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Fortsættes ...

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DESCRIPTION

BACKGROUND OF THE INVENTION

1. Field of the Invention

[0001] The invention generally relates to a variant human Arginase I protein which is suitable for use in the treatment of a cancer that does not express, or is otherwise deficient in, argininosuccinate synthetase (ASS), ornithine transcarbamylase (OTC), or other enzymes required for arginine biosynthesis,.

2. Description of the Related Art

[0002] It has been recognized for over 50 years that certain tumor cells have a high demand for amino acids, such as L-Arginine and are killed under conditions of L-Arginine depletion (Wheatley and Campbell, 2002). In human cells L-Arginine is synthesized in two steps; first argininosuccinate synthetase (ASS) converts L-Citrulline and aspartate to argininosuccinate, followed by conversion of argininosuccinate to L-Arginine and fumarate by argininosuccinate lyase. L-Citrulline itself is synthesized from L-Ornithine and carbamoyl phosphate by the enzyme ornithine transcarbamylase (OTC). A large number of hepatocellular carcinomas, melanomas and, as discovered recently, renal cell carcinomas (Ensor *et al.*, 2002; Feun *et al.*, 2007; Yoon *et al.*, 2007) do not express ASS and thus are sensitive to L-Arginine depletion. The molecular basis for the lack of ASS expression appears to be diverse and includes aberrant gene regulation and splicing defects. Whereas non-malignant cells enter into quiescence (G₀) when depleted of L-Arginine and thus remain viable for several weeks, tumor cells have cell cycle defects that lead to the re-initiation of DNA synthesis even though protein synthesis is inhibited, in turn resulting in major imbalances and rapid cell death (Shen *et al.*, 2006; Scott *et al.*, 2000). The selective toxicity of L-Arginine depletion for HCC, melanoma and other ASS-deficient cancer cells has been extensively demonstrated *in vitro*, in xenograft animal models and in clinical trials (ensor *et al.*, 2002; Feun *et al.*, 2007; Shen *et al.*, 2006; Izzo *et al.*, 2004). Recently Cheng *et al.* (2007) demonstrated that many HCC cells are also deficient in ornithine transcarbamylase expression and thus, they are also susceptible to enzymatic L-Arginine depletion.

[0003] There is interest in the use of L-Arginine hydrolytic enzymes for cancer therapy, especially the treatment of hepatocarcinomas, melanomas and renal cell carcinomas, which are common forms of cancer associated with high morbidity. Two L-Arginine degrading enzymes have been used for cancer therapy: bacterial arginine deiminase and human Arginases. Unfortunately, both of these enzymes display significant shortcomings that present major impediments to clinical use (immunogenicity and low catalytic activity and very

poor stability in serum, respectively). Thus, the therapeutic success of L-Arginine depletion therapy will rely on addressing these shortcomings.

SUMMARY OF THE INVENTION

[0004] The invention relates to an isolated human Arginase I protein which:

1. i. comprises the native Arginase I amino acid sequence, optionally without the N-terminal methionine residue; and
2. ii. comprises a non-native metal co-factor which is cobalt in the form of Co^{2+} for use in medicine.

[0005] A number of mutations have been found to increase the catalytic activity and drastically reduce the K_m for L-Arginine under physiological conditions. In some embodiments, not according to the claimed invention, mutations are substitution mutations selected from the group consisting of Asp181Ser, Ser230Cys, Ser230Gly, Cys303Phe, Cys303Ile, Glu256Gln, Asp181Glu and Ser230Ala. In some aspects, the present invention provides embodiments where two or more mutations are introduced in human arginase. In some embodiments, the human arginase protein comprises at least two amino acid substitutions. In a particular embodiment, the substitutions are Asp181Glu and Ser230Ala.

[0006] In some aspects, not according to the claimed invention, the present disclosure provides arginases comprising additional changes relative to the wild-type or native protein. In some embodiments, not according to the claimed invention, the changes include substitution, deletions (e.g. lacking part of the native sequence), truncations, or a combination thereof. In some embodiments, not according to the claimed invention, the present disclosure also contemplates native arginases, wherein the only amino acid sequence changes are deletions. In a particular embodiment, the present disclosure contemplates a human Arginase I protein, wherein the protein lacks an N-terminal methionine. Other and larger deletions are also contemplated for the various mutant arginases described herein, but are not according to the claimed invention. For example, truncated Arginase lacking the 14 C-terminal amino acids has been reported, leaving Arg-308 as the last residue in the sequence (Mora *et al.*, 2000). In yet another embodiment, not according to the claimed invention, arginase lacks the first 21 amino acids of the wild-type sequence.

[0007] In some aspects, the present invention also contemplates fusion proteins comprising an arginase linked to a non-arginase amino acid sequence. In one embodiment, the non-arginase sequence comprises at least a portion of the Fc region of an immunoglobulin, e.g., to increase the half-life of the arginase in serum when administered to a patient. The Fc region or portion thereof may be any suitable Fc region. In one embodiment, the Fc region or portion thereof is an IgG Fc region.. In one embodiment, a dimeric Fc-Arginase fusion protein is contemplated.

[0008] In one embodiment disclosed, the not according to the claimed invention, arginase lacks a portion of the wild-type sequence. In an embodiment of the invention, the arginase is Arginase I lacking an N-terminal methionine. In some embodiments, the not according to the claimed invention, arginase contains a substitution which is Asp181Ser, Ser230Cys, Ser230Gly, Cys303Phe or Cys303Ile. In one embodiment, the substitutions are Asp181Glu and Ser230Asp.

[0009] In some aspects, the not according to the claimed invention, present disclosure further contemplates nucleic acid encoding such arginases. In some cases, the nucleic acid has been codon optimized for expression in bacteria. In particular embodiments, the bacteria is *E. coli*. In other aspects, the present disclosure further contemplates vectors containing such nucleic acids. In particular cases, the nucleic acid encoding the mutant arginase is operably linked to a promoter, including but not limited to heterologous promoters. In still further aspects, the not according to the claimed invention, present disclosure further contemplates host cells comprising such vectors. In some cases, the host cells are transfected or transformed host cells expressing the mutant arginases. The proteins may be expressed in any suitable manner. In one case, the proteins are expressed in a host cell such that the protein is glycosylated. In another case, the proteins are expressed in a host cell such that the protein is aglycosylated.

[0010] The present invention also contemplates the arginase proteins of the present invention in particular for use in the treatment of cancer. In some embodiments, the cancer is one that does not express, or is otherwise deficient in, argininosuccinate synthetase (ASS) or ornithine transcarbamylase (OTC). In particular embodiments, the human cancer is an arginine auxotrophic cancer. In one embodiment, the not according to the claimed invention, present disclosure contemplates a formulation comprising a fusion protein as described above for use in the treatment of cancer in a human patient.

[0011] The cancer may be any type of cancer or tumor type. In some embodiments, the cancer is hepatocellular carcinoma, renal cell carcinoma, melanoma, prostate cancer, or pancreatic cancer. In some cases, the formulation is adapted for administration topically, intravenously, intradermally, intraarterially, intraperitoneally, intralesionally, intracranially, intraarticularly, intraprostatically, intrapleurally, intratracheally, intraocularly, intranasally, intravitreally, intravaginally, intrarectally, intramuscularly, subcutaneously, subconjunctival, intravesicularly, mucosally, intrapericardially, intraumbilically, orally, by inhalation, by injection, by infusion, by continuous infusion, by localized perfusion bathing target cells directly, *via* a catheter, or *via* a lavage. In one embodiment, to increase serum half-life, the arginase variants described herein are "pegylated."

[0012] All of the above mentioned arginases, variants and the like are contemplated in a preferred embodiment as purified or isolated proteins, and preferably monomeric proteins.

[0013] The use of the term "or" in the claims is used to mean "and/or" unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the

disclosure supports a definition that refers to only alternatives and "and/or."

[0014] Throughout this application, the term "about" is used to indicate that a value includes the standard deviation of error for the device or method being employed to determine the value.

[0015] Following long-standing patent law, the words "a" and "an," when used in conjunction with the word "comprising" in the claims or specification, denotes one or more, unless specifically noted.

[0016] The term "therapeutically effective" as used herein refers to an amount of cells and/or therapeutic composition (such as a therapeutic polynucleotide and/or therapeutic polypeptide) that is employed in methods of the present invention to achieve a therapeutic effect, such as wherein at least one symptom of a condition being treated is at least ameliorated, and/or to the analysis of the processes or materials used in conjunction with these cells.

[0017] Other objects, features and advantages of the present invention will become apparent from the following detailed description.

BRIEF DESCRIPTION OF THE FIGURES

[0018] The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1 shows Arginase I (SEQ ID NO:1) and Arginase II (SEQ ID NO:2) nucleic acid sequences.

FIG. 2 is a photograph of a SDS-PAGE showing purification steps for human Arginase I. L = Molecular Weight Ladder; WC = Whole Cell Fraction, SN = Supernatant; FT = Flow Through from IMAC Column; W = Column Wash; E = Arginase Elution Fraction.

FIG. 3 is a representative graph of steady-state kinetics of L-arginine hydrolysis by Co-hArgI (●) and Mn-hArgI (○) in a 100 mM Hepes buffer, pH 7.4, 37 °C. Co-hArgI had a k_{cat} of $240 \pm 14 \text{ s}^{-1}$, a K_M of $190 \pm 40 \text{ }\mu\text{M}$, and k_{cat}/K_M of $1,270 \pm 330 \text{ mM}^{-1} \text{ s}^{-1}$. Mn-ArgI had a k_{cat} of $300 \pm 12 \text{ s}^{-1}$, a K_M of $2,330 \pm 260 \text{ }\mu\text{M}$, and k_{cat}/K_M of $129 \pm 20 \text{ mM}^{-1} \text{ s}^{-1}$.

FIG. 4 is a plot of k_{cat}/K_M versus pH for Co-hArgI (●) with an ascending limb pK_a of 7.5 and Mn-hArgI (■) with an ascending limb pK_a of 8.5.

FIG. 5 is a graph showing the stability of Co-hArgI and Mn-hArgI (1 μM) incubated in pooled human serum at 37°C over time in pooled human serum. Aliquots were withdrawn over time

and assayed against 1 mM of L-Arg in a 100 mM Hepes buffer, pH 7.4, at 37 °C. Mn-hArgI (○) displayed an exponential loss of activity with a $T_{1/2}$ life of 4.8 ± 0.8 hrs. In contrast Co-hArgI (●) displayed a bi-phasic loss of activity with an apparent first $T_{1/2}$ of 6.1 ± 0.6 hrs followed by much longer second $T_{1/2}$ of 37 ± 3 hrs.

FIG. 6 is a graph showing HPLC traces of the 20 standard amino acids incubated with either Co-hArgI (Top panel) or dialysis buffer (Lower Panel). Co-hArgI incubated with the 20 standard amino acids resulted in the loss of a single peak at RT = 12.3 min, matching that of L-Arginine controls, and the appearance of a single new peak at RT = 18.8 min, matching that of L-Ornithine controls.

FIGS. 7A-B are graphs showing survival of HCC in tissue culture when treated with various arginase variants (along with controls). **FIG. 7A** demonstrates the survival of HCC tissue culture (Hep3b) when treated with 0-100 nM Arginase (Day 5). Mn-hArgI (A), resulted in an apparent IC_{50} of 5 ± 0.3 nM (~ 0.18 μ g/ml). Incubations with Co-hArgI (●) lead to a 15-fold increase in cytotoxicity with an apparent IC_{50} of 0.33 ± 0.02 nM (~ 0.012 μ g/ml). **FIG. 7B** is a graph showing the effect hArgI on the growth A375 melanoma cells (Day 5). Mn-hArgI (A), resulted in an apparent IC_{50} of 4.1 ± 0.1 nM (~ 0.15 μ g/ml). Incubation with Co-hArgI (●) lead to a 13-fold increase in cytotoxicity with an apparent IC_{50} of 0.32 ± 0.06 nM (~ 0.012 μ g/ml).

FIG. 8 is a photograph of a non-denaturing electrophoretic gel showing that the hArgI-E256Q variant is monomeric as opposed to trimeric wild-type h-ArgI.

FIG. 9 is a representative graph of steady-state kinetics of L-arginine hydrolysis by Co-hArg-II (●) and Mn-hArg-II (○). Cobalt substituted hArg-II (●) hydrolysis of L-Arg at pH 7.4 and 37°C, with a k_{cat} of 182 ± 7 s⁻¹, a K_M of 126 ± 18 μ M, and a k_{cat}/K_M of $1,440 \pm 260$ mM⁻¹s⁻¹. Manganese substituted hArg-II (○) hydrolysis of L-Arg at pH 7.4 and 37°C, with a k_{cat} of 48 ± 2 s⁻¹, a K_M of $2,900 \pm 300$ μ M, and k_{cat}/K_M of 17 ± 2 mM⁻¹ s⁻¹.

FIG. 10 is a graph showing serum L-arginine depletion in the mouse model. Serum L-Arg concentrations of Balb/c mice treated with a single IP dose of Co-hArgI are kept $\leq$ to 3-4 μ M for over 3 days.

FIG. 11 is a graph showing HCC tumor xenograft reduction when treated with Co-hArgI as compared to controls. Nude mice bearing a Hep3b tumor xenografts were treated twice by IP injection with either PBS (○) or Co-hArgI (●) at day 9 and at day 12. Tumor shrinkage was observed in the mice treated with Co-hArgI whereas PBS treated tumors grew unchecked.

FIG. 12 14-20 % SDS-PAGE showing hArgI conjugated to PEG MW 5000, with an apparent MW of ~ 150 kDa.

DESCRIPTION OF THE ILLUSTRATIVE EMBODIMENTS

[0019] The invention generally relates to an isolated human Arginase I protein as described above.

[0020] The invention also relates to a variant human Arginase I protein for use in the treatment of cancer. In some embodiments, the cancer is one that does not express, or is otherwise deficient in, argininosuccinate synthetase (ASS), ornithine transcarbamylase (OTC), or other enzymes required for arginine biosynthesis. The Arginase I protein may be fused to other polypeptides or conjugated to polymers that increase serum persistence, e.g., high molecular weight polyethylene glycol

I. Arginase

[0021] Arginase is a manganese-containing enzyme. It is the final enzyme of the urea cycle. Arginase is the fifth and final step in the urea cycle, a series of biophysical reactions in mammals during which the body disposes of harmful ammonia. Specifically, arginase converts L-arginine into L-ornithine and urea.

[0022] L-Arginine is the nitrogen donating substrate for nitric oxide synthase (NOS), producing L-Citrulline and NO. Although the K_M of Arginase (2-5 mM) has been reported to be much higher than that of NOS for L-Arginine (2-20 μ M), Arginase may also play a role in regulating NOS activity. Under certain conditions Arginase I is Cys-S-nitrosylated, resulting in higher affinity for L-Arginine and reduced availability of substrate for NOS.

[0023] Arginase is a homo-trimeric enzyme with an α/β fold of a parallel eight-stranded β -sheet surrounded by several helices. The enzyme contains a di-nuclear metal cluster that is integral to generating a hydroxide for nucleophilic attack on the guanidinium carbon of L-Arginine. The native metal for Arginase is Mn^{2+} . These Mn^{2+} ions coordinate water, orientating and stabilizing the molecule and allowing water to act as a nucleophile and attack L-arginine, hydrolyzing it into ornithine and urea.

[0024] Mammals have two Arginase isozymes (EC 3.5.3.1) that catalyze the hydrolysis of L-Arginine to urea and L-Ornithine. The Arginase I gene is located on chromosome 6 (6q.23), is highly expressed in the cytosol of hepatocytes, and functions in nitrogen removal as the final step of the urea cycle. The Arginase II gene is found on chromosome 14 (14q.24.1). Arginase II is mitochondrially located in tissues such as kidney, brain, and skeletal muscle where it is thought to provide a supply of L-Ornithine for proline and polyamine biosynthesis (Lopez *et al.*, 2005).

[0025] Arginases have been investigated for nearly 50 years as a method for degrading extracellular L-Arginine (Dillon *et al.*, 2002). Some promising clinical results have been achieved by introducing Arginase by transhepatic arterial embolisation; following which, several patients experienced partial remission of HCC (Cheng *et al.*, 2005). However, since Arginase

has a high K_M (~2-5 mM) and exhibits very low activity at physiological pH values, high dosing is required for chemotherapeutic purposes (Dillon *et al.*, 2002). While native Arginase is cleared from circulation within minutes (Savoca *et al.*, 1984), a single injection of PEG-Arginase MW5000 in rats was sufficient to achieve near complete arginine depletion for ~3 days (Cheng *et al.*, 2007).

[0026] Cheng *et al.* made the surprising observation that many human HCC cells lines do not express OTC (in addition to ASS) and thus they are susceptible to PEG-Arginase (Cheng *et al.*, 2007). In mice implanted with Hep3b hepatocarcinoma cells weekly administration of PEG-Arginase resulted in tumor growth retardation which was accentuated by co-administration of 5-fluorouracil (5-FU). However, PEG-Arginase was used at the very high doses that are impractical for human therapy, reflecting its lower physiological activity.

[0027] To address these issues a bacterial arginine hydrolyzing enzyme, Arginine Deiminase or ADI which displays good kinetics and stability has been tested *in vitro*. A PEGylated form of ADI is now undergoing Phase II/III clinical trials. Unfortunately ADI is a bacterial enzyme and therefore it induces strong immune responses and adverse effects in most patients. However, for those patients that do not develop significant adverse responses, an impressive percentage exhibit stable disease or remission. Nonetheless because of its unfavorable immunological profile it is unlikely that L-Arginine depletion by ADI will become a mainstream treatment for liver cancer.

[0028] For clinical use, it is essential that the arginase is engineered to allow it to persist for long times (e.g., days) in circulation. In the absence of any modification, human arginase has a half life of only a few minutes in circulation primarily because its size is not sufficiently large to avoid filtration through the kidneys. Unmodified human Arginase is very susceptible to deactivation in serum and it is degraded with a half life of only four hours. Therefore, the present invention developed novel and improved forms of arginase for clinical research and potential therapeutic use with improved circulation persistence.

II. Arginase Variants

[0029] Mammals have two Arginase isozymes (EC 3.5.3.1) that catalyze the hydrolysis of L-Arginine to urea and L-Ornithine. The Arginase I gene is located on chromosome 6 (6q.23), is highly expressed in the cytosol of hepatocytes, and functions in nitrogen removal as the final step of the urea cycle. The Arginase II gene is found on chromosome 14 (14q.24.1). Arginase II is mitochondrially located in tissues such as kidney, brain, and skeletal muscle where it is thought to provide a supply of L-Ornithine for proline and polyamine biosynthesis (Lopez *et al.*, 2005).

[0030] L-Arginine is the sole substrate for nitric oxide synthase (NOS), producing L-Citrulline and NO. Although the K_M of Arginase (2-5 mM) has been reported to be much higher than that

of NOS for L-Arginine (2-20 μM), Arginase may also play a role in regulating NOS activity (Durante *et al.*, 2007). Under certain conditions Arginase I is Cys-S-nitrosylated, resulting in higher affinity for L-Arginine and reduced availability of substrate for NOS (Santhanam *et al.*, 2007). Arginase is a homo-trimeric enzyme with an α/β fold of a parallel eight-stranded β -sheet surrounded by several helices. The enzyme contains a di-nuclear metal cluster that is integral to generating a hydroxide for nucleophilic attack on the guanidinium carbon of L-Arginine (Cama *et al.*, 2003; Dowling *et al.*, 2008). The native metal for Arginase is Mn^{2+} . Arginase with the native metal (*i.e.* Mn^{2+}) exhibits a pH optimum of 9. At physiological pH the enzyme exhibits more than a 10-fold lower $k_{\text{cat}}/K_{\text{M}}$ in the hydrolysis of L Arginine (FIG. 4). The low catalytic activity displayed by the authentic human arginase with the native Mn^{2+} enzyme presents a problem for human therapy since it means that impractical doses of the enzyme have to be used to achieve a therapeutically relevant reduction in L-Arginine plasma levels.

[0031] The present invention contemplates mutant arginases wherein the natural metal cofactor (Mn^{2+}) is replaced with cobalt (Co^{2+}). Incorporation of Co^{2+} in the place of Mn^{2+} in human Arginase I or human Arginase II results in dramatically higher activity at physiological pH. It was found that an enzyme containing Co^{2+} ("Co-hArgI") displayed a 10 fold increase in $k_{\text{cat}}/K_{\text{M}}$ in vitro at pH 7.4, which in turn translated into a 15 fold increase in HCC cytotoxicity and a 13-fold increase in melanoma cytotoxicity as compared to the human Arginase I which contains Mn^{2+} . It was also found that a pharmacological preparation of Co-hArgI could clear serum L-Arg for over 3 days in mice with a single injection. Furthermore, it was found that a pharmacological preparation of Co-hArgI could shrink HCC tumor xenografts in nude mice whereas Mn-hArgI only slowed tumor growth (Ensor, Holsberg *et al.*, 2002).

[0032] The present invention provides a human arginase protein comprising at least one amino acid substitution at the metal binding site. The structure of Arginase shows an active site cleft containing two Mn^{2+} ions, with the more deeply localized ion designated Mn_A coordinated to H101, D124, D128, D232 and bridging hydroxide. The other metal is designated Mn_B and is coordinated by H126, D124, D232, D234 and bridging hydroxide (Christianson and Cox, 1999). The residues comprising the metal binding site for the first shell of Arginase I are H101, D124, H126, D128, D232, and D234 and for the second shell are W122, D181, and S230. Similarly, the residues comprising the metal binding site for the first shell of Arginase II are H120, D143, H145, D147, D251, D253 and for the second shell are W141, D200, S249.

[0033] Arginase has been shown to require both Mn^{2+} ions for full activity, however Mn_A can be reversibly dissociated resulting in an enzyme with half its catalytic activity (Scolnick *et al.*, 1997). Metal (A) of hArgI is coordinated to the imidazole of H101, which is in turn hydrogen bonded to the hydroxyl of S230. Metal (B) of hArgI is coordinated to the imidazole of H126, which has a 2nd shell hydrogen bond with the carboxyl of D181. The positions involved in the binding of the metal were subjected to saturation mutagenesis and the resulting libraries were screened using a microtiter well plate assay for arginase activity (described in more detail

below in the examples) to isolate clones expressing proteins that display higher catalytic activity. Novel clones were identified by sequencing, re-transformed into *E. coli* (BL21) and purified and kinetically characterized as described below in the examples. Variants displaying apparent activity $\geq$ to wild-type were purified in larger scale and assayed for their steady-state kinetic parameters of k_{cat} & K_M . The following variants were found to have greater k_{cat}/K_M constants than Co-hArgI: D181S, D181E/S230A (double mutant containing two substitutions). Similarly the amino acid substitutions S230C and S230G were found to have a particularly important effect on catalytic activity and also on serum stability. Additionally, amino acids removed from the metal binding site were also subjected to combinatorial saturation mutagenesis. For example, it was found that a C303P substitution in Co-hArg I conferred a 10-fold higher k_{cat}/K_M relative to the native Mn-hArg I at pH 7.4. Many of these variant or mutant forms of the arginase are contemplated for use in the treatment of cancer, including where they are made as fusion proteins, e.g. with an Fc region (or portion thereof) of an immunoglobulin (in order to increase half-life).

[0034] The Cys₃₀₃ variants were also tested for serum stability. It was found that a C303P variant, i.e. a single amino acid substitution in Arginase, exhibits a ~ 60 % increase in serum stability which in turn translates into a 30 fold increase in HCC cytotoxicity as compared to the Mn substituted enzyme at pH 7.4. In one embodiment, the present invention contemplates treatment with this novel enzyme or this novel enzyme with further mutations. In a particular embodiment, this novel enzyme is employed for treatment as an Arginase-Fc protein fusion that capitalizes on the endosomal recycling of the IgG fc domain to ensure long serum persistence of the Arginase variant. Long serum persistence improves the use of Arginase as a therapeutic.

III. Pegylation

[0035] In certain aspects of the invention, methods and compositions related to pegylated arginase are disclosed. Specifically, pegylation of arginase at an engineered Cysteine residue (e.g., substituting the third residue of the N-terminal) may be used to produce a homogenous pegylated arginase composition. Methods for isolation of pegylated arginase based on temporary disruption of polymerization are also disclosed.

[0036] Pegylation is the process of covalent attachment of poly(ethylene glycol) polymer chains to another molecule, normally a drug or therapeutic protein. Pegylation is routinely achieved by incubation of a reactive derivative of PEG with the target macromolecule. The covalent attachment of PEG to a drug or therapeutic protein can "mask" the agent from the host's immune system (reduced immunogenicity and antigenicity), increase the hydrodynamic size (size in solution) of the agent which prolongs its circulatory time by reducing renal clearance. Pegylation can also provide water solubility to hydrophobic drugs and proteins.

[0037] The first step in pegylation is the suitable functionalization of the PEG polymer at one or

both terminals. PEGs that are activated at each terminus with the same reactive moiety are known as "homobifunctional", whereas if the functional groups present are different, then the PEG derivative is referred as "heterobifunctional" or "heterofunctional." The chemically active or activated derivatives of the PEG polymer are prepared to attach the PEG to the desired molecule.

[0038] The choice of the suitable functional group for the PEG derivative is based on the type of available reactive group on the molecule that will be coupled to the PEG. For proteins, typical reactive amino acids include lysine, cysteine, histidine, arginine, aspartic acid, glutamic acid, serine, threonine, tyrosine. The N-terminal amino group and the C-terminal carboxylic acid can also be used.

[0039] The techniques used to form first generation PEG derivatives are generally reacting the PEG polymer with a group that is reactive with hydroxyl groups, typically anhydrides, acid chlorides, chloroformates and carbonates. In the second generation pegylation chemistry more efficient functional groups such as aldehyde, esters, amides *etc.* made available for conjugation.

[0040] As applications of pegylation have become more and more advanced and sophisticated, there has been an increase in need for heterobifunctional PEGs for conjugation. These heterobifunctional PEGs are very useful in linking two entities, where a hydrophilic, flexible and biocompatible spacer is needed. Preferred end groups for heterobifunctional PEGs are maleimide, vinyl sulfones, pyridyl disulfide, amine, carboxylic acids and NHS esters.

[0041] The most common modification agents, or linkers, are based on methoxy PEG (mPEG) molecules. Their activity depends on adding a protein-modifying group to the alcohol end. In some instances polyethylene glycol (PEG diol) is used as the precursor molecule. The diol is subsequently modified at both ends in order to make a hetero- or homo-dimeric PEG-linked molecule (as shown in the example with PEG bis-vinylsulfone).

[0042] Proteins are generally PEGylated at nucleophilic sites such as unprotonated thiols (cysteinyll residues) or amino groups. Examples of cysteinyll-specific modification reagents include PEG maleimide, PEG iodoacetate, PEG thiols, and PEG vinylsulfone. All four are strongly cysteinyll-specific under mild conditions and neutral to slightly alkaline pH but each has some drawbacks. The amide formed with the maleimides can be somewhat unstable under alkaline conditions so there may be some limitation to formulation options with this linker. The amide linkage formed with iodo PEGs is more stable, but free iodine can modify tyrosine residues under some conditions. PEG thiols form disulfide bonds with protein thiols, but this linkage can also be unstable under alkaline conditions. PEG-vinylsulfone reactivity is relatively slow compared to maleimide and iodo PEG; however, the thioether linkage formed is quite stable. Its slower reaction rate also can make the PEG-vinylsulfone reaction easier to control.

[0043] Site-specific pegylation at native cysteinyll residues is seldom carried out, since these residues are usually in the form of disulfide bonds or are required for biological activity. On the

other hand, site-directed mutagenesis can be used to incorporate cysteinyl pegylation sites for thiol-specific linkers. The cysteine mutation must be designed such that it is accessible to the pegylation reagent and is still biologically active after pegylation.

[0044] Amine-specific modification agents include PEG NHS ester, PEG tresylate, PEG aldehyde, PEG isothiocyanate, and several others. All react under mild conditions and are very specific for amino groups. The PEG NHS ester is probably one of the more reactive agents; however, its high reactivity can make the pegylation reaction difficult to control at large scale. PEG aldehyde forms an imine with the amino group, which is then reduced to a secondary amine with sodium cyanoborohydride. Unlike sodium borohydride, sodium cyanoborohydride will not reduce disulfide bonds. However; this chemical is highly toxic and must be handled cautiously, particularly at lower pH where it becomes volatile.

[0045] Due to the multiple lysine residues on most proteins, site-specific pegylation can be a challenge. Fortunately, because these reagents react with unprotonated amino groups, it is possible to direct the pegylation to lower-pK amino groups by performing the reaction at a lower pH. Generally the pK of the alpha-amino group is 1-2 pH units lower than the epsilon-amino group of lysine residues. By PEGylating the molecule at pH 7 or below, high selectivity for the N-terminus frequently can be attained. However; this is only feasible if the N-terminal portion of the protein is not required for biological activity. Still, the pharmacokinetic benefits from pegylation frequently outweigh a significant loss of *in vitro* bioactivity, resulting in a product with much greater *in vivo* bioactivity regardless of pegylation chemistry.

[0046] There are several parameters to consider when developing a pegylation procedure. Fortunately, there are usually no more than four or five key parameters. The "design of experiments" approach to optimization of pegylation conditions can be very useful. For thiol-specific pegylation reactions, parameters to consider include: protein concentration, PEG-to-protein ratio (on a molar basis), temperature, pH, reaction time, and in some instances, the exclusion of oxygen. (Oxygen can contribute to intermolecular disulfide formation by the protein, which will reduce the yield of the PEGylated product.) The same factors should be considered (with the exception of oxygen) for amine-specific modification except that pH may be even more critical, particularly when targeting the N-terminal amino group.

[0047] For both amine- and thiol-specific modifications, the reaction conditions may affect the stability of the protein. This may limit the temperature, protein concentration, and pH. In addition, the reactivity of the PEG linker should be known before starting the pegylation reaction. For example, if the pegylation agent is only 70 percent active, the amount of PEG used should ensure that only active PEG molecules are counted in the protein-to-PEG reaction stoichiometry. How to determine PEG reactivity and quality will be described later.

IV. Proteins and Peptides

[0048] In certain embodiments, the present invention concerns novel compositions comprising

at least one protein or peptide, such as stabilized arginase multimers. These peptides may be comprised in a fusion protein or conjugated to an agent as described *supra*.

A. Proteins and Peptides

[0049] As used herein, a protein or peptide generally refers, but is not limited to, a protein of greater than about 200 amino acids, up to a full length sequence translated from a gene; a polypeptide of greater than about 100 amino acids; and/or a peptide of from about 3 to about 100 amino acids. For convenience, the terms "protein," "polypeptide" and "peptide are used interchangeably herein.

[0050] In certain embodiments the size of at least one protein or peptide may comprise, but is not limited to, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, about 110, about 120, about 130, about 140, about 150, about 160, about 170, about 180, about 190, about 200, about 210, about 220, about 230, about 240, about 250, about 275, about 300, about 325, about 350, about 375, about 400, about 425, about 450, about 475, about 500, about 525, about 550, about 575, about 600, about 625, about 650, about 675, about 700, about 725, about 750, about 775, about 800, about 825, about 850, about 875, about 900, about 925, about 950, about 975, about 1000, about 1100, about 1200, about 1300, about 1400, about 1500, about 1750, about 2000, about 2250, about 2500 or greater amino acid residues.

[0051] As used herein, an "amino acid residue" refers to any naturally occurring amino acid, any amino acid derivative or any amino acid mimic known in the art. In certain embodiments, the residues of the protein or peptide are sequential, without any non-amino acid interrupting the sequence of amino acid residues. In other embodiments, the sequence may comprise one or more non-amino acid moieties. In particular embodiments, the sequence of residues of the protein or peptide may be interrupted by one or more non-amino acid moieties.

[0052] Accordingly, the term "protein or peptide" encompasses amino acid sequences comprising at least one of the 20 common amino acids found in naturally occurring proteins, or at least one modified or unusual amino acid, including but not limited to those shown on Table 1 below.

Table 1 Modified and Unusual Amino Acids			
Abbr.	Amino Acid	Abbr.	Amino Acid
Aad	2-Aminoadipic acid	EtAsn	N-Ethylasparagine
Baad	3- Aminoadipic acid	Hyl	Hydroxylysine
Bala	β -alanine, β -Amino-propionic acid	AHyl	allo-Hydroxylysine

Table 1 Modified and Unusual Amino Acids			
Abbr.	Amino Acid	Abbr.	Amino Acid
Abu	2-Aminobutyric acid	3Hyp	3-Hydroxyproline
4Abu	4- Aminobutyric acid, piperidinic acid	4Hyp	4-Hydroxyproline
Acp	6-Aminocaproic acid	Ide	Isodesmosine
Ahe	2-Aminoheptanoic acid	Alle	allo-Isoleucine
Aib	2-Aminoisobutyric acid	MeGly	N-Methylglycine, sarcosine
Baib	3-Aminoisobutyric acid	Melle	N-Methylisoleucine
Apm	2-Aminopimelic acid	MeLys	6-N-Methyllysine
Dbu	2,4-Diaminobutyric acid	MeVal	N-Methylvaline
Des	Desmosine	Nva	Norvaline
Dpm	2,2'-Diaminopimelic acid	Nle	Norleucine
Dpr	2,3-Diaminopropionic acid	Orn	Ornithine
EtGly	N-Ethylglycine		

[0053] Proteins or peptides may be made by any technique known to those of skill in the art, including the expression of proteins, polypeptides or peptides through standard molecular biological techniques, the isolation of proteins or peptides from natural sources, or the chemical synthesis of proteins or peptides. The nucleotide and protein, polypeptide and peptide sequences corresponding to various genes have been previously disclosed, and may be found at computerized databases known to those of ordinary skill in the art. One such database is the National Center for Biotechnology Information's Genbank and GenPept databases (available on the world wide web at ncbi.nlm.nih.gov/). The coding regions for known genes may be amplified and/or expressed using the techniques disclosed herein or as would be known to those of ordinary skill in the art.

Alternatively, various commercial preparations of proteins, polypeptides and peptides are known to those of skill in the art.

B. Nucleic Acids and Vectors

[0054] In certain aspects of the disclosure, not according to the claimed invention, nucleic acid sequences encoding a fusion protein as a stabilized multimeric arginase may be disclosed. Depending on which expression system to be used, nucleic acid sequences can be selected based on conventional methods. For example, human arginase I and II contain multiple codons that are rarely utilized in *E. coli* that may interfere with expression, therefore the respective genes or variants thereof may be codon optimized for *E. coli* expression. Various vectors may be also used to express the protein of interest, such as a fusion multimeric arginase or a cysteine-substituted arginase. Exemplary vectors include, but are not limited,

plasmid vectors, viral vectors, transposon or liposome-based vectors.

C. Host cells

[0055] Host cells, preferably eukaryotic cells, useful in the present invention are any that may be transformed to allow the expression and secretion of arginase and fusion multimers thereof. The host cells may be bacteria, mammalian cells, yeast, or filamentous fungi. Various bacteria include *Escherichia* and *Bacillus*. Yeasts belonging to the genera *Saccharomyces*, *Kluyveromyces*, *Hansenula*, or *Pichia* would find use as an appropriate host cell. Various species of filamentous fungi may be used as expression hosts including the following genera: *Aspergillus*, *Trichoderma*, *Neurospora*, *Penicillium*, *Cephalosporium*, *Achlya*, *Podospora*, *Endothia*, *Mucor*, *Cochliobolus* and *Pyricularia*.

[0056] Examples of usable host organisms include bacteria, *e.g.*, *Escherichia coli* MC1061, derivatives of *Bacillus subtilis* BRB1 (Sibakov *et al.*, 1984), *Staphylococcus aureus* SAI123 (Lordanescu, 1975) or *Streptococcus lividans* (Hopwood *et al.*, 1985); yeasts, *e.g.*, *Saccharomyces cerevisiae* AH 22 (Mellor *et al.*, 1983) and *Schizosaccharomyces pombe*; filamentous fungi, *e.g.*, *Aspergillus nidulans*, *Aspergillus awamori* (Ward, 1989), *Trichoderma reesei* (Penttila *et al.*, 1987; Harkki *et al.*, 1989).

[0057] Examples of mammalian host cells include Chinese hamster ovary cells (CHO-K1; ATCC CCL61), rat pituitary cells (GH₁; ATCC CCL82), HeLa S3 cells (ATCC CCL2.2), rat hepatoma cells (H-4-II-E; ATCC CRL 1548) SV40-transformed monkey kidney cells (COS-1; ATCC CRL 1650) and murine embryonic cells (NIH-3T3; ATCC CRL 1658). The foregoing being illustrative but not limitative of the many possible host organisms known in the art. In principle, all hosts capable of secretion can be used whether prokaryotic or eukaryotic.

[0058] Mammalian host cells expressing the arginase and/or their fusion multimers are cultured under conditions typically employed to culture the parental cell line. Generally, cells are cultured in a standard medium containing physiological salts and nutrients, such as standard RPMI, MEM, IMEM or DMEM, typically supplemented with 5-10% serum, such as fetal bovine serum. Culture conditions are also standard, *e.g.*, cultures are incubated at 37°C in stationary or roller cultures until desired levels of the proteins are achieved.

D. Protein Purification

[0059] Protein purification techniques are well known to those of skill in the art. These techniques involve, at one level, the homogenization and crude fractionation of the cells, tissue or organ to polypeptide and non-polypeptide fractions. The protein or polypeptide of interest may be further purified using chromatographic and electrophoretic techniques to achieve partial or complete purification (or purification to homogeneity) unless otherwise specified.

Analytical methods particularly suited to the preparation of a pure peptide are ion-exchange chromatography, gel exclusion chromatography, polyacrylamide gel electrophoresis, affinity chromatography, immunoaffinity chromatography and isoelectric focusing. A particularly efficient method of purifying peptides is fast performance liquid chromatography (FPLC) or even high performance liquid chromatography (HPLC).

[0060] A purified protein or peptide is intended to refer to a composition, isolatable from other components, wherein the protein or peptide is purified to any degree relative to its naturally-obtainable state. An isolated or purified protein or peptide, therefore, also refers to a protein or peptide free from the environment in which it may naturally occur. Generally, "purified" will refer to a protein or peptide composition that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term "substantially purified" is used, this designation will refer to a composition in which the protein or peptide forms the major component of the composition, such as constituting about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, or more of the proteins in the composition.

[0061] Various techniques suitable for use in protein purification are well known to those of skill in the art. These include, for example, precipitation with ammonium sulphate, PEG, antibodies and the like, or by heat denaturation, followed by: centrifugation; chromatography steps such as ion exchange, gel filtration, reverse phase, hydroxylapatite and affinity chromatography; isoelectric focusing; gel electrophoresis; and combinations of these and other techniques. As is generally known in the art, it is believed that the order of conducting the various purification steps may be changed, or that certain steps may be omitted, and still result in a suitable method for the preparation of a substantially purified protein or peptide.

[0062] Various methods for quantifying the degree of purification of the protein or peptide are known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the amount of polypeptides within a fraction by SDS/PAGE analysis. A preferred method for assessing the purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific activity of the initial extract, and to thus calculate the degree of purity therein, assessed by a "-fold purification number." The actual units used to represent the amount of activity will, of course, be dependent upon the particular assay technique chosen to follow the purification, and whether or not the expressed protein or peptide exhibits a detectable activity.

[0063] There is no general requirement that the protein or peptide always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products may have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater "-fold" purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product,

or in maintaining the activity of an expressed protein.

[0064] In certain embodiments a protein or peptide may be isolated or purified, for example, a stabilized arginase multimeric fusion protein, or an arginase prior or post pegylation. For example, a His tag or an affinity epitope may be comprised in such a arginase variant to facilitate purification. Affinity chromatography is a chromatographic procedure that relies on the specific affinity between a substance to be isolated and a molecule to which it can specifically bind. This is a receptor-ligand type of interaction. The column material is synthesized by covalently coupling one of the binding partners to an insoluble matrix. The column material is then able to specifically adsorb the substance from the solution. Elution occurs by changing the conditions to those in which binding will not occur (*e.g.*, altered pH, ionic strength, temperature, *etc.*). The matrix should be a substance that itself does not adsorb molecules to any significant extent and that has a broad range of chemical, physical and thermal stability. The ligand should be coupled in such a way as to not affect its binding properties. The ligand should also provide relatively tight binding. And it should be possible to elute the substance without destroying the sample or the ligand.

[0065] Size exclusion chromatography (SEC) is a chromatographic method in which molecules in solution are separated based on their size, or in more technical terms, their hydrodynamic volume. It is usually applied to large molecules or macromolecular complexes such as proteins and industrial polymers. Typically, when an aqueous solution is used to transport the sample through the column, the technique is known as gel filtration chromatography, versus the name gel permeation chromatography which is used when an organic solvent is used as a mobile phase.

[0066] The underlying principle of SEC is that particles of different sizes will elute (filter) through a stationary phase at different rates. This results in the separation of a solution of particles based on size. Provided that all the particles are loaded simultaneously or near simultaneously, particles of the same size should elute together. Each size exclusion column has a range of molecular weights that can be separated. The exclusion limit defines the molecular weight at the upper end of this range and is where molecules are too large to be trapped in the stationary phase. The permeation limit defines the molecular weight at the lower end of the range of separation and is where molecules of a small enough size can penetrate into the pores of the stationary phase completely and all molecules below this molecular mass are so small that they elute as a single band.

[0067] High-performance liquid chromatography (or High pressure liquid chromatography, HPLC) is a form of column chromatography used frequently in biochemistry and analytical chemistry to separate, identify, and quantify compounds. HPLC utilizes a column that holds chromatographic packing material (stationary phase), a pump that moves the mobile phase(s) through the column, and a detector that shows the retention times of the molecules. Retention time varies depending on the interactions between the stationary phase, the molecules being analyzed, and the solvent(s) used.

V. Pharmaceutical Compositions

[0068] It is contemplated that the novel arginases of the present invention can be administered systemically or locally to inhibit tumor cell growth and, most preferably, to kill cancer cells in cancer patients with locally advanced or metastatic cancers. They can be administered intravenously, intrathecally, and/or intraperitoneally. They can be administered alone or in combination with anti-proliferative drugs. In one embodiment, they are administered to reduce the cancer load in the patient prior to surgery or other procedures. Alternatively, they can be administered after surgery to ensure that any remaining cancer (e.g. cancer that the surgery failed to eliminate) does not survive.

[0069] It is not intended that the present invention be limited by the particular nature of the therapeutic preparation. For example, such compositions can be provided in formulations together with physiologically tolerable liquid, gel or solid carriers, diluents, and excipients. These therapeutic preparations can be administered to mammals for veterinary use, such as with domestic animals, and clinical use in humans in a manner similar to other therapeutic agents. In general, the dosage required for therapeutic efficacy will vary according to the type of use and mode of administration, as well as the particularized requirements of individual subjects.

[0070] 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.

[0071] Where clinical applications are contemplated, it may be necessary to prepare pharmaceutical compositions--expression vectors, virus stocks, proteins, antibodies and drugs--in a form appropriate for the intended application. Generally, pharmaceutical compositions of the present invention comprise an effective amount of one or more arginase variants or additional agent dissolved or dispersed in a pharmaceutically acceptable carrier. The phrases "pharmaceutical or pharmacologically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, such as, for example, a human, as appropriate. The preparation of an pharmaceutical composition that contains at least one arginase variant, such as a stabilized multimeric arginase or a pegylated arginase isolated by the method disclosed herein, or additional active ingredient will be known to those of skill in the art in light of the present disclosure, as exemplified by Remington's Pharmaceutical Sciences, 18th Ed., 1990. Moreover, for animal (e.g., human) administration, it will be understood that preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biological Standards.

[0072] As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, surfactants, antioxidants, preservatives (e.g., antibacterial agents, antifungal agents), isotonic agents, absorption delaying agents, salts, preservatives, drugs, drug stabilizers, gels, binders, excipients, disintegration agents, lubricants, sweetening agents, flavoring agents, dyes, such like materials and combinations thereof, as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 18th Ed., 1990). Except insofar as any conventional carrier is incompatible with the active ingredient, its use in the pharmaceutical compositions is contemplated.

[0073] The present invention may comprise different types of carriers depending on whether it is to be administered in solid, liquid or aerosol form, and whether it need to be sterile for such routes of administration as injection. The present invention can be administered intravenously, intradermally, transdermally, intrathecally, intraarterially, intraperitoneally, intranasally, intravaginally, intrarectally, topically, intramuscularly, subcutaneously, mucosally, orally, topically, locally, inhalation (e.g., aerosol inhalation), injection, infusion, continuous infusion, localized perfusion bathing target cells directly, via a catheter, via a lavage, in lipid compositions (e.g., liposomes), or by other method or any combination of the foregoing as would be known to one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 18th Ed., 1990).

[0074] The arginase variants may be formulated into a composition in a free base, neutral or salt form. Pharmaceutically acceptable salts, include the acid addition salts, e.g., those formed with the free amino groups of a proteinaceous composition, or which are formed with inorganic acids such as for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric or mandelic acid. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as for example, sodium, potassium, ammonium, calcium or ferric hydroxides; or such organic bases as isopropylamine, trimethylamine, histidine or procaine. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amounts as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as formulated for parenteral administrations such as injectable solutions, or aerosols for delivery to the lungs, or formulated for alimentary administrations such as drug release capsules and the like.

[0075] Further in accordance with the present invention, the composition of the present invention suitable for administration is provided in a pharmaceutically acceptable carrier with or without an inert diluent. The carrier should be assimilable and includes liquid, semi-solid, i.e., pastes, or solid carriers. Except insofar as any conventional media, agent, diluent or carrier is detrimental to the recipient or to the therapeutic effectiveness of a the composition contained therein, its use in administrable composition for use in practicing the methods of the present invention is appropriate. Examples of carriers or diluents include fats, oils, water, saline solutions, lipids, liposomes, resins, binders, fillers and the like, or combinations thereof. The composition may also comprise various antioxidants to retard oxidation of one or more component. Additionally, the prevention of the action of microorganisms can be brought about by preservatives such as various antibacterial and antifungal agents, including but not limited

to parabens (e.g., methylparabens, propylparabens), chlorobutanol, phenol, sorbic acid, thimerosal or combinations thereof.

[0076] In accordance with the present invention, the composition is combined with the carrier in any convenient and practical manner, *i.e.*, by solution, suspension, emulsification, admixture, encapsulation, absorption and the like. Such procedures are routine for those skilled in the art.

[0077] In a specific embodiment of the present invention, the composition is combined or mixed thoroughly with a semi-solid or solid carrier. The mixing can be carried out in any convenient manner such as grinding. Stabilizing agents can be also added in the mixing process in order to protect the composition from loss of therapeutic activity, *i.e.*, denaturation in the stomach. Examples of stabilizers for use in the composition include buffers, amino acids such as glycine and lysine, carbohydrates such as dextrose, mannose, galactose, fructose, lactose, sucrose, maltose, sorbitol, mannitol, *etc.*

[0078] In further embodiments, the present invention may concern the use of a pharmaceutical lipid vehicle compositions that include arginase variants, one or more lipids, and an aqueous solvent. As used herein, the term "lipid" will be defined to include any of a broad range of substances that is characteristically insoluble in water and extractable with an organic solvent. This broad class of compounds are well known to those of skill in the art, and as the term "lipid" is used herein, it is not limited to any particular structure. Examples include compounds which contain long-chain aliphatic hydrocarbons and their derivatives. A lipid may be naturally occurring or synthetic (*i.e.*, designed or produced by man). However, a lipid is usually a biological substance. Biological lipids are well known in the art, and include for example, neutral fats, phospholipids, phosphoglycerides, steroids, terpenes, lysolipids, glycosphingolipids, glycolipids, sulphatides, lipids with ether and ester-linked fatty acids and polymerizable lipids, and combinations thereof. Of course, compounds other than those specifically described herein that are understood by one of skill in the art as lipids are also encompassed by the compositions and methods of the present invention.

[0079] One of ordinary skill in the art would be familiar with the range of techniques that can be employed for dispersing a composition in a lipid vehicle. For example, the stabilized multimeric arginase or pegylated arginase may be dispersed in a solution containing a lipid, dissolved with a lipid, emulsified with a lipid, mixed with a lipid, combined with a lipid, covalently bonded to a lipid, contained as a suspension in a lipid, contained or complexed with a micelle or liposome, or otherwise associated with a lipid or lipid structure by any means known to those of ordinary skill in the art. The dispersion may or may not result in the formation of liposomes.

[0080] The actual dosage amount of a composition of the present invention administered to an animal patient can be determined by physical and physiological factors such as body weight, severity of condition, the type of disease being treated, previous or concurrent therapeutic interventions, idiopathy of the patient and on the route of administration. Depending upon the dosage and the route of administration, the number of administrations of a preferred dosage and/or an effective amount may vary according to the response of the subject. The practitioner

responsible for administration will, in any event, determine the concentration of active ingredient(s) in a composition and appropriate dose(s) for the individual subject.

[0081] In certain embodiments, pharmaceutical compositions may comprise, for example, at least about 0.1% of an active compound. In other embodiments, an active compound may comprise between about 2% to about 75% of the weight of the unit, or between about 25% to about 60%, for example, and any range derivable therein. Naturally, the amount of active compound(s) in each therapeutically useful composition may be prepared in such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

[0082] In other non-limiting examples, a dose may also comprise from about 1 microgram/kg/body weight, about 5 microgram/kg/body weight, about 10 microgram/kg/body weight, about 50 microgram/kg/body weight, about 100 microgram/kg/body weight, about 200 microgram/kg/body weight, about 350 microgram/kg/body weight, about 500 microgram/kg/body weight, about 1 milligram/kg/body weight, about 5 milligram/kg/body weight, about 10 milligram/kg/body weight, about 50 milligram/kg/body weight, about 100 milligram/kg/body weight, about 200 milligram/kg/body weight, about 350 milligram/kg/body weight, about 500 milligram/kg/body weight, to about 1000 mg/kg/body weight or more per administration, and any range derivable therein. In non-limiting examples of a derivable range from the numbers listed herein, a range of about 5 mg/kg/body weight to about 100 mg/kg/body weight, about 5 microgram/kg/body weight to about 500 milligram/kg/body weight, *etc.*, can be administered, based on the numbers described above.

VII. Definitions

[0083] The term "aa" refers to amino acid(s). Amino acid substitutions are indicated by the amino acid position, *e.g.* 303, in the molecule using a letter code (the letter in front of the number indicates the amino acid being replaced, while the letter after the number indicates the amino acid being introduced).

[0084] As used herein the term "portion" when in reference to a protein (as in "a portion of a given protein") refers to fragments of that protein. The fragments may range in size from four amino acid residues to the entire amino acid sequence minus one amino acid.

[0085] As used herein the terms "protein" and "polypeptide" refer to compounds comprising amino acids joined via peptide bonds and are used interchangeably.

[0086] As used herein, the term "fusion protein" refers to a chimeric protein containing the protein of interest (*i.e.*, a human arginase or variant thereof) joined (or operably linked) to an

exogenous protein fragment (the fusion partner which consists of a non-arginase protein). The fusion partner may enhance serum half-life, solubility, or both. It may also provide an affinity tag (e.g. his-tag) to allow purification of the recombinant fusion protein from the host cell or culture supernatant, or both.

[0087] The terms "in operable combination", "in operable order" and "operably linked" refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

[0088] The term " K_m " as used herein refers to the Michaelis-Menton constant for an enzyme and is defined as the concentration of the specific substrate at which a given enzyme yields one-half its maximum velocity in an enzyme catalyzed reaction.

[0089] The term k_{cat} as used herein refers to the turnover number or the number of substrate molecule each enzyme site converts to product per unit time, and in which the enzyme is working at maximum efficiency.

[0090] The term K_{cat}/K_m as used herein is the specificity constant which is a measure of how efficiently an enzyme converts a substrate into product.

[0091] The term "Mn-hArgI" refers to human Arginase I with an Mn(II) cofactor. The term "Co-hArgI" refers to human Arginase I (mutant or native) with a Co(II) cofactor.

[0092] The term " IC_{50} " is the half maximal (50%) inhibitory concentration (IC) and thus a measure of effectiveness.

[0093] The term "pegylated" refers to conjugation with polyethylene glycol (PEG), which has been widely used as a drug carrier, given its high degree of biocompatibility and ease of modification. (Harris *et al.*, 2001). Attachment to various drugs, proteins, and liposomes has been shown to improve residence time and decrease toxicity. (Greenwald *et al.*, 2000; Zalipsky *et al.*, 1997). PEG can be coupled (e.g. covalently linked) to active agents through the hydroxyl groups at the ends of the chain and via other chemical methods; however, PEG itself is limited to at most two active agents per molecule. In a different approach, copolymers of PEG and amino acids have been explored as novel biomaterials which would retain the biocompatibility properties of PEG, but which would have the added advantage of numerous attachment points per molecule (providing greater drug loading), and which can be synthetically designed to suit a variety of applications (Nathan *et al.*, 1992; Nathan *et al.*, 1993).

[0094] The term "gene" refers to a DNA sequence that comprises control and coding sequences necessary for the production of a polypeptide or precursor thereof. The polypeptide can be encoded by a full length coding sequence or by any portion of the coding sequence so long as the desired enzymatic activity is retained.

[0095] The term "subject" refers to animals, including humans.

[0096] The term "wild-type" refers to a gene or gene product which has the characteristics of that gene or gene product when isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designated the "normal" or "wild-type" form of the gene. In contrast, the term "modified" or "variant" or "mutant" refers to a gene or gene product which displays modifications in sequence and or functional properties (*i.e.*, altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally-occurring mutants can be isolated; these are identified by the fact that they have altered characteristics when compared to the wild-type gene or gene product.

VIII. Kits

[0097] The present invention provides kits, such as therapeutic kits. For example, a kit may comprise one or more pharmaceutical composition as described herein and optionally instructions for their use. Kits may also comprise one or more devices for accomplishing administration of such compositions. For example, a subject kit may comprise a pharmaceutical composition and catheter for accomplishing direct intravenous injection of the composition into a cancerous tumor. In other embodiments, a subject kit may comprise pre-filled ampoules of a stabilized multimeric arginase or isolated pegylated arginase, optionally formulated as a pharmaceutical, or lyophilized, for use with a delivery device.

[0098] Kits may comprise a container with a label. Suitable containers include, for example, bottles, vials, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container may hold a composition which includes an antibody that is effective for therapeutic or non-therapeutic applications, such as described above. The label on the container may indicate that the composition is used for a specific therapy or non-therapeutic application, and may also indicate directions for either *in vivo* or *in vitro* use, such as those described above. The kit of the invention will typically comprise the container described above and one or more other containers comprising materials desirable from a commercial and user standpoint, including buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

IX. Examples

[0099] The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof. In the experimental disclosure which follows, the following abbreviations apply: eq (equivalents); M (Molar); μ M (micromolar); mM (millimolar); N (Normal); mol (moles); mmol (millimoles); μ mol

(micromoles); nmol (nanomoles); g (grams); mg (milligrams); μ g (micrograms); L (liters); ml (milliliters); μ l (microliters); cm (centimeters); mm (millimeters); μ m (micrometers); nm (nanometers); EC (degrees Centigrade); MW (molecular weight); PBS (phosphate buffered saline); min (minutes).

EXAMPLE 1

GENE SYNTHESIS AND EXPRESSION OF HUMAN ARGINASE I & II

[0100] The human Arginase I and II genes both contain mutiple codons that are rarely utilized in *E. coli* that can interfere with expression. Thus, in order to optimize protein expression in *E. coli*, the respective genes were assembled with codon optimized oligonucleotides designed with DNA-Works software (Hoover *et al.*, 2002). Each construct contains an N-terminal NcoI restriction site, an in-frame N-terminal His₆ tag followed by a Tobacco Etch Virus (TEV) protease site and a C-terminal BamHI site for simplifying cloning. Cleavage by TEV protease removes the His₆ peptide and the N-terminal Met of arginase. An Arginase II gene was designed with a TEV protease cleavage site and without the first native 21 aa. The first 21 aa are a putative mitochondrial-targeting sequence and its removal results in greater protein yield and stability (Colleluori *et al.*, 2001). After cloning into a pET28a vector (Novagen), *E. coli* (BL21) containing an appropriate Arginase expression vector were grown at 37°C using Terrific Broth (TB) media containing 50 μ g/ml kanamycin in shake flasks at 250 rpm until reaching an OD₆₀₀ of 0.5-0.6. At that point the cultures were transferred to 25°C, induced with 0.5 mM IPTG and allowed to express protein for an additional 12 hrs. Cell pellets were then collected by centrifugation and resuspended in an IMAC buffer (10 mM NaPO₄/10 mM imidazole/ 300 mM NaCl, pH 8). After lysis by a French pressure cell, lysates were centrifuged at 20,000 x g for 20 min at 4°C, and the resulting supernatant was applied to a cobalt or nickel IMAC column, washed with 10-20 column volumes of IMAC buffer, and then eluted with an IMAC elution buffer (50 mM NaPO₄/ 250 mM imidazole/ 300 mM NaCl, pH 8). The desired divalent metal cation is incorporated by incubation with 10 mM metal (CoCl₂ or MnSO₄) for 15 min at 50°-55°C, followed by filtration through a 0.45 μ m syringe filter. Using a 10,000 MWCO centrifugal filter device (Amicon), proteins were then buffer-exchanged several times into a 100 mM Hepes, 10 % glycerol, pH 7.4 solution. Aliquots of Arginase enzyme were then flash frozen in liquid nitrogen and stored at - 80°C. Arginase purified in this manner is > 95 % homogeneous as assessed by SDS-PAGE and coomassie staining (FIG. 2). The yield is calculated to be ~ 200 mg/L culture based upon the calculated extinction coefficient, ϵ_{280} = 24,180 M⁻¹cm⁻¹ in a final buffer concentration of 6 M guanidinium hydrochloride, 20 mM phosphate buffer, pH 6.5 (Gill and von Hippel, 1989).

EXAMPLE 2

INCORPORATING AND DETERMINING METAL CONTENT IN ARGINASE I

[0101] As mentioned in Example 1, incorporation of Mn^{2+} and Co^{2+} can be achieved by purifying Arginase, followed by an incubation step with 10 mM metal at 50°C for 10 min. In order to determine the final metal content and identity of the Arginase preps, protein samples of Mn-hArgI (145 μM), Co-hArgI (182 μM) and associated dialysis buffers (100 mM Hepes, pH 7.4) were diluted in 2% nitric acid and analyzed by inductively coupled plasma mass spectrometry (ICP-MS, Department of Geological Sciences, University of Texas at Austin) to quantify the protein's cobalt, iron, manganese and zinc content by subtracting the concentration of metals found in dialysis buffer from the metal concentration of the final protein samples and dividing by protein concentration. To determine protein concentrations, an extinction coefficient was calculated for hArgI based on amino acid sequence (Gill and von Hippel, 1989). All protein concentrations for Arginase I were calculated based upon the calculated $\epsilon_{280} = 24,180 \text{ M}^{-1}\text{cm}^{-1}$ in a final buffer concentration of 6 M guanidinium hydrochloride, 20 mM phosphate buffer, pH 6.5. For comparison, Arginase concentration was also calculated by BCA assay using dilutions of BSA as a standard. Using this method it was found that Arginase samples incubated with Co^{2+} contain 2.1 ± 0.5 equivalents Co and 0.4 ± 0.1 equivalents Fe, with no detectable amounts of Zn or Mn. Samples incubated with Mn^{2+} contain 1.5 ± 0.2 equivalents Mn and 0.4 ± 0.1 equivalents Fe, and no detectable amounts of Zn or Co. Thus, heat incubation is an efficient method for incorporation of Cobalt.

REFERENCE EXAMPLE 3

INCORPORATING AND DETERMINING METAL CONTENT IN ARGINASE II

[0102] Efficient metal incorporation into Arginase II was achieved by culturing *E.coli* harboring the ArgII gene in minimal media until an OD_{600} of 1 is reached, whereupon the protein was expressed with 1 mM IPTG and 100 μM CoCl_2 for an additional 12 hrs. In order to determine the final metal content and identity of the Arginase preps, protein samples of Co-hArgII (290 μM) and associated dialysis buffers (100 mM Hepes, pH 7.4) were diluted in 1% nitric acid and analyzed by inductively coupled plasma mass spectrometry (ICP-MS, Department of Geological Sciences, University of Texas at Austin) to quantify the protein's cobalt, iron, manganese and zinc content by subtracting the concentration of metals found in dialysis buffer from the metal concentration of the final protein samples and dividing by protein concentration. To determine protein concentrations, an extinction coefficient was calculated for hArgII based on amino acid sequence (Gill and von Hippel, 1989). All protein concentrations for Arginase II were calculated based upon the calculated $\epsilon_{280} = 22,900 \text{ M}^{-1}\text{cm}^{-1}$ in a final buffer concentration

of 6 M guanidinium hydrochloride, 20 mM phosphate buffer, pH 6.5. For comparison, Arginase concentration was also calculated by BCA assay using dilutions of BSA as a standard. Using this method it was found that Arginase samples expressed with Co^{2+} contain 1.35 ± 0.1 equivalents Co and 0.63 ± 0.1 equivalents Fe, with no detectable amounts of Zn or Mn.

EXAMPLE 4

STEADY STATE KINETICS OF COBALT ARGINASE I AT PHYSIOLOGICAL PH

[0103] Diacetylmonoxine (DAMO) derivitization of urea products in the presence of strong acids, thiosemicarbazide and Fe^{3+} with heating to produce a chromophore with a λ_{max} of ~ 530 nm, was used to monitor urea production following hydrolysis of L-Arginine by arginase. The dye structure is not definitively known, but the reaction is hypothesized to be a condensation of DAMO and urea/uriedo that is possibly stabilized by Fe^{3+} ions (Beale and Croft, 1961). A standard curve of urea vs. A_{530} was constructed that was found to be linear between 0 - 300 μM urea with a lower detection limit of 1-2 μM . The steady-state kinetics of Co-hArgI, and Mn-hArgI were examined over a range of L-arginine concentrations (0-40 mM) in a 100 mM Hepes buffer pH 7.4, 37°C. Typically reactions were performed by equilibrating 200 μL in 1.5 ml eppendorf tubes at 37°C in a heat block, starting the reaction by adding 5 μL of enzyme for 30 sec and quenching with 15 μL of 12 N HCl. Reactions and blanks were then mixed with 800 μL of color developing reagent (COLDER) (Knipp and Vasak, 2000) and boiled for 15 min. After cooling for 10 min, the samples were transferred to cuvettes and read at 530 nm on a spectrophotometer. L-Arginine has a background absorbance that makes correction necessary, so L-Arginine blanks were included for all concentrations used. The resulting data is then corrected for background and the concentrations of product formed calculated from the standard curve. The product is then divided by the time and the concentration of enzyme used and $v_o/[E]$ is plotted vs. substrate concentration and fit directly to the Michaelis-Menten equation (FIG. 3), where $v_o/[E] = k_{\text{cat}}[S]/([S] + K_M)$. With this method, Co-hArgI had a k_{cat} of $240 \pm 14 \text{ s}^{-1}$, a K_M of $190 \pm 40 \mu\text{M}$, and k_{cat}/K_M of $1,270 \pm 330 \text{ mM}^{-1} \text{ s}^{-1}$, as compared to Mn-hArgI, which had a k_{cat} of $300 \pm 12 \text{ s}^{-1}$, a K_M of $2,330 \pm 260 \mu\text{M}$, and k_{cat}/K_M of $129 \pm 20 \text{ mM}^{-1} \text{ s}^{-1}$. The use of Cobalt as a cofactor at physiological pH leads to 10 fold increase in the specificity constant.

REFERENCE EXAMPLE 5

STEADY STATE KINETICS OF COBALT ARGINASE II AT PHYSIOLOGICAL PH

[0104] Arginase II purified as described in Example 3 and characterized as described in Example 4 was found to have a k_{cat} of $182 \pm 7 \text{ s}^{-1}$, a K_{M} of $126 \pm 18 \text{ }\mu\text{M}$, and a $k_{\text{cat}}/K_{\text{M}}$ of $1,440 \pm 260 \text{ mM}^{-1}\text{s}^{-1}$ as compared to Mn-hArgII where we found a k_{cat} of $48 \pm 2 \text{ s}^{-1}$, a K_{M} of $2,900 \pm 300 \text{ }\mu\text{M}$, and $k_{\text{cat}}/K_{\text{M}}$ of $17 \pm 2 \text{ mM}^{-1} \text{ s}^{-1}$. The use of Cobalt as a cofactor at physiological pH leads to 80 fold increase in the specificity constant for Arginase II.

EXAMPLE 6

96-WELL PLATE SCREEN FOR ARGINASE ACTIVITY AND RANKING CLONES

[0105] Arginase hydrolysis of L-Arginine produces L-Ornithine and urea. The L-Arginine hydrolysis assay of Example 3 was adapted to 96-well plate format for the detection of urea and used for screening libraries of protein mutants and for rank-ordering clones with the greatest Arginase activity. Clones displaying greater than 2-fold increase in activity were selected for further characterization. The assay was shown to have a dynamic range of ~ 5 -200 μM for ureido product detection. More than 500 clones can easily be screened per day *via* manual screening. The signal output, *i.e.*, color intensity, reflects three main parameters, namely the specificity constant ($k_{\text{cat}}/K_{\text{M}}$), the enzyme concentration [Enz], and time (t). If necessary, enzyme levels in individual clones can be detected by ELISA; however, generally enzyme expression of Arginase varies less than two-fold and therefore expression differences do not constitute a significant issue.

[0106] Single colonies were picked into 96-well culture plates containing 75 μL of TB medialwell containing 50 $\mu\text{g}/\text{ml}$ kanamycin. These cultures were then grown at 37°C on a plate shaker until reaching an OD_{600} of 0.8-1, cooled to 25°C , whereupon an additional 75 μL of media containing 50 $\mu\text{g}/\text{ml}$ kanamycin, and 0.5 mM IPTG was added. Expression was performed at 25°C with shaking for ~ 2 hrs, following which 100 μL of culture/well was transferred to a 96 well assay plate. The assay plates were then centrifuged to pellet the cells, the media was removed, and the cells were lysed by addition of 50 $\mu\text{L}/\text{well}$ of B-PER protein extraction reagent (Pierce). An additional 50 $\mu\text{L}/\text{well}$ of 200 μM L-Arginine, 1 mM CoCl_2 , in a 100 mM HEPES buffer, pH 7.4 was subsequently added and allowed to react at 37°C . After reacting ~ 1 -2 min, 100 $\mu\text{L}/\text{well}$ of color developing reagent are added and the plate was processed (Knipp and Vasak, 2000). Colonies having the ability to produce urea resulted in formation of a bright red dye with a λ_{max} of 530 nm.

EXAMPLE 7

PH RATE DEPENDENCE OF COBALT ARGINASE AND MANGANESE ARGINASE

[0107] To examine the pH dependence of $k_{\text{cat}}/K_{\text{M}}$ of cobalt and manganese substituted Arginase, the steady-state kinetic constants were determined across a broad range of pH values. The following buffers were used: sodium acetate (pH 5-5.5), MES (pH 6-6.5), HEPES (pH 7-7.8), Tris (pH 8-9), Capso (pH 9-10.5), all at a 100 mM concentration. All kinetics were determined in at least triplicate at 37°C. After fitting the kinetic data to the Michaelis-Menten equation, the $k_{\text{cat}}/K_{\text{M}}$ values were calculated and fit to a Henderson-Hasselbach equation to determine $\text{p}K_{\text{a}}$ values. Because fits to two $\text{p}K_{\text{a}}$ values closer than 3.5 units tend to underestimate y_{max} , Segel's method was used to calculate corrected $\text{p}K_{\text{a}}$ values for each limb of the $k_{\text{cat}}/K_{\text{M}}$ profiles (Segel, 1975). Adjusted fits of $k_{\text{cat}}/K_{\text{M}}$ vs. pH resulted in a bell shaped curve with Co-hArgl having an ascending limb $\text{p}K_{\text{a}}$ of 7.5 ± 0.1 and a descending limb $\text{p}K_{\text{a}}$ of 9.8 ± 0.1 . Mn-hArgl also had a bell shaped curve with an ascending limb $\text{p}K_{\text{a}}$ of 8.5 ± 0.1 and a descending limb displaying an apparent $\text{p}K_{\text{a}}$ value of 10.1 ± 0.1 (Figure 4). Mn-hArgl and Co-hArgl enzymes exhibited a $\Delta \text{p}K_{\text{a}}$ of 1 pH units. This shift in $\text{p}K_{\text{a}}$ upon Co substitution likely imparts much of the observed improvement in the specificity constant. At physiological pH, approximately 44 % of Co-hArgl would have hydroxide bound as opposed to 7 % with Mn-hArgl.

EXAMPLE 8

THE EFFECT OF MUTATIONS AT POSITION 303

[0108] An NNS codon saturation library at position 303 was constructed and screened using the following mutagenic primers: Forward '5-cgatcacggttagcaNNSttcggttagcccg (SEQ ID NO:3), and reverse '5- CGGGCTAAACCGAAsnnTGCTAACGTGATCG (SEQ ID NO:4), using the hArgl gene as template DNA and specific end primers; forward "5-GATATACCATGGGTTCTTCTCACCATCATCACCACCACAGCTCTGGCG (SEQ ID NO:5) and; reverse '5-CGAATTCGGATCCTCACTTCGGTGGATTGAGATAATCAATT (SEQ ID NO:6). The PCR product digested with NcoI and BamHI and ligated into pET28a vector with T4 DNA ligase. The resulting ligation was transformed directly into *E.coli* (BL21), plated on LB-kanamycin plates for subsequent screening as described in Example 4. Clones exhibiting highest activity were isolated and the DNA was sequenced. The respective enzyme variants were purified as described in Example 1 and heat incubated with Cobalt as described in Example 2. All proteins were purified to > 95 % homogeneity as assessed by SDS-PAGE. Arginine hydrolysis kinetics were determined with a range of L-arginine concentrations (0-2 mM) at 37°C in a 100 mM Hepes buffer pH 7.4, and the resulting data fit to the Michaelis-Menten equation in Kaleidagraph. Cys₃₀₃ substituted with Phe or Ile lead to a 2 fold & 1.6 fold increase in $k_{\text{cat}}/K_{\text{M}}$ respectively as compared to wild-type Co-hArgl. Leu, Pro, His, and Arg

substitutions had about 90 % of wild-type activity.

[0109] The Cys₃₀₃ variants were also tested for serum stability at 37°C as follows: Purified enzymes were added to pooled human serum (Innovative) at a concentration of 1 μ M and incubated at 37°C. Every ~24 hours, aliquots were withdrawn and tested in triplicate for their ability to hydrolyze 1 mM L-arginine in a 100 mM Hepes buffer pH 7.4. After ~ 4 days the resulting data was fit to either an exponential or logistic decay model to calculate $T_{1/2}$ values. The stability of the wild-type enzyme was used as a standard and was calculated to be a $T_{1/2}$ of 33 ± 3 hrs. Enzymes substituted with Phe, Ile, Leu, and His were only about half as stable as wild-type. Mn-hArgI (◊) displayed an exponential loss of activity with a $T_{1/2}$ life of 4.8 ± 0.8 hrs. In contrast Co-hArgI (●) displayed a bi-phasic loss of activity with an apparent first $T_{1/2}$ of 6.1 ± 0.6 hrs followed by much longer second $T_{1/2}$ of 37 ± 3 hrs.

EXAMPLE 9

SUBSTRATE SPECIFICITY

[0110] The selectivity of the engineered human Arginase for the hydrolysis of arginine was evaluated. Co-hArgI (1 μ M) or dialysis buffer was incubated with all 20 aa (5 mM each) for 12 hr at 37°C in a 220 mM phosphate buffer pH 7.4. Standards, controls and experiments were derivatized with OPA and FMOc (Agilent) and separated on a C18 reverse phase HPLC column (Agilent) (5 μ m, 4.6 x 150 mm) essentially as described by Agilent Technologies (Publication Number: 5980-3088) except for modification of the separation protocol slightly by reducing the flow rate by $\frac{1}{2}$ and doubling the acquisition time to get better peak separation. The 20 standard amino acids incubated with dialysis buffer showed 20 peaks with good resolution, and the 20 standard amino acids incubated with Co-hArgI showed 20 peaks (FIG. 6) with disappearance of the L-Arginine peak (RT = 12.3 min) and appearance of one new peak with a retention time matching that of L-Ornithine (RT = 18.8 min). None of the other amino acids were observed to be affected by Co-hArg I.

EXAMPLE 10

HIGH THROUGHPUT PURIFICATION AND KINETIC SCREENING OF VARIANTS

[0111] A small-scale purification scheme was developed to rapidly purify dozens of proteins at once and carry out high throughput enzyme kinetics analysis. 50 ml cultures of hArg I were expressed in 125 ml shake flasks as described in Example 1. 5 ml of the resulting culture were collected by centrifugation. The cell pellets were then lysed with 400 μ L of B-PER protein

extraction reagent (Pierce). The soluble fraction was mixed with 500 μ L IMAC lysis buffer and 100 μ L of IMAC beads in a 1.5 ml Eppendorf tube, incubated for two minutes and centrifuged at 3000 rpm for 20 s in a table top centrifuge. The supernatant was discarded and the beads are washed with 2 x 1 ml IMAC lysis buffer by mixing/centrifugation and discarding the supernatant. hArg I was then eluted from the beads by addition of 300 μ L of IMAC elution buffer and another centrifuge step. The resulting hArg I containing supernatant was subjected to buffer exchange twice with a 100 mM Hepes, pH 7.4 buffer using a 10,000 MWCO centrifugal concentration device (YM-10 Amicon). The protein was then quantified by A_{280} , heated in the presence of Cobalt as above, and the resulting Co-hArg I was assessed by SDS-PAGE as described in Example 1. This method allows purification of 12 - 16 proteins in ~ 2 hrs with a yield of 200 - 300 μ g protein at 90 - 95 % purity as assessed by SDS-PAGE.

[0112] The enzyme variants were then tested for their ability to hydrolyze L-Arginine by incubating 24 nM of enzyme with 200 μ M of L-arginine in microtiter plate wells. Aliquots were collected at different time points and directly quenched into the acidic color-developing reagent (COLDER). After developing the dye and reading the absorption, the progress curve data was fit to an exponential equation to estimate an apparent k_{cat}/K_M value.

EXAMPLE 11

ENGINEERING THE 2ND SHELL METAL LIGANDS OF ARGINASE FOR OPTIMAL ACTIVITY

[0113] The catalytic power of a metallohydrolase stems in part from its remarkable ability to depress the normal pK_a value of water (~16) to a much lower value and coordinate the highly reactive hydroxide ion for attack on substrate. Both the kind of metal and its local environment comprising of 1st and 2nd shell ligands influence the pK_a of the nucleophilic water/hydroxide molecule (Christianson and Cox, 1999). Metal (A) of hArgI is coordinated to the imidazole of H101, which is in turn hydrogen bonded to the hydroxyl of S230. Metal (B) of hArgI is coordinated to the imidazole of H126, which has a 2nd shell hydrogen bond with the carboxyl of D181. An NNS codon saturation library at position 181 and 230 was constructed using the following mutagenic primers: (D181) Forward "5-cattggcttacgtNNSgtcgacccagg (SEQ ID NO:7), reverse '5-CCTGGGTCGACSNACGTAAGCCAATG (SEQ ID NO:8); (S230) forward '5-cgtccaatccatctgNNSttcgatgttgacg (SEQ ID NO:9), reverse '5-CGTCAACATCGAASNACAGATGGATTGGACG (SEQ ID NO:10), along with the hArgI gene and specific end primers via overlap extension PCR. After cloning, the library was transformed in *E. coli* (BL21) and screened as described in Example 4. Novel clones were identified by sequencing, re-transformed into *E. coli* (BL21) and purified and kinetically characterized as described in Example 8. Variants displaying apparent activity $\geq$ to wild-type were purified in larger scale and assayed for their steady-state kinetic parameters of k_{cat} & K_M . The following variants were found to have greater k_{cat}/K_M constants than Co-hArgI: hArg I D181S: $1420 \pm$

200 s⁻¹ mM⁻¹, hArg I D181E/S230A: 1,450 ± 200 s⁻¹ mM⁻¹, hArg I S230C: 2,290 ± 200 s⁻¹ mM⁻¹, and hArg I S230G: 2,340 ± 70 s⁻¹ mM⁻¹.

EXAMPLE 12

CYTOTOXICITY OF CO-ARG AND ITS VARIANTS TOWARDS HEPATOCELLULAR CARCINOMA CELLS AND METASTATIC MELANOMAS

[0114] In order to test the *in vitro* cytotoxicity of engineered Arginase, varying concentrations (0 - 100 nM) of Mn-ArgI, Co-ArgI, or Co-hArgI variants were incubated with HCC (Hep 3b) cells or melanoma (A375) cells (American Type Culture Collection) in 96-well plates at a seeding density of 500 cells/well, in DMEM media supplemented with fetal bovine serum. After 24 hours of incubation at 37°C, the cells were treated with Arginase containing media in triplicate at various concentrations. The control solution was a balanced salt solution in media. The treated cells were maintained at 37°C and 5% CO₂. Cells were tested by standard MTT assay (Sigma-Aldrich) on days 1, 3, 5, & 7 by addition of 100 µL/well of MTT (5mg/mL), and incubated for 4 hours with gentle agitation one to two times per hour. Following this, the solution was aspirated and 200 µL of DMSO was then added to each well. Absorbance at 570 nm was interpreted for each well using an automated plate reader to determine the relative number of surviving cells compared to controls. The resulting data was fit to an exponential equation to determine an apparent IC₅₀ value for Arginase cytotoxicity. FIG. 7A shows the effect of Mn-ArgI, or Co-ArgI addition on % MTT absorbance of HCC cells. The IC₅₀ values from day 5 were calculated, yielding an IC₅₀ value for Mn-hArgI of 5 ± 0.3 nM (~ 0.18 µg/ml) and a value of 0.33 ± 0.02 nM for Co-hArgI (~ 0.012 µg/ml). Thus, the Co-ArgI enzyme appears to be 15 fold more cytotoxic than the Mn substituted enzyme against HCC. Against the metastatic melanoma cell line (A375) Mn-hArgI (FIG. 7B) resulted in an apparent IC₅₀ of 4.1 ± 0.1 nM (~ 0.15 µg/ml). Incubation with Co-hArgI lead to a 13-fold increase in cytotoxicity with an apparent IC₅₀ of 0.32 ± 0.06 nM (~ 0.012 µg/ml).

EXAMPLE 13

ENGINEERING AN FC-ARGINASE FUSION PROTEIN FOR ENHANCED *IN VIVO* HALF-LIFE

[0115] Fusion to the IgG Fc domain has been employed extensively for prolonging the *in vivo* half-lives of therapeutic polypeptides such as the TNF-α inhibitor etanercept (Enbrel®). The Fc domain binds to the FcγRn receptor, which is expressed on vascular endothelium and many other tissues (Roopenian and Akilesh, 2007). The affinity of FcγRn for the IgG Fc domain is

strongly pH dependent. Binding occurs at the acidic pH of endosomal compartments allowing the protein to be recycled onto the cell surface and thus escape proteolytic degradation. At the cell surface, the Fc domain is released from FcγRn because the binding affinity is very low at physiological pH. Endosomal recycling via FcγRn is estimated to increase the serum half-life of immunoglobulins at least 4-7 fold, to about 7-14 days in humans. Fc fusions exploit this property to endow short lived molecules with a long half life. However, the human Arginase is a homotrimer and therefore if fused to the IgG Fc, which itself is a dimer, the resulting Fc-Arginase polypeptide will likely form high molecular weight aggregates.

[0116] This problem was avoided by employing mutant forms of Arginase that disrupt trimerization and are stable in the monomeric form. The trimerization and subunit interface of Arginase I have been studied in some detail (Lavulo *et al.*, 2001). A single aa substitution at Glu256Gln has been shown to disrupt trimerization resulting in the formation of monomeric Arginase I enzyme (Sabio *et al.*, 2001). This mutation was introduced into hArgI by the use of two mutagenic primers: Forward '5-ggtttaacgtatcgcCAGggcctgtatatcacgg (SEQ ID NO:11) and Reverse '5-CCGTGATATACAGGCCCTGGCGATACGTTAAACC (SEQ ID NO: 12), and two specific end primers (Example 1) through overlap extension PCR, and cloning into a pET28a vector. After expression and purification of this variant, the steady-state kinetic analysis revealed nearly identical activity compared to Co-hArgI with a k_{cat}/K_M of $1,320 \text{ s}^{-1} \text{ mM}^{-1}$. FIG. 8 shows a non-denaturing PAGE gel showing that Co-hArgI-E256Q is a monomer, as expected.

[0117] This construct was then cloned into Fc expression vectors available. The Fc expression vector is a construct based on a pTRC99a plasmid (Amersham) that contains a DsbA leader sequence followed by the IgG Fc coding region, an EcoRI restriction site and a stop codon. The monomeric Arginase gene was placed in frame behind the Fc coding region by digesting both vector and gene with EcoRI, and was subsequently ligated and transformed into *E. coli* (BL21) for sequencing and expression. Since the IgG Fc is normally a glycosylated protein, expression of recombinant IgGs or of Fc fusions has so far been carried out in recombinant mammalian cells that, unlike bacteria, are capable of N-linked glycosylation. However, while glycosylation at Asn297 is critical for the binding to the activating and inhibitory Fcγ receptors (FcγRI-III in humans) it does not have a noticeable effect on the affinity or pH dependent binding to FcγRn (Tao and Morrison, 1989; Simmons *et al.*, 2002). Thus, aglycosylated IgG antibodies expressed in bacteria exhibit serum persistence in primates nearly indistinguishable from that of fully glycosylated antibodies expressed in mammalian cells (Simmons *et al.*, 2002). In contrast to prevailing earlier notions, IgG antibodies and Fc proteins can be expressed efficiently in *E.coli* up to g/L levels in fermenters. *E.coli* expression is technically much simpler and faster. In addition, since the resulting protein is aglycosylated, it does not display glycan heterogeneity, an important issue in the expression of therapeutic glycoproteins (Jefferis, 2007). The fusion protein is purified by Protein A chromatography and the yield of correctly folded, dimeric Fc-Arginase fusion relative to polypeptides that fail to dimerize is quantified by FPLC gel filtration chromatography. This formulation has lead to a highly active and very stable form of human Arginase, suitable for *in vivo* trials.

EXAMPLE 14

PEGYLATION OF ARGINASE

[0118] Arginase was purified as described in Example 10 with one exception: after binding to the IMAC column, the protein was washed with extensively (80-90 column volumes) with an IMAC buffer containing 0.1 % Triton 114 (This step removes most of the endotoxin), 10-20 column volumes of IMAC buffer, and then eluted with an IMAC elution buffer (50 mM NaPO₄/ 250 mM imidazole/ 300 mM NaCl, pH 8). Arginase was buffer exchanged into a 100 mM NaPO₄ buffer at pH 8.3 using a 10,000 MWCO filtration device (Amicon). Using a small reaction jar, Methoxy PEG Succinimidyl Carboxymethyl Ester 5000 MW (JenKem Technology) was added to Arginase at 40:1 molar ratio and allowed to react for 1 hr at 25°C under constant stirring. The resulting mixture was then made 10 mM with CoCl₂ and heated at 50°C for 10 minutes. After centrifuging to remove any precipitates, the PEG-5000 Arginase was extensively buffer exchanged (PBS with 10 % glycerol) using a 100,000 MWCO filtration device (Amicon), and sterilized with a 0.2 micron syringe filter (VWR). All pegylated enzyme was analyzed for lipopolysaccharide (LPS) content using a Limulus Amebocyte Lysate (LAL) kit (Cape Cod Incorporated).

[0119] Pegylated Co-hArgl was found to have nearly identical serum stability to wt enzyme and displayed a k_{cat}/K_M value of $1690 \pm 290 \text{ s}^{-1}\text{mM}^{-1}$. Figure 12 shows a denaturing gel of the final product with an apparent MW of ~ 150 kDa.

EXAMPLE 15

SERUM DEPLETION OF L-ARG IN THE MOUSE MODEL

[0120] Balb/c mice were treated by single IP injection with 500 µg of pharmacologically prepared, pegylated Co-hArgl or an equal volume of PBS. Mice were sacrificed by cardiac veni-puncture for blood collection at the time points of 0, 48, 72, and 96 hrs. Blood samples were immediately mixed 50:50 (v/v) with a 400 mM sodium citrate buffer pH 4 allowed to clot for 30 min and centrifuged for serum separation. The resulting serum was then filtered on 10,000 MWCO device (Amicon) for the removal of large proteins and precipitates and the flow-through was collected for analysis. L-arginine standards, control mouse serum and experiments were derivatized with OPA (Agilent) and separated on a C18 reverse phase HPLC column (Agilent) (5 µm, 4.6 x 150 mm) essentially as described by Agilent Technologies (Publication Number: 5980-3088) except for modification of the separation protocol slightly by reducing the flow rate by ½ and doubling the acquisition time to get better peak separation. An L-arginine standard curve was constructed by plotting L-Arg peak area versus concentration in

order to quantify serum L-Arg levels. A single dose of pharmacologically prepared Co-hArgl was sufficient to keep L-Arg at or below detection limits for over 3 days (FIG. 10).

EXAMPLE 16

HCC TUMOR XENOGRAFT TREATMENT WITH CO-HARGI

[0121] Nude mice were injected subcutaneously in the flank with $\sim 10^6$ HCC cells collected from a 75 % confluent tissue culture. After the HCC xenografted tumors had grown to $\sim 0.5 \text{ cm}^3$ in diameter (Day 9), mice were sorted into two groups. The experimental group received a 500 μg IP injection of pharmacologically optimized Co-hArgl at day 9 and at day 12. The control group received IP injections of PBS at days 9 and 12. As can be seen in FIG. 11, the PBS treated tumors had increased 3-fold in size by day 15. In stark contrast, Co-hArgl treated tumors had *decreased* in size by day 15. Mn-hArgl treated tumors had only been shown to be retarded in growth rate (Cheng *et al.*, 2007). Co-hArgl appears to be a highly effective chemotherapeutic agent against HCCs both *in vitro* and *in vivo*.

[0122] All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved.

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PATENTKRAV

1. Isoleret humant arginase I-protein, der:

i. omfatter den naturlige arginase I-aminosyresekvens, eventuelt uden den N-terminale methioninrest, og

ii. omfatter en ikke-naturlig metal-cofaktor, der er cobalt i form af Co^{2+} , til anvendelse i medicin.

2. Isoleret humant arginase I-protein til anvendelse ifølge krav 1, hvor det humane arginase I-protein omfatter en aminosyresekvens, der kodes for af SEQ ID NO:1, eventuelt uden den N-terminale methioninrest.

3. Isoleret humant arginase I-protein til anvendelse ifølge krav 1 eller 2, hvor det humane arginase I-protein ikke har en N-terminal methionin.

4. Isoleret humant arginase I-protein til anvendelse ifølge et hvilket som helst af kravene 1 til 3, hvor det humane arginase I-protein er kovalent bundet til polyethylenglycol.

5. Isoleret humant arginase I-protein til anvendelse ifølge et hvilket som helst af kravene 1 til 4, hvor det humane arginase I-protein udviser en k_{cat}/K_m , der er større end $400 \text{ mM}^{-1}\text{s}^{-1}$ ved 37°C og pH 7,4.

6. Isoleret humant arginase I-protein til anvendelse ifølge et hvilket som helst af kravene 1 til 5, hvor det humane arginase I-protein kan opnås ved ekspresion i en værtscelle, der omfatter en vektor, der indeholder en nukleinsyre, der koder for den naturlige arginase I-aminosyresekvens, eventuelt uden den N-terminale methioninrest.

7. Farmaceutisk sammensætning til anvendelse i medicin, hvor den farmaceutiske sammensætning omfatter et isoleret humant arginase I-protein som defineret i et hvilket som helst af kravene 1 til 6 sammen med en farmaceutisk acceptabel bærer.

8. Farmaceutisk sammensætning til anvendelse ifølge krav 7, hvor den farmaceutisk acceptable bærer er saltvandsopløsning.

DRAWINGS

Arginase I Synthetic Sequence

GATATACCATGGGTTCTTCTCACCATCATCACCACCACAGCTCTGGCGAGAACCTGTACTTCCAGTCTGC
 GAAGAGCCGTACGATCGGCATTATTGGTGCGCCGTTCTCTAAAGGTCAGCCACGCGGTGGTGTGGAAGAG
 GGTCCGACGGTTCTGCGTAAGGCCGTTTATTAGAAAAGCTGAAAGAGCAGGAGTGGGACGTTAAGGACT
 ACGGTGACTTACCATTGCGGACATCCCGAATGATAGCCGTTCCAAATCGTTAAGAATCCGCGCTCTGT
 GGGTAAAGCAAGCGAGCAGTTAGCAGGTAAGGTGGCCGAGGTCAAGAAAAACGGTCGTATTAGCCTGGTT
 TTAGGCGGTGATCATAGCTTAGCAATTGGCTCTATCTCTGGTCATGCCCGTGTGCACCCAGATTAGGTG
 TCATTTGGGTTGACGCCCATACGGATATCAATACGCCATTAAACGACCACCAGCGCAATCTGCATGGCCA
 GCCGGTTAGCTTCTTACTGAAGGAGCTGAAGGGTAAAATTCCAGATGTTCCGGGCTTTAGCTGGGTACG
 CCATGTATTTCTGCCAAGGATATCGTGTACATTGGCTTACGTGACGTCGACCCAGGTGAGCACTACATCT
 TAAAGACCCCTGGGTATCAAGTATTTACGATGACGGAAGTGGACCGCTTAGGCATCGGCAAGGTGATGGA
 GGAGACGCTGAGCTATCTGCTGGGCCGTAAAGAACGTCCAATCCATCTGAGCTTCGATGTTGACGGCTTA
 GACCCGAGCTTTACGCCAGCCACCGGCACGCCGGTCTGTTGGTGGTTTAAACGTATCGCGAAGGCCTGTATA
 TCACGGAGGAAATCTATAAGACGGGTTTACTGAGCGGTCTGGACATTATGGAGGTTAATCCAAGCTTAGG
 TAAGACGCCGGAAGAAGTTACCCGTACCGTTAACACGGCGGTGCGGATCACGTTAGCATGTTTCGGTTTA
 GCGCGAGGGCAACCATAAACCAATTGATTATCTGAATCCACCGAAGTGAGGATCCGAATTCG

Arginase II Synthetic Sequence

GATATACCATGGGCAGCAGCCATCATCACCACCATCACAGCTCTGGTGAAAACTTATACTTCCAAAGCGT
 CCATAGCGTCGCGAGTGATTGGTGCCCCGTTTAGCCAAAGGTCAAAAACGCAAGGGTGTGAACATGGTCCG
 GCAGCGATCCGCGAAGCAGGTTTAAATGAAGCGTTTAAAGCAGCTTAGGCTGTCACTTAAAGGATTTCCGCTG
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 CTCTGAGGAGGAAGCGAAGACGACCGCCAACCTGGCGGTGGACGTCATCGCCTCTTCTTTCCGCCAGACG
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 CACGTGTCCGTATTTGAGGATCCGAATTCG

FIG. 1

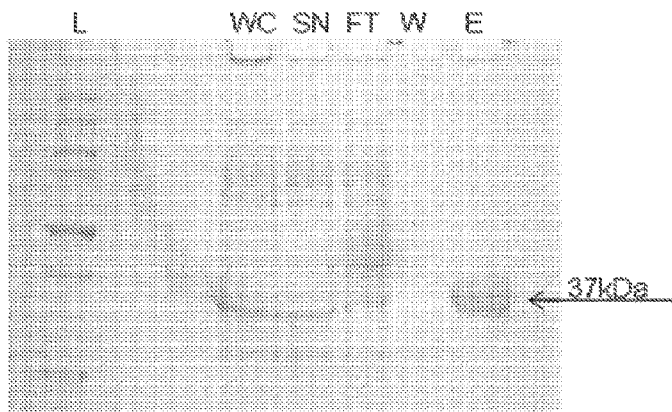


FIG. 2

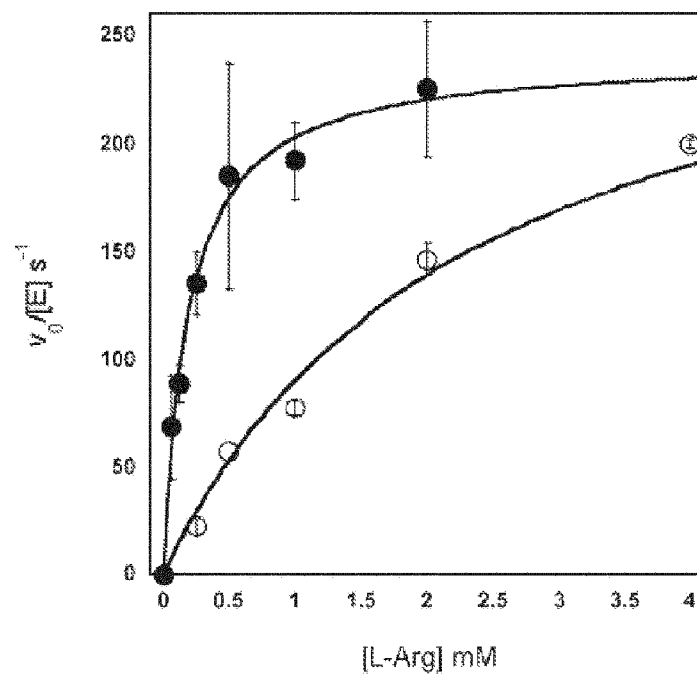


FIG. 3

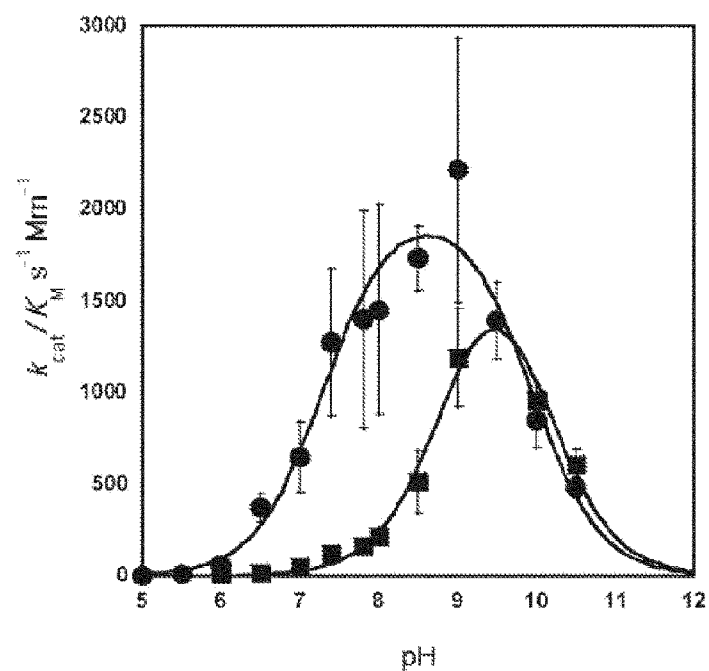


FIG. 4

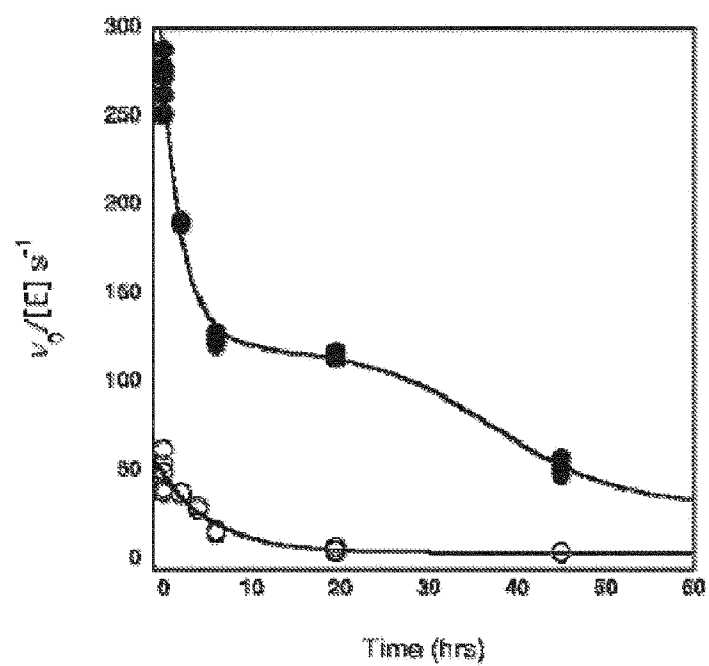
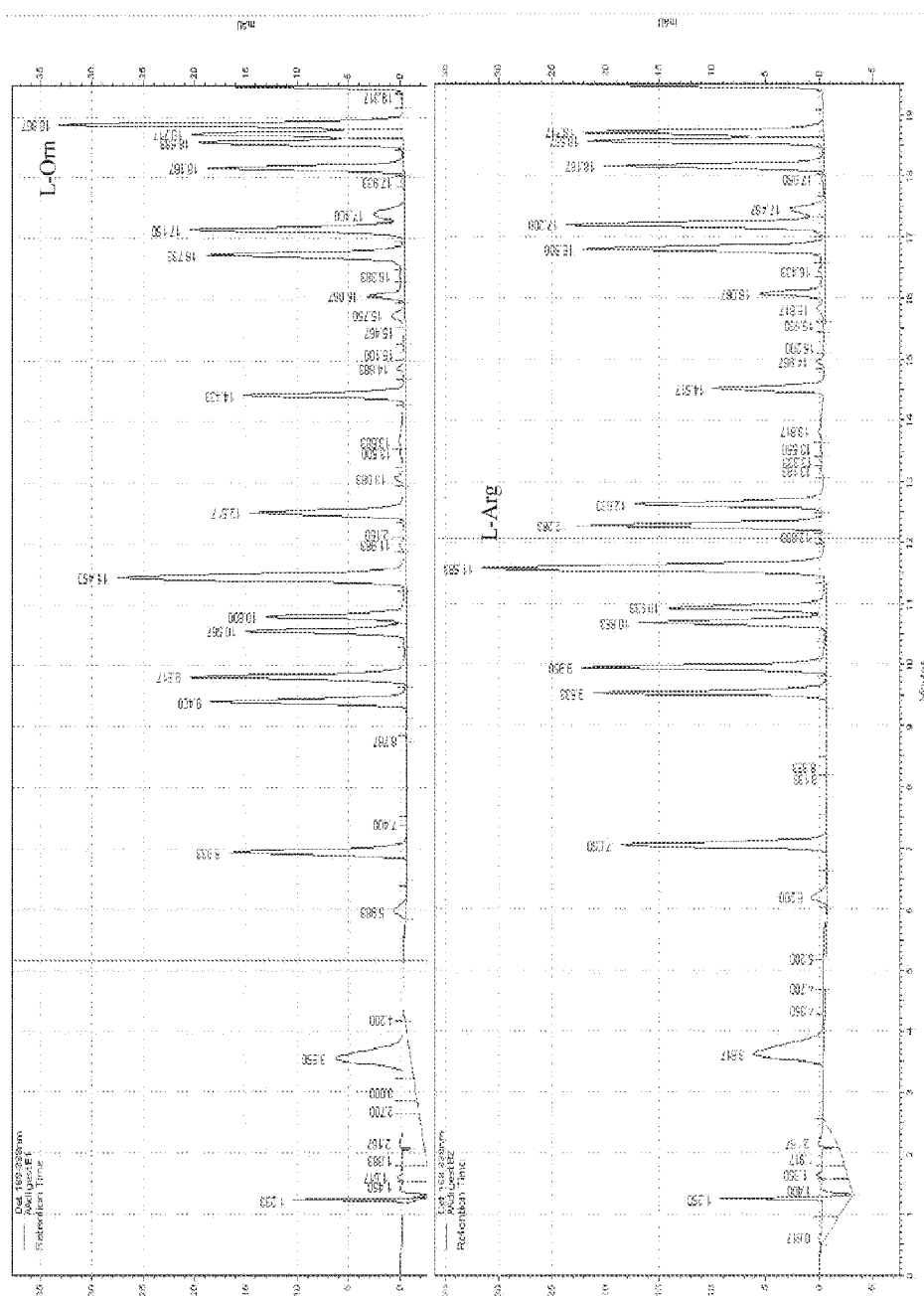


FIG. 5



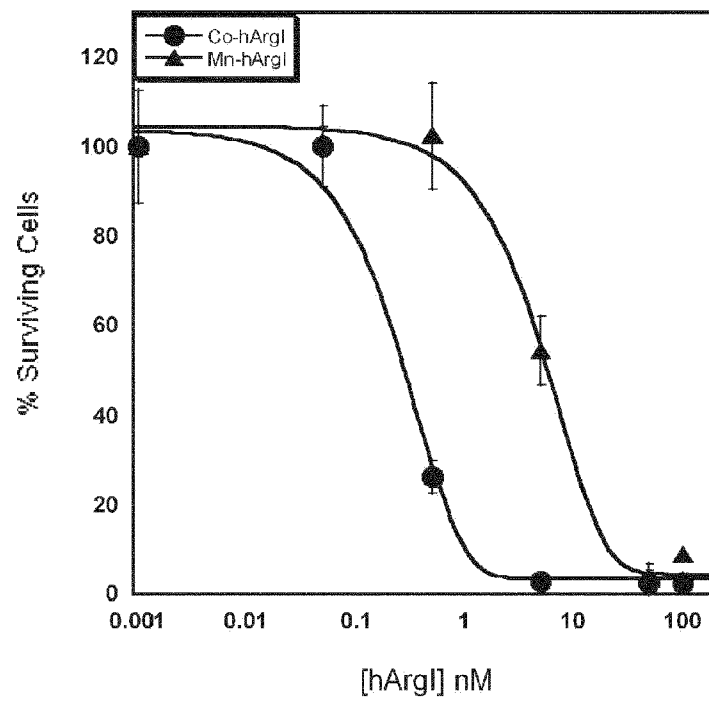


FIG. 7A

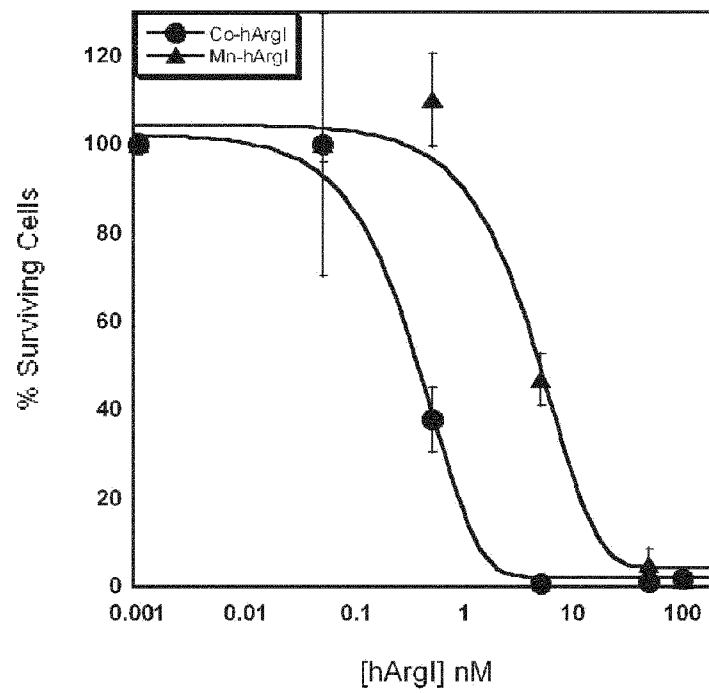


FIG. 7B

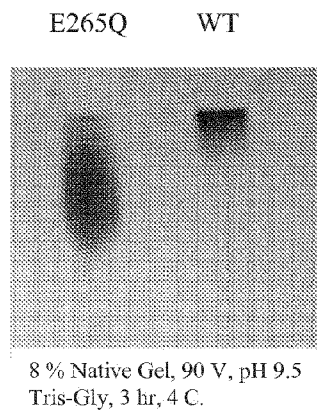


FIG. 8

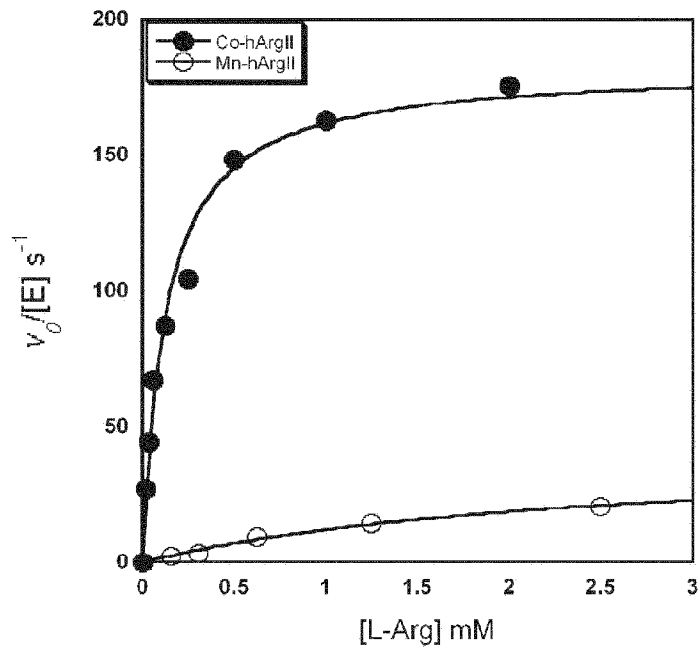


FIG. 9

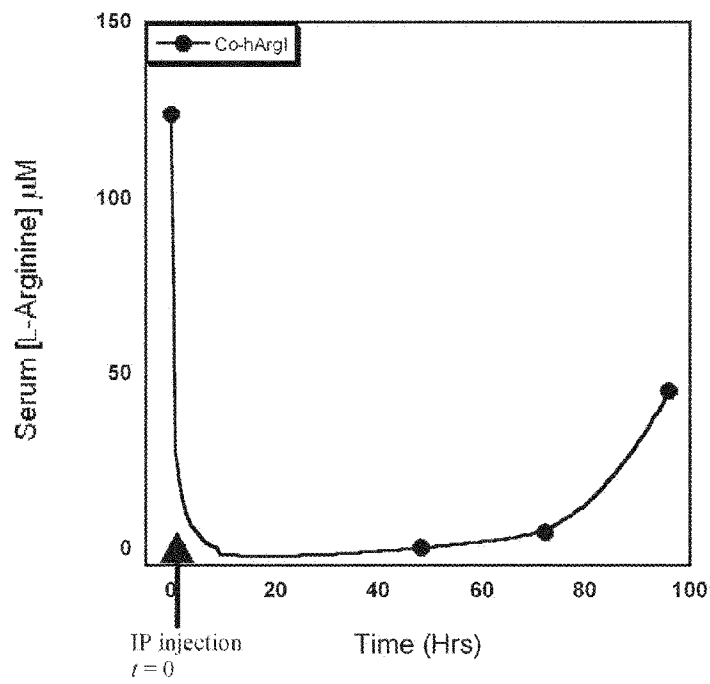


FIG. 10

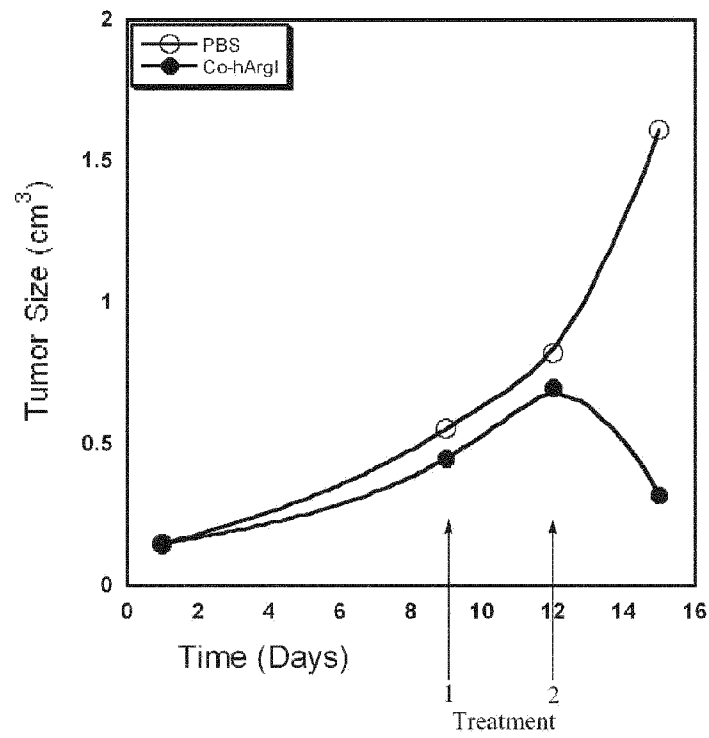


FIG. 11

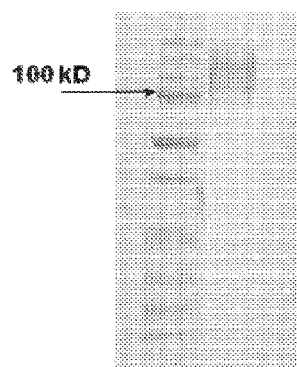


FIG. 12

SEKVENSLISTE

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