzilarginase and any potential adverse effects on the breastfed child from the drug.

### **Description**

[00138] Pegzilarginase is a cobalt substituted, recombinant human arginase I enzyme that is covalently conjugated to monomethoxy polyethylene glycol (mPEG) that acts by catalyzing the same reaction as arginase I, converting arginine into ornithine and urea. Human arginase I is a binuclear manganese metalloenzyme. To produce pegzilarginase, the manganese cofactor is replaced with cobalt to yield Co-Arginase I. The substitution of the native manganese (Mn+2) with cobalt (Co+2) in the active site of arginase I enhances the stability and catalytic activity at physiological pH. Pegylation extends the circulating half-life. The average molecular weight of pegzilarginase is approximately 284 kDa. Pegzilarginase has a specific activity ranging from approximately 320-600 units per mg of protein content. One

activity unit is defined as the amount of enzyme required to convert 1 micromole of arginine to ornithine per minute at 37°C.

[00139] Pegzilarginase is intended for intravenous or subcutaneous infusion and is supplied as a sterile, clear, colorless to slightly yellow or slightly pink solution formulated at a 1 mg/mL and at a 5 mg/mL concentration in a buffer containing 50 mM sodium chloride, 5 mM potassium phosphate, and 1.5% w/v glycerol, at a pH of 7.4. It is provided as a preservative-free, sterile solution in a clear, single-use, glass vial. Each vial of 1 mg/mL pegzilarginase drug product contains 5 mL of drug product (5 mg pegzilarginase per vial). Each vial of 5 mg/mL pegzilarginase drug product contains 5 mL of drug product (25 mg pegzilarginase per vial). Vials are stoppered with a coated rubber stopper and sealed with an aluminum flip off seal and are stored frozen at  $\leq -60^{\circ}\text{C}$  and thawed before use.

### Pharmacodynamics

[00140] pegzilarginase treatment of adults and pediatric patients with Arginase 1 Deficiency resulted in the reduction of blood arginine concentrations from pre-treatment baseline values into the normal blood arginine range of 40 to 115 micromole/L. Maximum suppression of L-arginine was observed at approximately 8 hours post-dose, decreasing in a dose dependent manner with recovery to pre-dose levels occurring by 168 hours post dose. A strong correlation was observed between pegzilarginase and arginine, with an immediate suppressive effect on arginine following IV administration, and the maximum decrease of arginine concentration being reached within 24 hours post-dose.

### Pharmacokinetics

[00141] Following IV administration to 14 subjects, pharmacokinetic samples were collected throughout the dosing interval from 0-168 hours to characterize the relationship between pegzilarginase pharmacokinetics and arginine. Across the dose range (0.015 mg/kg — 0.2 mg/kg), pegzilarginase exposure, as measured by  $C_{\text{max}}$  and  $\text{AUC}_{0-168}$ , increased approximately proportional to dose, with a 13-fold increase in dose resulting in a 14-fold increase in  $C_{\text{max}}$  and  $\text{AUC}_{0-168}$ . No accumulation of pegzilarginase was observed following a once weekly IV dosing regimen, with a  $T_{1/2}$  of approximately 30 hours across the dose range, and low to moderate inter-subject variability (13 – 46 % CV) in the exposure metrics.

**Animal Toxicology and/or Pharmacology**

[00142] The pharmacologic effects of pegzilarginase on arginine levels were assessed in a neonatal transgenic mouse model of Arginase I and a tamoxifen-induced arginase deficiency model in adult mice. These models mimic the human disease in that a significant excess of circulating arginine and catabolites of arginine are present; however, unlike humans with Arginase I Deficiency, these animals develop severe and generally lethal hyperammonemia. Pharmacologic effects also were assessed in a rat arginine-induced model of hyperargininemia. Pegzilarginase reduced plasma arginine levels in a dose-dependent manner.

[00143] The potential toxicity and TK of pegzilarginase were evaluated in postnatal day (PND) 21 (equivalent to a 2-year old human) juvenile rats administered once weekly IV bolus injections at 0.1, 0.3, and 1.0 mg/kg for 6 months followed by a 6-week recovery period. Pegzilarginase was well tolerated, with no test article-related mortality and no significant test article effects observed on: food consumption, coagulation, urinalysis, ophthalmoscopic examinations, sexual maturation, growth hormone analyses, bone marrow analyses, functional observation battery (FOB) evaluations and neurobehavioral testing (auditory startle habituation, motor activity, or Morris water swim maze). There were no pegzilarginase-related macroscopic findings at the end of the 6-month terminal and 6-week recovery intervals. Adverse microscopic changes were limited to the testes and epididymides and correlated with reduced weight of male reproductive organs and adverse sperm analyses findings at 0.3 and 1.0 mg/kg. At 1.0 mg/kg, an adverse effect was observed on sperm analyses with reduced sperm motility, lower caudal epididymal sperm counts, decreased sperm concentration, and increased percentage of abnormal sperm observed. These observations were considered a direct treatment-related effect and correlated with microscopic changes of subtle tubular degeneration in the testes at 0.3 mg/kg and 1.0 mg/kg. Following the 6-week recovery period for the control and 1.0 mg/kg groups, these changes were overall reversible with the exception of the increased percentage of abnormal sperm and sperm counts. The partial reversibility after 6 weeks was not unexpected because the normal sperm development cycle is approximately 9 weeks or longer than the 6-week recovery period.

[00144] Importantly, there were no apparent PEGylation effects observed by histopathology. Toxicokinetic data indicated that pegzilarginase exposure was maintained throughout the study. In conclusion, the NOAEL in females was 1.0 mg/kg. In males, the

NOAEL was 0.1 mg/kg based on microscopic changes in the testes at 0.3 mg/kg and 1.0 mg/kg.

[00145] The potential toxicity and TK of pegzilarginase were evaluated following once weekly intravenous bolus injection to cynomolgus monkeys at doses of 0.1, 0.3, and 1.0 mg/kg for 13 weeks followed by a 4-week recovery period. Clinical signs observed at 1.0 mg/kg included decreased body weight, increased incidences of sparse hair (entire body), dry/discolored skin (entire body), tremors, inappetence, watery feces, decreased activity, ataxia, muscle wasting, and/or unkempt/hunched appearance. No treatment-related effects were noted in clinical pathology parameters (coagulation, growth hormone, and urinalysis), ECG and ophthalmic examinations, respiratory rate, and blood pressure assessments.

#### How Supplied/Storage and Handling

[00146] Pegzilarginase is supplied as a solution for injection.

[00147] Pegzilarginase is supplied frozen ( $\leq -60^{\circ}\text{C}$ ). Diluted pegzilarginase should be used immediately. If immediate use is not possible, diluted pegzilarginase may be stored for up to 8 hours at  $2^{\circ}\text{C}$  to  $8^{\circ}\text{C}$  ( $36^{\circ}\text{F}$  to  $46^{\circ}\text{F}$ ) during administration.

#### [00148] EXAMPLES

[00149] Before describing several exemplary embodiments of the disclosure, it is to be understood that the disclosure is not limited to the details of construction or process steps set forth in the following description. The disclosure is capable of other embodiments and of being practiced or being carried out in various ways.

[00150] In the experimental disclosure which follows, the following abbreviations apply: eq (equivalents); M (Molar);  $\mu\text{M}$  (micromolar); mM (millimolar); N (Normal); mol (moles); mmol (millimoles);  $\mu\text{mol}$  (micromoles); nmol (nanomoles); g (grams); mg (milligrams);  $\mu\text{g}$  (micrograms); L (liters); ml (milliliters);  $\mu\text{l}$  (microliters); cm (centimeters); mm (millimeters);  $\mu\text{m}$  (micrometers); nm (nanometers); MW (molecular weight); PBS (phosphate buffered saline); min (minutes).

#### Example 1: Cation Exchange Column Chromatography (Column 1)

[00151] In a preferred embodiment, Arginase 1 is captured on a cation exchange column (CEX) to reduce product-related impurities and process-related impurities such as host cell proteins (HCP), DNA, and endotoxin (see Figure 3 for an overview of the purification

process). In a particular embodiment, the first column (Column 1) chromatography step in the Arginase 1 purification process uses SP Sepharose FF resin and an inlet heat exchanger. Column 1 uses cation-exchange chromatography to bind Arginase 1 in the absence of salt at pH 7.6, and elute with a buffer of increased salt concentration (Figure 4(a)). In one  
5 embodiment the salt is NaCl and the elution from Column 1 is performed with 25 mM HEPES, 0.1M NaCl, pH 7.2-7.6 at room temperature. However, alternative embodiments are possible such as application of a NaCl gradient to Column 1.

[00152] Figure 4(a) shows a representative purification of Arginase 1 on Column 1. Approximately three liters of clarified *E. coli* lysate were loaded on the cation exchange  
10 column. As can be seen from the high level of absorbance at 280 nm, a large amount of protein did not bind onto the column and is detected in the flow through. The column was then washed with approximately two liters of column wash solution. As detected by absorbance at 280 nm, a fraction enriched for Arginase 1 was then eluted with 0.1 M NaCl (final peak).

## 15 Example 2: Cobalt Substitution

[00153] In a preferred embodiment, the Arginase 1 native manganese co-enzyme is replaced by cobalt. During cobalt substitution (also called cobalt loading), one or both of the two manganese ions normally present in Arginase 1 are replaced with cobalt ions. A wide variety of temperatures can be used for the cobalt substitution step as well as a wide  
20 concentration of cobalt (See Table 2). Incubation times for cobalt substitution can be as short as 10 minutes and be performed at over 50 °C. Conversely, cobalt loading temperature can be as low as 1°C or 5°C and performed for over 8 hours. Also, the higher proportion of cobalt loaded into Arginase 1 leads to a higher specific activity.

[00154] The Arginase 1 eluted from Column 1 (also called Column 1 Pool) can be held  
25 at room temperature for the cobalt substitution step. In one embodiment, Cobalt Chloride Stock Solution (0.5 M CoCl<sub>2</sub>) is diluted 50-fold by adding it to Column 1 Pool at a defined rate, with final cobalt chloride concentration at 10 mM. The cobalt substitution is then mixed for two hours at 20°C. In another embodiment, Arginase 1 cobalt loading is performed in a solution of 10mM CoCl<sub>2</sub> for about 2 hours to about 8 hours at room temperature.

30 [00155] An overview of the cobalt loading step can be seen in Table 3.

[00156] Table 3

Co-Arginase I Cobalt Loading

| Identity        | Co (mM) | Temp (°C) | Time (Min) | Total Co (µg/mg Arginase) | Total Mn (µg/mg Arginase) | Specific Activity (U/mg) |
|-----------------|---------|-----------|------------|---------------------------|---------------------------|--------------------------|
| APO-Arginase I* | NA      | NA        | NA         | <0.025                    | 0.008                     | 24                       |
| APO Loading 1*  | 0.1     | 5         | 15         | 0.3                       | ND                        | 117                      |
| Coh-Arg I*      | 10      | 20        | 60         | 2                         | 0.06                      | 410                      |
| APO Loading 2*  | 1       | 5         | 15         | 2.4                       | ND                        | 395                      |
| APO Loading 3*  | 10      | 20        | 15         | 2.8                       | ND                        | 493                      |
| APO Loading 4*  | 10      | 20        | 60         | 2.9                       | ND                        | 489                      |
| APO Loading 5   | 10      | 37        | 15         | 2.8                       | ND                        | NT                       |
| APO Loading 6   | 10      | 53        | 15         | 2.6                       | ND                        | NT                       |
| Co-ArgI-PEG     | 10      | 53        | 15         | 3                         | ND                        | 500                      |
| Theoretical     |         |           |            | 3.4                       |                           |                          |

\*Graphed

**Example 3: Ultrafiltration/Diafiltration 1 (UF/DF 1)**

[00157] UF/DF 1 removes free cobalt ions and exchanges the Co-Arginase 1 into a solution in preparation for anion exchange chromatography on. The UF/DF1 step uses membranes with a molecular weight cutoff of 30 kDa. One important function of this step is to reduce the levels of free cobalt and buffer exchange the Co-Arginase 1 Pool prior to anion exchange chromatography. Membranes are sanitized with cleaning solution (0.5 N NaOH) and rinsed with water. A normalized water permeability test (NWP) is performed followed by equilibration prior to use in production. Once the UF/DF system is equilibrated, the Co-Arginase 1 Pool is diafiltered against 25 mM HEPES, 0.1 M NaCl, pH 7.6, for three diavolumes, followed by four diavolumes of 50 mM Tris, pH 8.4. After diafiltration, the pool is recirculated and recovered from the system using two times the system's hold-up volume with 50 mM Tris, pH 8.4.

[00158] The UF/DF1 membranes are cleaned by performing a 2 M NaCl flush followed by a denaturing cleaning step using 0.5 N NaOH with a 30 minute recirculation. The system is flushed with purified water and the NWP tested to assess the effectiveness of the cleaning procedures. Membranes may be stored in 0.1 N NaOH.

[00159] In an alternative embodiment, the first exchange of buffer is into 25mM HEPES, 0.1M NaCl, pH 7.2-7.6 and the second exchange is into 50mM Tris, pH 8.1-8.5.

**Example 4: Anion Column Chromatography (Column 2)**

[00160] A preferred embodiment of the Arginase 1 purification uses another column which is an anion exchange column chromatography ("Column 2"). One embodiment of Column 2 is a Q Sepharose FF resin. One function of this Column 2 step is to reduce process-related impurities such as host-cell DNA and endotoxin from the UF/DF1 pool. Column 2  
5 binds these impurities while Co-Arginase 1 flows through and is collected in the column effluent during the load and wash steps. In one embodiment, the anion exchange flow-through chromatography for Column 2 is performed with Q Sepharose FF, and up to 40 g protein/L resin is loaded onto the column with buffer 50mM Tris, pH 8.1-8.5.

[00161] In another embodiment of the methods, the First Protein Product is loaded onto  
10 an anion exchange column to capture impurities while Arginase 1 is retrieved in the flow through. Figure 4(b) is a representative chromatogram of Arginase 1 purification over an anion exchange column (Column 2). As can be seen from absorbance at 280 nm a large amount of protein is detected in the flow through. Impurities are captured on Column 2 and not eluted into the Column 2 Pool (also called Second Protein Product) which is further enriched for  
15 Arginase 1.

#### **Example 5: Capto Multimodal Column Chromatography (Column 3)**

[00162] In a preferred embodiment, the Arginase purification process uses a third column chromatography column (Column 3). In one embodiment, Column 3 is a Capto multimodal chromatography (MMC) column or alternatively a size exclusion column.  
20 Embodiments that use MMC capture Arginase 1 on the column while process-related impurities such as host cell proteins (HCP), DNA, and endotoxin are washed out in the flow through. In this embodiment, Co-Arginase 1 can be captured by the column in the absence of salt at pH 8.4 and then Co-Arginase 1 eluted with a buffer of increased salt concentration. A  
25 representative example of Capto Multimodal Cation exchange chromatography column is shown in Figure 4(c).

[00163] In one embodiment, MMC chromatography (Column 3) uses approximately 15 column volumes to load up to 30 g protein/L resin, and the high salt step elution is performed with 50mM Tris, 250mM NaCl, pH 8.1-8.5. In several embodiments, the flow through from  
30 the anion exchange column (Column 2) is loaded onto a Capto MMC column at pH 8.4, washed, then the bound Co-Arginase 1 is eluted using 50 mM tromethamine, 250 mM sodium chloride.

**Example 6: Ultrafiltration/Diafiltration 2 (UF/DF 2)**

[00164] UF/DF 2 concentrates Arginase 1 and exchanges the protein into a pre-PEGylated intermediate. The UF/DF2 step uses membranes with a molecular weight cutoff of 30 kDa. An important function of this step is to buffer exchange the Column 3 Pool of the un-PEGylated Co-Arginase 1 intermediate prior PEGylation (or prior to additional filtration and storage). Membranes are sanitized with cleaning solution (0.5 N NaOH) and rinsed with water. Once the UF/DF system is equilibrated, Column 3 Pool (also called Third Protein Product) is diafiltered against 20 mM sodium phosphate, 50 mM sodium chloride, 1.5% (w/v) glycerol, pH 7.4, for five diavolumes. If the Column 3 pool concentration is < 8 g/L, the pool is further concentrated to 8 g/L. After diafiltration (and concentration, if necessary), the pool is recirculated and recovered from the system using two times the system's holdup volume with 20 mM sodium phosphate, 50 mM sodium chloride, 1.5% (w/v) glycerol, pH 7.4. After recovery, a two-step dilution with diafiltration solution may be employed. The first dilution has a concentration target of 6 g/L and the second step has a concentration target of 5 g/L. Two steps may be used to reach the target. The second step may not be necessary if the concentration following the first dilution is within the targeted range.

**Example 7: Intermediate Filtration and UF/DF 3**

[00165] Prior to the PEGylation reaction, the cobalt-containing Arginase 1 can be stored long-term, including frozen long-term. The intermediate Co-Arginase can be filtered through a 0.2 µm filter and can be frozen for long term storage.

[00166] The UF/DF3 step uses membranes with a molecular weight cutoff of 30 kDa. One function of this step is to buffer exchange and concentrate the filtered UF/DF2 pool (fresh or thawed) to provide conditions optimal for PEGylation. If frozen Co-Arginase 1 intermediate is used as the starting material, thawing will be done at room temperature for up to 36 hours. The membranes are sanitized with cleaning solution (0.5 N NaOH) and rinsed with water. A normalized water permeability test (NWP) is performed followed by equilibration prior to use in production. Once the UF/DF system is equilibrated, the Co-Arginase 1 Intermediate is diafiltered against 0.1 M sodium phosphate, pH 8.4, for five diavolumes. After diafiltration, the pool is concentrated, recirculated, and recovered from the system using two times the system's hold-up volume with 0.1 M sodium phosphate, pH 8.4. After recovery, a two-step dilution with

diafiltration solution is employed. The first dilution has a concentration target of 11 g/L and the second step has a concentration target of 10 g/L. Two steps are utilized to facilitate the target is levels. The second step may not be necessary if the concentration following the first dilution is within the targeted range.

5 [00167] Regarding the UF/DF 2 and UF/DF 3 steps, the first buffer exchange can be into 20mM Sodium Phosphate, 50mM NaCl, 1.5% Glycerol, pH 7.4,  $\geq 5$ DV, and protein concentrated to approximately 5.0 mg/mL. The second buffer exchange can be made into 0.1M sodium phosphate, pH 8.1-8.5, and protein concentrated to approximately 10.0 mg/mL (in preparation for PEGylation of the drug substance).

10

**Example 8: PEGylation of Arginase 1**

[00168] PEGylation covalently attaches PEG (polyethylene glycol) to the Co-Arginase 1 (drug substance) molecule (see Table 4 for a representative embodiment of the PEGylation step). In one embodiment, the PEGylation reaction covalently binds 5000 Da PEG molecules  
15 to Co-Arginase 1. In alternative embodiments, PEGylation can be performed prior to cobalt substitution of Arginase 1 or at other points in the production process. In one embodiment, the PEG conjugation reaction can use solid methoxy PEG succinimidyl carboxymethyl ester which reacts with sterically available lysines on Co-Arginase 1. The resulting PEGylated protein (Co-rhARG1-PEG) has a molecular weight of approximately 280 kDa. The PEGylated  
20 pool can be filtered and stored at 2-8°C until UF/DF4 operations.

[00169] Table 4: PEGylation Process for Co-rhARG1 Drug Substance

| Step | Process Operation                                                                                                  |
|------|--------------------------------------------------------------------------------------------------------------------|
| 1    | Thaw intermediate (if applicable)                                                                                  |
| ↓    |                                                                                                                    |
| 2    | Ultrafiltration/diafiltration                                                                                      |
| ↓    |                                                                                                                    |
| 3    | Add solid methoxy PEG succinimidyl carboxymethyl ester (MW 5000) and incubate for greater than 15 min<br>pH 8.4    |
| ↓    |                                                                                                                    |
| 4    | Ultrafiltration/diafiltration                                                                                      |
| ↓    |                                                                                                                    |
| 5    | Filter the (Co-rhARG1-PEG) drug substance, fill in polycarbonate bottles, and freeze at $\leq -60^{\circ}\text{C}$ |

[00170] In one embodiment, solid methoxy PEG succinimidyl carboxymethyl ester (MW 5000) can be added to the Arginase 1 containing solution at a 19.3x molar excess and incubation 0.5- 4.0 hours, pH 8.4.

[00171] Following PEGylation, ultrafiltration/diafiltration removes unbound PEG, exchanges the Arginase 1 into a formulation buffer and concentrates the Arginase 1 for the formulation step. This UF/DF4 step uses membranes with a molecular weight cutoff of 100 kDa. One function of this step is to buffer exchange the PEG pool into the final formulation while removing free PEG. Membranes used for this purpose are sanitized with cleaning solution (0.5 N NaOH) and rinsed with water. Once the UF/DF system is equilibrated, the PEG Pool is diafiltered against 5 mM potassium phosphate, 50 mM sodium chloride, 1.5% (w/v) glycerol, pH 7.4 for ten diavolumes. After diafiltration, the pool is recovered from the system with pressure. The recovered UF/DF4 Pool is diluted to 5 g/L with 5 mM potassium phosphate, 50 mM sodium chloride, 1.5% (w/v) glycerol, pH 7.4, prior to the final filtration and fill steps. IN an alternative embodiment, Arginase 1 is exchanged into 20mM Sodium Phosphate, 50mM NaCl, 1.5% Glycerol, pH 7.4, and adjusted to a protein concentration of about 5.0 mg/mL.

[00172] In some embodiments, the formulation buffer 5 mM potassium phosphate, 50 mM sodium chloride, 1.5% glycerol, pH 7.4 was found to enhance the stability upon storage of Arginase 1 compared other buffers such as sodium phosphate buffer. In one or more embodiments, the buffer 5 mM potassium phosphate comprises 1 mM  $K_2HPO_4$  and 4 mM  $KH_2PO_4$ .

[00173] Drug substance (Co-rhARG1-PEG) is a PEGylated cobalt-substituted human Arginase 1 made by conjugating activated PEG molecules with the  $\epsilon$ -amino group of lysines and the amine group of N-terminal amino acid. A dye-based fluorescent assay is used to determine the molar ratio of PEG molecules per protein using ortho phthaldialdehyde. Ortho-phthaldialdehyde reacts in the presence of thiols, specifically with primary amines, to form fluorescent derivatives. Measurement of the fluorescent signal allows for the quantitation of reactive free amines present in a protein molecule. Quantitation is based on a standard curve using N-acetyl lysine. The number of PEGylated amines per protein can be determined by subtracting the number of free amines as measured by the fluorescent assay of the PEGylated drug substance from the theoretical number of free amines present in the unconjugated Co-Arginase 1. The theoretical number of free amines from lysine residues plus the N-terminal amino acid is 25. Free unconjugated PEG in the drug substance is measured by SEC-HPLC

with detection by refractive index. Results can be expressed as  $\mu\text{g/mL}$  of free PEG (see Table 5).

**[00174]** Table 5: SEC-HPLC Method Parameters Free PEG Co-rhARG1-PEG Drug Substance

| Description             | Method parameter                                      |
|-------------------------|-------------------------------------------------------|
| Column                  | TOSOH G3000PWxl, 7.8 x 300 mm, 7 $\mu\text{m}$ column |
| Detector                | Refractive Index Detector                             |
| Injection volume        | 10 $\mu\text{L}$                                      |
| Flow rate               | 0.8 mL/min                                            |
| Autosampler temperature | 5°C                                                   |
| Column temperature      | 20°C                                                  |
| Mobile phase A          | 1X PBS                                                |
| Stop time               | 20 minutes                                            |

5

#### Example 9: CIEX-HPLC Characterization of Drug Intermediate

**[00175]** During *E. coli* fermentation, various Arginase 1 charge variants may be produced. Charge variants can be analyzed by a cation exchange HPLC (CIEX-HPLC) method using a TSK gel cation exchange column. This type of analysis uses a mobile phase (A) of 20 mM MES, pH 6.0 and a mobile phase B of 20 mM MES, 500 mM NaCl pH 6.0; flow rate of 1.0 mL/minutes; run time of 40.0 minutes; column temperature of 22°C; and mobile phase gradient according to Table 6.

10

**[00176]** Table 6: CIEX-HPLC Charge Variants Co-Arginase 1 Intermediate Gradient Program

| Minutes | % Mobile Phase B |
|---------|------------------|
| 0.00    | 0                |
| 1.95    | 0                |
| 17.00   | 30               |
| 23.00   | 60               |
| 25.00   | 100              |
| 30.00   | 100              |
| 30.10   | 0                |
| 40.00   | 0                |

15

**[00177]** Samples are diluted with formulation buffer prior to analysis. The results are described as percent charge variant distribution. A representative chromatogram is shown in

Figure 5(a) where six predominant peaks are typically observed for Co Arginase 1 intermediate.

#### Example 10: iCIEF Characterization of Drug Substance

[00178] The drug intermediate is PEGylated to form the drug substance. PEGylation of the drug intermediate renders the use of the drug intermediate CIEX-HPLC method less suitable than other embodiments developed as part of this invention. An anion IEX-HPLC was evaluated but did not give an adequate separation. Alternatively, an imaging capillary isoelectric focusing (iCIEF) method was developed to analyze charge variants of the drug substance.

[00179] Analytes in imaging capillary isoelectric focusing (iCIEF) migrate through a capillary by the counter-migration of hydronium ions (anolyte), and hydroxyl ions (catholyte) in the presence of an applied electric field. The sample is diluted in a matrix containing carrier ampholytes and pI markers. Separation of proteins occurs in two focusing steps. An initial prefocusing step establishes the pH gradient. Charge variants are more sharply focused and separated during a second higher voltage focusing step. An image of UV light absorption of the entire capillary is digitally captured every 30 seconds and after completion of the focusing steps.

[00180] The results can be expressed as percent charge variant distribution. A representative electropherogram is shown in Figure 5(b) where nine predominant peaks are observed for drug substance. Peaks 3 and 4 are integrated together because the resolution between those peaks has been shown to be variable. The relative areas of these peaks are provided in Table 7:

[00181] Table 7: iCIEF Characterization of Charge Variants of Co-rhARG1-PEG

|    | Name     | pI    | Area   | % Area |
|----|----------|-------|--------|--------|
| 1  | Marker   | 4.220 |        |        |
| 2  | Peak 1   | 6.111 | 130195 | 12     |
| 3  | Peak 2   | 6.268 | 245568 | 22     |
| 6  | Peak 3+4 | 6.360 | 255343 | 23     |
| 7  | Peak 5   | 6.501 | 226011 | 20     |
| 8  | Peak 6   | 6.604 | 156066 | 14     |
| 9  | Peak 7   | 6.708 | 71805  | 6      |
| 10 | Peak 8   | 6.794 | 27780  | 2      |
| 11 | Peak 9   | 6.866 | 8847   | 1      |
| 12 | Marker   | 7.650 |        |        |

**Example 11: Enzyme Activity of Co-Arginase 1 Intermediate and Drug Substance**

[00182] The enzymatic assay used to measure activity and to establish identity of Co-Arginase 1 intermediate and Co-rhARG1-PEG drug substance monitors the conversion of arginine to ornithine. The reaction mixtures have one enzyme concentration tested at seven  
 5 different arginine substrate concentrations over a range of 0 - 2 mM. The reactions are conducted for a fixed time at 37°C. The reaction time has been established to ensure that there is less than 10% consumption of substrate at any given substrate concentration. The reaction is quenched and the product, ornithine, is derivatized and quantified by reverse phase-UPLC.

[00183] Examples of plots of reaction velocity vs substrate concentration are shown in  
 10 Figure 8(a) (Co-Arginase 1 Intermediate) and Figure 8(b) (Co-rhARG1-PEG drug substance) along with representative  $K_{cat}$ ,  $K_m$ , and  $K_{cat}/K_m$  values.

**Example 12: Analysis of Cobalt and Manganese**

[00184] Cobalt, residual manganese, and free cobalt were measured using inductively  
 15 coupled plasma mass spectrometry (ICP-MS). Samples were digested by microwaving and using 1% nitric acid and 6% hydrogen peroxide to release all the metals from the matrix. The resulting digestion was analyzed by ICP-MS. Cobalt and residual manganese samples were digested without any sample treatment. Free cobalt was measured on permeate samples that have been ultrafiltered to separate the enzyme from the permeate to measure cobalt that is not  
 20 associated with the enzyme. Table 8 summarizes some of the characteristics of Co-Arginase 1 Intermediate.

[00185] Table 8: Typical Co-Arginase 1 Intermediate Characteristics

| Test               | Purpose  | Analytical Principle | Typical Values                                                                                      |
|--------------------|----------|----------------------|-----------------------------------------------------------------------------------------------------|
| CIEX HPLC          | Charge   | HPLC                 | Peak 1: 0-4%<br>Peak 2: 2-8%<br>Peak 3: 10-20%<br>Peak 4: 16-30%<br>Peak 5: 40-52%<br>Peak 6: 4-15% |
| SDS-PAGE (reduced) | Purity   | Charge mobility      | ≥95%                                                                                                |
| $K_m$<br>$k_{cat}$ | Activity | HPLC                 | 0.15-0.24 mM<br>200-380/sec                                                                         |
| Specific activity  | Activity | HPLC                 | 320-600 U/mg                                                                                        |
| Cobalt             | Activity | ICP-MS               | 7-13 µg/mL                                                                                          |

|                    |                    |        |             |
|--------------------|--------------------|--------|-------------|
| Free cobalt        | Stability          | ICP-MS | ≤0.05 µg/mL |
| Residual manganese | Impurity, activity | ICP-MS | ≤1 µg/mL    |
| pH                 | Acidity            | pH     | 7.0-7.8     |

**[00186]** Table 9: Typical Co-rhARG1-PEG Drug Substance Characteristics

| Test                     | Purpose    | Analytical Principle                           | Typical Values                                                                                                                           |
|--------------------------|------------|------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------|
| Charge heterogeneity     | Quality    | Imaging capillary isoelectric focusing (ICIEF) | Peak 1: <20%<br>Peak 2: <30%<br>Peaks 3 and 4: 10-30%<br>Peak 5: 15-30%<br>Peak 6: 10-25%<br>Peak 7: <25%<br>Peak 8: <15%<br>Peak 9: <8% |
| $K_m$<br>$k_{cat}$       | Activity   | HPLC                                           | 0.15-0.24 mM<br>200-380/sec                                                                                                              |
| Specific activity        | Activity   | HPLC                                           | 320-600 U/mg                                                                                                                             |
| Total Cobalt             | Activity   | ICP-MS                                         | 7-13 µg/mL                                                                                                                               |
| Residual Manganese       | Activity   | ICP-MS                                         | <1 µg/mL                                                                                                                                 |
| Free Cobalt              | Stability  | ICP-MS                                         | ≤0.05 µg/mL                                                                                                                              |
| PEG: Protein Molar Ratio | PEGylation | HPLC                                           | 8-16 moles/mole                                                                                                                          |
| Free PEG                 | Impurities | HPLC                                           | <500 µg/mL                                                                                                                               |
| pH*                      | Acidity    | pH                                             | 7.0-7.8                                                                                                                                  |

**Example 13: Co-Arginase 1 Intermediate Post-Translational Modifications**

**[00187]** Co-Arginase 1 intermediate post-translational modifications were detected using a variety of techniques such as peptide mapping, LC-MS intact mass spectrometry, and reverse phase LC/MS. The summary of all identified modifications is listed in Table 10.

**[00188]** Table 10: Identified Modifications of Co-Arginase 1 Intermediate

| Modification                               | Method of Detection                                       |
|--------------------------------------------|-----------------------------------------------------------|
| N-terminal gluconoylation / Gluconoylation | Peptide mapping (Glu-C digestion) RP-HPLC-MS/MS and LC-MS |
|                                            | RP-UPLC/MS                                                |
| Phosphogluconoylation                      | RP-UPLC/MS                                                |
| Di-Gluconoylation                          | RP-UPLC/MS                                                |
| N-terminal methionine-truncation           | Peptide mapping (Glu-C digestion) RP-HPLC-MS/MS and LC-MS |
| Acetylation                                | Peptide mapping (Glu-C digestion) RP-                     |

|                                         |                                                               |
|-----------------------------------------|---------------------------------------------------------------|
|                                         | HPLC-MS/MS and LC-MS                                          |
| N-deamidation/<br>succinimide formation | Peptide mapping (Glu-C digestion) RP-<br>HPLC-MS/MS and LC-MS |

[00189] Characterization determined that when Co-Arginase 1 intermediate modification was present, the predominant modification was N-terminal gluconoylation (confirmed by peptide mapping). Additional characterization of Arginase 1 modified species was performed by testing samples taken at three time points (fermentation, post-Column 1, and on drug intermediate from Column 3). The analytical methods typically require Arginase to be dissociated and analyzed as a monomer. Arginase 1 N-terminal gluconoylated (analyzed as a monomer) was typically 10.8 to 13.9%. Other modifications were N-terminal phosphogluconoylated monomer (4.3 to 6.5%), and di-gluconoylated monomer (0.7 to 1.2%), across the three timepoint samples. In a sample used from a standardized reference production run, the levels of un-modified Co-Arginase 1 (monomer) and Co-Arginase 1 intermediate were comparable at 80.6% to 83.6%, respectively. The standard condition used for the purification process (i.e. no salt gradient applied to Column 1) moderately altered the relative level of un-modified monomer carried through to Co-Arginase 1 intermediate (81.1 to 83.6%).

[00190] Table 11: Co-Arginase 1 Characterization LC/MS Results

| Sample ID              | Arginase 1 Monomer (Unmodified) (%) | Gluconoylated Arginase 1 Monomer (%) | Phosphogluconoylated Arginase 1 Monomer (%) | Di-Gluconoylated Arginase 1 Monomer (%) |
|------------------------|-------------------------------------|--------------------------------------|---------------------------------------------|-----------------------------------------|
| Standardized Reference | 80.6                                | 13.9                                 | 4.3                                         | 1.1                                     |
| Fermentation           | 81.1                                | 12.4                                 | 6.5                                         | 1.2                                     |
| Post-Column            | 81.4                                | 12.2                                 | 5.4                                         | 0.9                                     |
| Drug Substance         | 83.6                                | 10.8                                 | 4.9                                         | 0.7                                     |

#### Example 14: Variation of Column 1 Conditions

[00191] In alternative embodiments, a NaCl gradient can be applied to Column 1. Using a NaCl gradient over Column 1 enables separation of different Arginase 1 variants to select for preferred embodiments. Figure 7 shows a gradient from 0.0 - 0.2 M NaCl applied to Column 1. Individual fractions collected from the Column 1 elution were assayed by SE-HPLC, CEX-HPLC, and RP-HPLC.

[00192] An analytical CEX-HPLC method was used that assigns Arginase 1 charge variants Peak Numbers of 1 through 6 (see Figure 5(c)). The Peak Numbers align with the

various gluconoylation states as well as un-gluconoylated Arginase 1. This analysis showed six peaks in the Arginase 1 eluted from the NaCl gradient. Arginase 1 variants assigned Peak Numbers 1, 2, and 3 eluted early in the Column 1 elution peak. Peak 4 eluted through the highest concentration portion of the eluted Arginase 1 and peak 5 (unmodified Arginase 1) and peak 6 eluted later in the elution peak. Thus, the 0.0 – 0.2 M NaCl successfully separated different charge variants of Arginase 1.

[00193] Alternative NaCl gradients can be used for Column 1 elution such as 0 – 0.5 M NaCl. The use of a NaCl gradients was found to reproducibly separate Arginase 1 into six distinct peaks enabling selection of specific Arginase 1 variants for further processing in the manufacture of drug substance or drug product.

[00194] Further analysis of the first protein product (and the Arginase 1 variants) was also analyzed by LC/MS (see Figure 6). The LC/MS analysis identifies specific types of gluconylation generated by production of Arginase 1 in *E. coli*. The LC/MS analysis identifies unmodified Arginase 1, gluconylated Arginase 1, phosphogluconylated Arginase 1, and 2 times (2X) gluconylated Arginase 1.

[00195] Table 12 shows that the application of a 0-0.2 M NaCl gradient (and the corresponding fractionated CEX Peaks 1 - 6) produces fractions that have differing levels of gluconylation. Each of Peak Numbers 1-6 were analyzed by LC/MS. The data show that the dominant peak (Peak 5) has a high percentage of non-gluconylated Arginase 1 as well as high specific activity. Depending upon the desired characteristics different fractions (corresponding to Peaks 1-6) can be collected for further processing.

[00196] Table 12: LC/MS analysis of drug intermediate Peaks 1 – 6.

| Fraction      | Concentration (mg/mL) | % Non-Gluconylated Arginase 1 Monomer Mass by MS | Specific Activity (U/mg) |
|---------------|-----------------------|--------------------------------------------------|--------------------------|
| Peak Number 1 | 0.2                   | 28.4                                             | 118                      |
| Peak Number 2 | 0.9                   | 37.8                                             | 286                      |
| Peak Number 3 | 3.4                   | 56.2                                             | 298                      |
| Peak Number 4 | 6.5                   | 68.2                                             | 349                      |
| Peak Number 5 | 12.0                  | 96.6                                             | 329                      |
| Peak Number 6 | 2.2                   | 97.1                                             | 198                      |

[00197] In addition to varying NaCl concentrations on Column 1, different amounts of protein can be loaded on Column 1 to enhance purification of non-gluconylated Arginase 1 species.

[00198] Varying the load factor of Column 1 and using a NaCl gradient over Column 1 can compensate for unexpected perturbations experienced during *E. coli* fermentation that produce gluconylated Arginase 1 species.

#### Example 15: Variation in Fermentation Conditions

[00199] Experiments were performed to determine the robustness of fermentation conditions for production of Arginase 1. Table 13 shows that fermentation of *E. coli* at the at sub-optimal pH of 7.6 produces more gluconylation than at the preferred pH 7.2 fermentation. Vessels B1, B8, and B12 used optimal conditions of fermentation: pH 7.2, Dissolved Oxygen 30%, feed rate of media 0.06 mL/min. Vessel B3 was used to ferment Arginase 1 expressing *E. coli* at pH 7.6 (a pH that is higher than optimal conditions). The increase in pH resulted in a higher proportion of phospho-gluconylated adducts (23% vs 10-12% in control runs)

[00200] Table 13: Gluconylated Arginase 1 Observed in Fermentation Vessels

| Vessel  | Native Arg 1 | Gluconylated | Phospho-gluconylated | 2X Gluconylated | Gluconylated and Phospho-gluconylated |
|---------|--------------|--------------|----------------------|-----------------|---------------------------------------|
| B1      | 88.5         | 6.2          | 5.3                  | -               | --                                    |
| B8      | 89.2         | 6.3          | 4.6                  | --              | --                                    |
| B12     | 89.0         | 5.9          | 5.1                  | --              | --                                    |
| Average | 88.9         | 6.1          | 5.0                  | --              | --                                    |
| B3      | 76.7         | 15.0         | 5.8                  | 1.8             | 0.6                                   |

#### Example 16: Variation of Load Factor on Column 1

[00201] Different amounts of *E. coli* cell lysate were applied to Column 1 to determine the effect on purification of Arginase 1 charge variants, as well as yield and purity. Load factors of 15 - 60 g protein/L resin were used under various conditions showing a shift in the CIEEX charge species profiles (Table 14). Higher load factors resulted in better separation of

gluconylated variants (but depending on which fractions were collected a trade-off in yield may result). For example, with a load factor of 20 mg protein/mL resin peak 5 was 45.8% whereas with a load factor of 40 mg/mL this increased to 50.0%.

5 [00202] Table 14: Effect of Column 1 Load Factor on Protein Product 1

| Sample      | Peak | Area (%) | Step Yield (%) |
|-------------|------|----------|----------------|
| 40 mg/mL LF | 1    | 1.2      | 95.1           |
|             | 2    | 1.6      |                |
|             | 3    | 11.6     |                |
|             | 4    | 21.2     |                |
|             | 5    | 50.0     |                |
|             | 6    | 15.5     |                |
| 30 mg/mL LF | 1    | 1.1      | 101.0          |
|             | 2    | 4.1      |                |
|             | 3    | 14.0     |                |
|             | 4    | 21.1     |                |
|             | 5    | 46.2     |                |
|             | 6    | 13.5     |                |
| 20 mg/mL LF | 1    | 1.6      | 93.2           |
|             | 2    | 4.5      |                |
|             | 3    | 14.3     |                |
|             | 4    | 20.9     |                |
|             | 5    | 45.8     |                |
|             | 6    | 12.8     |                |

#### Example 17: Phase 1/2 Clinical Investigation

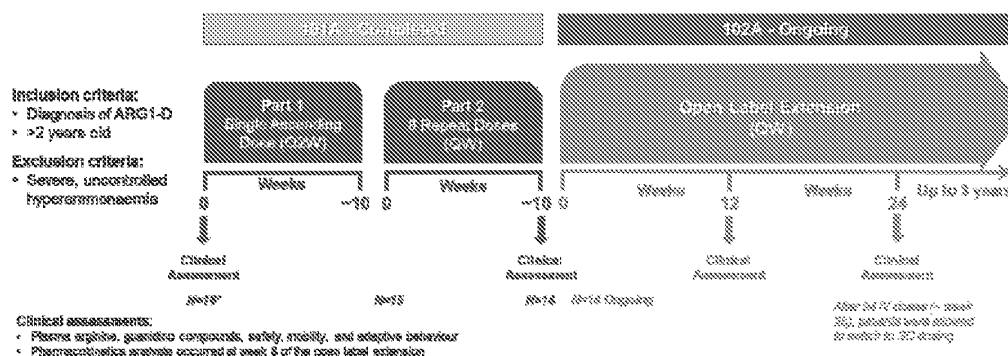
[00203] The drug product produced by the methods of this invention was used in a Phase 1/2, open-label study to evaluate administration of Co-rhARG1-PEG in Arginase 1  
 10 deficiency and hyperargininemia. The primary endpoint of this study was to evaluate the safety

and tolerability of intravenous (IV) administration of Co-rhARG1-PEG in subjects with hyperargininemia/Arginase 1 deficiency. The secondary endpoints were: to determine the effects of study drug administered IV on plasma arginine concentrations; to determine the effects of study drug administered IV on plasma guanidino compounds (GCs); and to characterize the pharmacokinetic (PK) profile of study drug administered IV. Other endpoints include evaluation of clinical outcome assessments in capturing clinical benefit such as: 6-Minute Walk Test (6MWT), Gross Motor Function Measure (GMFM) Parts D and E, and Adaptive Behaviour Assessment System (ABAS).

**[00204]** The Phase 1/2 data demonstrated that Co-rhARG1-PEG was highly effective in sustainably lowering plasma arginine. In addition, the control of plasma arginine levels in patients was accompanied by clinically meaningful responses in mobility and adaptive behavior. The treatment was generally well tolerated. Hypersensitivity reactions were infrequent and manageable with standard measures.

**[00205]** The Co-rhARG1-PEG drug product supplied for the study was as a liquid formulation in 10 mL single-use glass vials containing 5 mL of formulated drug product at a concentration of 1 mg/mL. The drug was formulated in 50 mM NaCl, 1 mM K<sub>2</sub>HPO<sub>4</sub>, 4 mM KH<sub>2</sub>PO<sub>4</sub>, and 1.5% w/v glycerol.

**[00206]** The Phase 1/2 study was conducted in two parts: Part 1 (Single Ascending Dose Escalation) and Part 2 (Repeated Dosing). The study design for this phase 1/2 trial 101A and the 102A open label extension is shown in the graphic below:



**[00207]** Part 1 introduced the patient to the drug and was focused upon safety. Part 2 was designed to settle the patient on a consistent dose and look for markers of clinical effectiveness. Each part was preceded by a baseline assessment of arginine levels. All patients

who participated in Part 1 could continue Arginase 1 dosing in Part 2 if they qualified for continued dosing.

[00208] In the study, each patient received a starting dose that could escalate in Part 1 with a 2-week washout/observation period between each successive dose level. The possible  
5 doses for each patient in Part 1 were 0.015, 0.03, 0.06, 0.10, 0.15, 0.20, and 0.30 mg/kg, at 2-week intervals as needed to optimize plasma arginine. Any particular dose can be repeated, or a dose increased/decreased between the specified dose levels if emerging data from prior dose levels met certain criteria. For example, the escalation of dose may cease if one or more of the following dose escalation stopping criteria were met: the patient's plasma arginine level was <  
10 40  $\mu$ M for at least 40 ( $\pm$  2) consecutive hours post-dosing for all samples collected during that time period or the patient's plasma arginine level averaged <115  $\mu$ M for at least 112 ( $\pm$  2) consecutive hours post-dosing for all samples collected during that time period.

[00209] If none of these events occurred, the patient could be escalated to the next higher dose level of Arginase 1 every 2 weeks until any dose escalation stopping criterion was  
15 reached or the patient had received the highest dose under this protocol of 0.30 mg/kg. Ultimately, for treatment purposes, it is also possible that the dosing might increase over 0.30 mg/kg.

[00210] Part 2 was a repeat-dosing period for patients who completed Part 1. Part 2 found a dose and regimen for each patient that safely optimized plasma arginine in the range of  
20 about 40  $\mu$ M to about 115  $\mu$ M during repeat-dose administration, with emphasis on maintaining pre-dose levels below 150–200  $\mu$ M. Several dose levels could have been used in Part 2 if the data indicated a potential to better investigate a dose-response outcome during repeat-dose administration. On-treatment arginine levels were also compared with arginine levels determined prior to treatment.

25 [00211] Patients who completed Part 2 of 101A were eligible to participate in a long-term open-label extension (OLE) trial (NCT03378531). Treatment with 24 weekly IV doses with the option to switch to subcutaneous dosing for the remainder of the 3-year OLE period.

## Results

30 [00212] Increases in mean  $C_{\max}$  and mean  $AUC_{0-168}$  appeared dose proportional in all patients. Mean ( $\pm$  SD)  $C_{\max}$  was  $0.428 \pm 0.0915$ ,  $0.723 \pm 0.247$ ,  $1.73 \pm 0.538$ ,  $2.27 \pm 0.238$ , and 6.13 (N=1)  $\mu$ g/mL for Co-rhARG1-PEG dose levels of 0.015, 0.03, 0.06, 0.1 and 0.2 mg/kg,

respectively. There was a slight anti-drug antibody (ADA) impact on mean  $C_{\max}$  in ADA positive vs. ADA negative patients (Figure 9).

[00213] Changes in the AUCs ( $AUC_{0-168}$ ,  $AUC_{0-\infty}$ ) appeared dose proportional across the studied dose range, noting that there no notable change between 0.06 and 0.1 mg/kg (with the data that was available). Mean clearance (CL) estimates ranged from 0.789 to 1.57 mL/hr/kg in all patients and 0.776 to 1.33 mL/hr/kg in ADA negative patients. The mean volume of distribution (Vss) estimates ranged from 35.3 to 52.1 mL/kg in all patients and 32.8 to 52.1 mL/kg in ADA negative patients.

[00214] Part 1 of the study helped select an optimal (individual) starting dose for each patient for Part 2 using the observed PD (arginine) response. In Week 1 of Part 2, there was a trend for increasing mean circulating drug concentrations in all patients with escalating doses of Co-rhARG1-PEG across the dose range evaluated. After the first dose of Co-rhARG1-PEG in Part 2, increases in mean  $C_{\max}$  appeared dose proportional in all patients. Mean ( $\pm$  SD)  $C_{\max}$  was 0.292 (N=1), 0.395 (N=1),  $1.01 \pm 0.221$ ,  $1.75 \pm 0.391$ ,  $1.99$  (N=1),  $2.34$  (N=1), and  $2.87 \pm 0.626$   $\mu$ g/mL for Co-rhARG1-PEG dose levels of 0.015, 0.03, 0.04, 0.06, 0.09, 0.1 and 0.12 mg/kg, respectively.

[00215] In Part 2, Week 8, mean circulating drug concentrations in all patients generally increased with escalating doses of Co-rhARG1-PEG. There was no notable ADA impact in the available PK concentrations at Week 8. As a result of the data, achievement of steady state was assumed for most (13/14) patients at this time. After the 8th QW dose of Co-rhARG1-PEG, increases in mean  $C_{\max}$  and  $AUC_{0-168}$  appeared dose proportional in all patients.

[00216] In addition to pharmacokinetic data, pharmacodynamic (arginine) data was collected (Figure 10). Patients with Arginase 1 deficiency were administered (weekly) QW IV doses of Co-rhARG1-PEG in Part 2 and the starting dose was selected in Part 1 based on the observed PD (arginine) response. After the first QW IV dose of Co-rhARG1-PEG, there were notable reductions in circulating arginine levels, particularly for Co-rhARG1-PEG doses at or above 0.04 mg/kg. In several instances, the individual arginine concentrations dropped below 40  $\mu$ M. In addition, recoveries to starting arginine levels were incomplete in most patients at doses  $\geq 0.04$  mg/kg and just prior to administration of the second QW (weekly) Co-rhARG1-PEG dose.

[00217] Overall, exposure to Co-rhARG1-PEG generally increased and there was increased arginine suppression with escalating dose. Individualized dose optimization was

undertaken in Part 1 such that there were a range of doses with varying numbers of patients per dose level in Weeks 1 and 8 of Part 2.

#### **Example 18: Subcutaneous Administration**

5 [00218] After Part 2 of the Phase 1/2 study of Example 17 was complete, some patients were switched from IV administration of Co-rhARG1-PEG to subcutaneous administration. Surprisingly, subcutaneous administration of Co-rhARG1-PEG gave a pharmacodynamic profile that appeared superior to IV administration. Also unexpectedly, the same formulation as for IV administration was successfully used for subcutaneous administration of Co-rhARG1-  
10 PEG.

[00219] The subcutaneous administration of Co-rhARG1-PEG maintained patient arginine levels within the preferred (healthy) target range for plasma arginine concentration longer than IV administration (Figure 11). The preferred optimized plasma arginine concentration in a patient is in the range of about 40  $\mu$ M to about 115  $\mu$ M (during repeat-dose  
15 administration), with emphasis on maintaining levels below the pre-dose 150–200  $\mu$ M. As can be seen in Figure 11, subcutaneous administration of Co-rhARG1-PEG results in arginine concentrations above the lower level of 40  $\mu$ M and below the upper level of 115  $\mu$ M. Surprisingly, subcutaneous administration gave arginine concentrations that are entirely in the preferred range. This means the patient will stay in the appropriate plasma range of arginine  
20 concentrations until another weekly Co-rhARG1-PEG dose is received.

#### **Example 19: Pharmacodynamic and Clinical Responses From Phase 1/2 Clinical Study and Open Label Extension**

[00220] 16 patients (11 paediatric and 5 adult) were enrolled into 101A Part 1 and 15  
25 patients advanced into 101A Part 2. 2 patients withdrew from the trial for personal reasons (1 patient after Part 1 dose 3 and 1 patient after Part 2 dose 3). All 14 patients completing 101A Part 2 advanced into the OLE trial.

[00221] Baseline characteristics for the patients are shown in Table 15.

30 [00222] Table 15: Baseline Characteristics

|                            |           |
|----------------------------|-----------|
| Age, years, median (range) | 15 (5–31) |
|----------------------------|-----------|

|                                          |               |
|------------------------------------------|---------------|
| Female, n (%)                            | 11 (69)       |
| Arginine, $\mu\text{M}$ , median (range) | 389 (238–566) |
| Ammonia, $\mu\text{M}$ , median (range)  | 38 (9–77)     |
| ALT (U/L), median (range)                | 34 (15–171)   |
| ARG1 mutation, n (%)                     | 11 (68.8)     |
| Homozygotes                              | 5 (31.2)      |
| Compound Heterozygotes                   |               |

**[00223]** An analysis of plasma arginine and guanidino compound levels found marked and sustained reductions in plasma arginine levels (Figure 12 (a)) were demonstrated with a median reduction of 274  $\mu\text{M}$  from baseline after 20 doses of pegzilarginase. Reductions in plasma arginine from baseline to dose 1, dose 8, and OLE were statistically significant ( $p < 0.001$ ). Plasma arginine reductions were accompanied by decreases in plasma guanidino compound (GC) levels. Figure 12(b) shows plasma levels for guanidinoacetic acid (GAA), N- $\alpha$ -acetyl-L-arginine (NAA),  $\alpha$ -keto- $\delta$ -guanidinovaleric acid (GVA) and argininic acid (ARGA) at baseline and the reduction of plasma GC levels during the OLE.

**[00224]** 15 of 16 patients completed all mobility assessments at baseline (patient 13 was confined to a wheelchair) (Figure 13(a)). Deficits were defined as: 6MWT: less than the lower 5<sup>th</sup> percentile; GMFM Part D:  $< 35$  of 39; GMFM Part E:  $< 68$  of 72; ABAS-3:  $\leq 85$ . 88% (14 of 16) of patients had at least one mobility deficit at baseline; 88%, 50% and 56% of the 16 patients were classified as having a baseline deficit in the 6MWT, GMFM Part D and GMFM Part E, respectively. Adaptive behaviour ABAS-3 assessments were available for 10 patients at baseline. 6 patients were not tested for technical reasons, including constraints due to language, age and cognitive impairment 8 of 10 (80%) patients had baseline deficiencies in 1 or more domains assessed by ABAS-3.

**[00225]** The overall clinical response showed that 11 of 14 patients (79%) were defined as responders at dose 20 based on a  $\geq 1$  MCID improvement in at least one of the 6MWT, GMFM-D or GMFM-E assessments (Figure 13(b)). 20-dose data demonstrated that 6MWT, GMFM-D, and GMFM-E are sufficiently sensitive to change for capturing clinical benefit in ARG1-D patients. The percentage of overall responders increased substantially from dose 8 to dose 20. All 5 patients (100%) who reached dose 44 maintained their dose 20 overall clinical response status as responders.

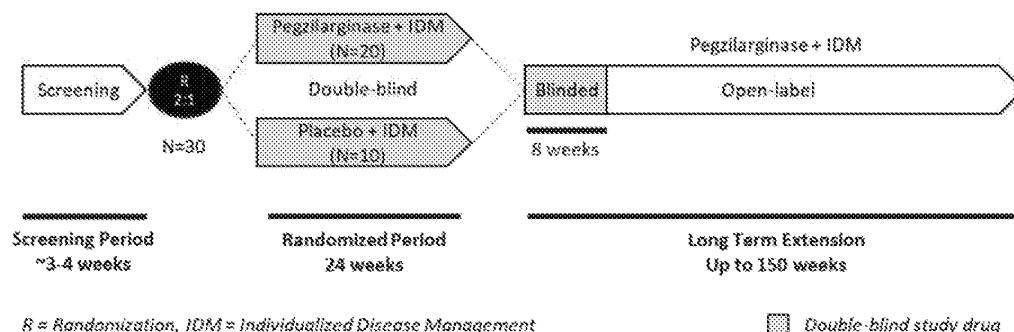
[00226] All responders for individual components had a  $\geq 1$  MCID improvement (Figure 13(b) and Figure 14). For the 6MWT, 7 of 13 (54%) patients were responders on this component alone. Mean change in 6MWT was 32 meters across all patients and 66 meters in the 7 responders. For GMFM-D: 5 of 8 (63%) patients with a baseline deficit were responders on this component alone (mean MCID 1.84, range 1.21 to 3.33). For GMFM-E: 5 of 8 (63%) patients with a baseline deficit were responders on this component alone (mean MCID 4.79, range 1.67 to 8.33). The percentages of responders for the individual mobility components were substantially greater at dose 20 relative to dose 8.

[00227] Data from all patients following 20 doses of pegzilarginase demonstrated marked and sustained reductions in plasma arginine, improvements in important disease manifestations, and a clinical responder rate of 79%. The Phase1/2 and OLE trials demonstrate the value of utilizing the 6MWT, GMFM-D, or GMFM-E as tools for capturing the clinical benefit of pegzilarginase. Pegzilarginase was well tolerated and the rates of treatment-related adverse events decreased over time. The improvements in arginine control and evidence of clinical benefit following pegzilarginase treatment provide further validation of the key endpoints and design elements of the pivotal Phase 3 PEACE trial (NCT03921541).

#### **Example 20: Phase 3 Clinical Trial Design**

[00228] A randomized, double-blind, placebo-controlled Phase 3 study of the efficacy and safety of Co-rhARG1-PEG in children and adults with Arginase 1 deficiency is being performed using the Co-rhARG1-PEG produced by the methods of this invention. This trial is currently underway as Pegzilarginase Effect on Arginase 1 Deficiency Clinical Endpoints, or PEACE (CAEB1102-300A; NCT03921541).

[00229] The study design for this Phase 3 trial is shown in the graphic below:



**[00230]** *Key inclusion criteria*

- a. Aged  $\geq 2$  years with a diagnosis of ARG1-D and with plasma arginine levels  $\geq 250$   $\mu\text{mol/L}$  to allow statistical testing of the proportion of patients who achieve plasma arginine below medical guidance of 200  $\mu\text{mol/L}$
- b. Able to maintain a stable consistent diet for the duration of the blinded period
- c. Able to remain on stable doses of ammonia scavengers, anti-epileptic therapy and/or medications for spasticity during the blinded period
- d. Able to perform and successfully complete clinical assessments and must have a baseline deficit in one of the components of the secondary clinical response endpoint as shown in Table 16.

**[00231]** *Key exclusion criteria*

- a. Hyperammonaemic episode requiring hospitalisation within 6 weeks before starting treatment
- b. Active infection within 3 weeks prior to receiving the first pegzilarginase dose
- c. Extreme mobility deficit, defined as inability to be assessed on the Gillette Functional Assessment Questionnaire (GFAQ) or a score of 1 on the GFAQ (cannot take any steps at all)
- d. Participated in previous interventional studies with pegzilarginase or currently participating in other clinical trials
- e. History of hypersensitivity to polyethylene glycol

[00232] Table 16: Definition of Baseline Deficits for Key Clinical Response Endpoints

| Assessment                    | Component | Definition of Baseline Deficit                     |
|-------------------------------|-----------|----------------------------------------------------|
| Timed walk test*              | 2MWD (m)  | Bottom 15 <sup>th</sup> Percentile for Age and Sex |
| Gross Motor Function Measure* | Part D    | <35 of 39                                          |
|                               | Part E    | <68 of 72                                          |

2MWD = 2-minute walk distance; GMFM = Gross Motor Function Measure; Part D = standing; Part E = walking, running, jumping  
 \*NIH Toolbox (US Department of Health and Human Services, Washington, DC, USA) motor domain dataset (2-minute Walk Endurance Test)

[00233] The primary endpoint of this Phase 3 trial is plasma arginine reduction (change from baseline in plasma arginine levels at week 24 based on the treatment means of the change from baseline in individual patients for the active and placebo arms).

[00234] Secondary endpoint measures include:

- a. Clinical Response Endpoint: A clinical responder is defined as a patient with an improvement in at least one of the 2MWT, GMFM-D, or GMFM-E clinical response endpoints at Week 24 as defined in Table 17
- b. Response rates for each individual component of the clinical response endpoint
- c. Other clinical outcome assessments
  - i. Functional Mobility Scale 5, 50, 500 meters
  - ii. Gillette Functional Assessment Questionnaire (GFAQ)
  - iii. Vineland Adaptive Behaviour Scales – II
- d. Evaluation of safety, including immunogenicity
- e. Proportion of patients with plasma arginine <200 µM and within normal range (40 - 115 µM)
- f. characterisation the pharmacokinetic profile of pegzilarginase

Table 17: Clinical Response Endpoints Defining a Clinical Responder

| Assessment | Component       | Definition of Response                          |
|------------|-----------------|-------------------------------------------------|
| 2MWT       | Distance walked | Improvement by ≥9%                              |
| GMFM       | Part D          | Improvement by 1.5 to 3.3 points based on GMFCS |
|            | Part E          | Improvement by 1.8 to 4.0 points based on GMFCS |

[00235] The total duration of the study is expected to be approximately 178 weeks per subject, including the long-term open-label extension period (3- to 4-week screening period, 24-week treatment period, followed by an open-label extension period of up to 150 weeks). Subjects will receive weekly IV infusions (approximately 30 minutes) of Co-rhARG1-PEG or volume-adjusted placebo once weekly. Dose modifications of Co-rhARG1-PEG based solely on plasma arginine values will be implemented by the unblinded pharmacist and/or physician, according to a dosing algorithm. After the first 8 weeks of the blinded long-term extension period, subjects will have the option to receive Co-rhARG1-PEG by subcutaneous administration, with investigator and sponsor approval. The initial mg/kg subcutaneous dose may be the same as the IV dose.

[00236] Subjects assigned to Co-rhARG1-PEG begin at Dose Level 2 (see Table 18 below), 0.10 mg/kg. Beginning with Visit 5, dose modifications, if required, based on plasma arginine values will be implemented by an unblinded physician, according to the following dosing algorithm:

- If the plasma arginine level is  $>150 \mu\text{M}$ , a single 168-hour sample will be used to increase the dose by 2 dose levels (not to exceed 0.20 mg/kg) if the 2 doses prior to this sample were a) the same dose level in mg/kg, and b) consecutive (with no missed doses).
- If the plasma arginine levels from 2 sequential 168-hour samples (regardless of missed doses) are both  $<50 \mu\text{M}$ , the dose is decreased by 1 dose level (see Table 17), not to decrease below 0.05 mg/kg.

Table 18: Dose Adjustments for Co-rhARG1-PEG

| Dose Level <sup>(a)</sup> | Dose       |
|---------------------------|------------|
| 1 (Minimum Possible Dose) | 0.05 mg/kg |
| 2 (Starting Dose)         | 0.10 mg/kg |
| 3                         | 0.15 mg/kg |
| 4 (Maximum Possible Dose) | 0.20 mg/kg |

(a) Pegzilarginase dosing starts at level 2, 0.10 mg/kg. Dose increases, when required, are by 2 dose levels. Dose decreases are by 1 dose level.

[00237] *Statistical Considerations*

[00238] The primary analysis will compare the mean decrease from baseline in plasma arginine levels in patients treated with pegzilarginase vs. placebo after 24 weekly doses, based

on the average of the last four plasma arginine measurement results which satisfy strict pre-specified criteria.

[00239] Sample sizes of 10 and 20 patients randomized to placebo and pegzilarginase, respectively, achieve 98% power to demonstrate a difference in mean plasma arginine levels of 200  $\mu$ M at the 0.05 significance level using a two-sided Mann-Whitney-Wilcoxon Test, assuming a common SD of 120  $\mu$ M.

[00240] Additionally, this number of subjects provides more than 80% power to detect a statistically significant difference of 40% between the group proportions for the clinical response endpoint at the 0.05 significance level using a Fisher's Exact Test.

#### Example 21: Site-Specific PEGylation Analysis

[00241] An exemplary sites-specific PEGylation analysis is provided in Figure 15. Three Co-rhARG1-PEG batches were analyzed. Peptide mapping was performed by denaturing Co-rhARG1-PEG Drug Substance, Co-Arginase 1 Intermediate (non-pegylated) Lots and the corresponding Reference Standards, with guanidine-HCl, reduced with DTT, and alkylated with iodoacetamide. These samples were diluted with 50 mM Tris Buffer pH 8.0. Each sample was digested with sequencing-grade trypsin for about 5 h at 37°C. The resulting peptides were resolved on a Waters Acquity BEH 300 C18 column, 2.1 X 150 mm, Waters C/N 186003687 using an acetonitrile gradient in 0.05% Trifluoro Acetic acid. LC-MS and MS/MS fragmentation of the peptides was obtained on a Waters Xevo G2-XS QTOF MS/MS.

[00242] As can be seen, none of the three batches had PEGylation at sites K3, K149, K190, K195, K29, K265 or K283. Also, all three batches had PEGylation at sites K16, K32, K38, K40, K47, K67, K74, K82, L87, K88, K152, K154, K171, K312 and K321. Some batches had a low frequency of PEGylation at sites K222 and K223.

[00243] Reference throughout this specification to "one embodiment," "certain embodiments," "various embodiments," "one or more embodiments" or "an embodiment" means that a particular feature, structure, material, or characteristic described in connection with the embodiment is included in at least one embodiment of the disclosure. Thus, the appearances of the phrases such as "in one or more embodiments," "in certain embodiments," "in various embodiments," "in one embodiment" or "in an embodiment" in various places throughout this specification are not necessarily referring to the same embodiment of the

disclosure. Furthermore, the particular features, structures, materials, or characteristics may be combined in any suitable manner in one or more embodiments.

**[00244]** Although the disclosure herein provided a description with reference to particular embodiments, it is to be understood that these embodiments are merely illustrative of the principles and applications of the disclosure. It will be apparent to those skilled in the art that various modifications and variations can be made to the present disclosure without departing from the spirit and scope thereof. Thus, it is intended that the present disclosure include modifications and variations that are within the scope of the appended claims and their equivalents.