UNITED STATES PATENT

Leung et al.

SITE-DIRECTED PEGYLATION OF ARGINASES AND THE USE THEREOF AS ANTI-CANCER AND ANTI-VIRAL AGENTS

Inventors: Yun Chung Leung, Hong Kong (CN); Wai-hung Lo, Hong Kong (CN)

Assignee: The Hong Kong Polytechnic University, Hunghom, Kowloon (HK)

Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 572 days.

Appl. No.: 12/732,188
Filed: Mar. 26, 2010
Prior Publication Data

Provisional application No. 61/163,863, filed on Mar. 26, 2009.

Int. Cl. C12N 9/78 (2006.01) C07K 14/00 (2006.01) C12P 21/00 (2006.01)

U.S. Cl. USPC .......... 435/227; 435/69.1; 530/350; 930/240; 930/320

Field of Classification Search USPC ................. 435/227; 530/350; 930/240, 320
See application file for complete search history.

References Cited
U.S. PATENT DOCUMENTS
5,766,897  A  6/1998 Braxton

FOREIGN PATENT DOCUMENTS
WO WO-03/063780 * 8/2003
WO WO-2011006895 A2 1/2011

OTHER PUBLICATIONS


Primary Examiner — Delia Ramirez
Attorney, Agent, or Firm — Ella Cheong Hong Kong; Sam T. Yip

ABSTRACT

Mono-pegylated arginase conjugate and method producing thereof. The mono-pegylated arginase is homogeneous in molecular weight and shows therapeutic effect for treating cancers and viral infections. The method of producing such arginase conjugate has a main step of genetically modifying the gene encoding an arginase so that the PEG moiety can attach to the enzyme at a predetermined, specific intended site. This is achieved by removing the PEG attaching amino acid residues at undesirable sites while keeping (or adding, if necessary) the one at the desirable site of the enzyme. Two exemplary mono-pegylated arginase conjugates so produced are human arginase 1 (HAI) where a polyethylene glycol (PEG) moiety is site-specific covalently bonded to Cys55 of the enzyme and Bacillus caldovelox arginase (BCA) where a polyethylene glycol (PEG) moiety is site-specific covalently bonded to Cys215 of the enzyme.

10 Claims, 30 Drawing Sheets
FIG. 1A

ATGAGCCCAAGTCGCAAGGACATATGGAATTATTGGAGCTCTTTTCTTAAAGGAGCACAGCA

CGAGGGGGGGGAGAAAGGCCTACAGTTATTTGGAGAATAGGCTGTGCTCTTGAAGAGCTT

AAAGAACAGAGCTGTATGGAGAGATTTCTGGGAAGCTGCTGCTGATCCTCATTAA

GACGTCCTCCTTTGAGAATTCGAGAACGTTGTCGCTGAGTTTACCTTGAGCTTTGAGA

GCTGGCAAGGGCAGAAATAGCATGAGAAGACGAGGCATCGCTGAGGCTGAGGAGAC

CAGAGTTTATGGAATGGGAGCATCTGTCTGCTGAGCTGAGTTTTGAGGAGAC

ACTGGGGTGGATTTTCTCAGACTATATCAGATTATATGGGAGCTGAGGCTGAGGAGAC

GAGGGGGGGGAGAAAGGGCTGCTGCTGCTGCTGAGTTTACCTTGAGCTTTGAGA

FIG. 1b

ATGAGCCCAAGTCGCAAGGACATATGGAATTATTGGAGCTCTTTTCTTAAAGGAGCACAGCA

CGAGGGGGGGGAGAAAGGCCTACAGTTATTTGGAGAATAGGCTGTGCTCTTGAAGAGCTT

AAAGAACAGAGCTGTATGGAGAGATTTCTGGGAAGCTGCTGCTGATCCTCATTAA

GACGTCCTCCTTTGAGAATTCGAGAACGTTGTCGCTGAGTTTACCTTGAGCTTTGAGA

GCTGGCAAGGGCAGAAATAGCATGAGAAGACGAGGCATCGCTGAGGCTGAGGAGAC

CAGAGTTTATGGAATGGGAGCATCTGTCTGCTGAGCTGAGTTTTGAGGAGAC

ACTGGGGTGGATTTTCTCAGACTATATCAGATTATATGGGAGCTGAGGCTGAGGAGAC

GAGGGGGGGGAGAAAGGGCTGCTGCTGCTGCTGAGTTTACCTTGAGCTTTGAGA

CCTAAAGTAA 969
FIG. 1c

ATGAAGCCAAATTTCAATTATCGGGTTCCGATGGATTTAGGGCAGACACGG 50
CCGGCGCTTGATATGCGGCAGCGCAATCGGTATAGCGGCTAGCTCG 100
AACGCTCTGGAGCTTCTTCTATAGCTTATTTTGAGATATGACCCGCA 150
ATTGGAAGAGCGAGCGGTTGCGAGAAGGAGATTGCTAGGTCGGCGCAG 200
TTTGAAGCCCCTGTTGCGGAAGCAGGAAATGTTATGCGGCGCTCACC 250
AGTCGGTCAGCAGGCGAGTTCTCCCGTGTGTTGCGGCGGACCAAGAG 300
ATGCCCAATTCGGCACTCGGCCGGGTTGCGGAAAAATTATAGCGGCTTGG 350
AGGTGACCTCGGGCTAGCAGGCGAGATTGCTATTGAATCTACCCGGA 400
CGTCCGGAAACTTCTAGGCAATGCGGCTTCGCGGCAGCTCCGGTGTGGCC 450
CATCCTGCGGCTGCGCAAATGCGGCAATCGGCGGCTACGCCGAAATACGCCGGA 500
ACATGTCGCTGTTGATAAGCGGTCCGATCTGCTCCTTGTAGGAGAGGAGAAAG 550
TTATGGCAGCGGTGATGAGTTATGAGGTTGAGTGGAGGAAACCAAGTCATGTA 600
CTGGAATAGCAGCGGCTGATGAGGGAGAACAGATAGCCCTTTTAAAGAAGAG 650
AACGGAAGGGGCTCTCCATTTTGTCGATTAGGATGCGCTCGTACCACCGG 700
ACGCCACGCGGATGTCGAGGCGATTCCGTGTTGAGTGGCCCGGCCAAGCAGT 750
AGCCATTTGGCTGAGATGCTGCGGAGCGCAGGACAATCACTCATCJGCAG 800
GGAAATTTGCTAGGAATTGCCAGCTTGGGATAGCGCCGCGGGCCAAACACACGAGT 850
CAGTGCGGTAGCGCAGTGGTGCTGTTTGGTGGGAGAAAAACTCATGTAAG 900

FIG. 1d

ATGAAGCCAAATTTCAATTATCGGGTTCCGATGGATTTAGGGCAGACACGG 50
CCGGCGCTTGATATGCGGCAGCGCAATCGGTATAGCGGCTAGCTCG 100
AACGCTCTGGAGCTTCTTCTATAGCTTATTTTGAGATATGACCCGCA 150
ATTGGAAGAGCGAGCGGTTGCGAGAAGGAGATTGCTAGGTCGGCGCAG 200
TTTGAAGCCCCTGTTGCGGAAGCAGGAAATGTTATGCGGCGCTCACC 250
AGTCGGTCAGCAGGCGAGTTCTCCCGTGTGTTGCGGCGGACCAAGAG 300
ATGCCCAATTCGGCACTCGGCCGGGTTGCGGAAAAATTATAGCGGCTTGG 350
AGGTGACCTCGGGCTAGCAGGCGAGATTGCTATTGAATCTACCCGGA 400
CGTCCGGAAACTTCTAGGCAATGCGGCTTCGCGGCAGCTCCGGTGTGGCC 450
CATCCTGCGGCTGCGCAAATGCGGCAATCGGCGGCTACGCCGAAATACGCCGGA 500
ACATGTCGCTGTTGATAAGCGGTCCGATCTGCTCCTTGTAGGAGAGGAGAAAG 550
TTATGGCAGCGGTGATGAGTTATGAGGTTGAGTGGAGGAAACCAAGTCATGTA 600
CTGGAATAGCAGCGGCTGATGAGGGAGAACAGATAGCCCTTTTAAAGAAGAG 650
AACGGAAGGGGCTCTCCATTTTGTCGATTAGGATGCGCTCGTACCACCGG 700
ACGCCACGCGGATGTCGAGGCGATTCCGTGTTGAGTGGCCCGGCCAAGCAGT 750
AGCCATTTGGCTGAGATGCTGCGGAGCGCAGGACAATCACTCATCJGCAG 800
GGAAATTTGCTAGGAATTGCCAGCTTGGGATAGCGCCGCGGGCCAAACACACGAGT 850
CAGTGCGGTAGCGCAGTGGTGCTGTTTGGTGGGAGAAAAACTCATGTAAG 900
CACCATCACCACATCACA 918
FIG. 3b

atgcatactccatatcatcacatcagtgacgccagtcctcgaagacctagattttagtgagct
MHHHHHHHHHMSAKSRTIGIGA
cttttcctcaaggacgccacgaggaggggtgagaagaggccctacagttattgagaag
FSKKGQPRGGVEEGPTVLRK
gctgtctctgtcttgaagaaaccttaaaaccaaaagagatgtgatgatgatgagatatttgaggacctt
AGLLEKLEKQECDEVDKVDYGDL
cctttgtgtcagactctccactaaatgcacagtccctctttcctcaattgtgagaagataccaaacaggtcttg
PFADIPNDSFPOIVKKNPRS
egaagacacacacacagcagctgctggcaggaaggtgcaagaagtgcacaagaagacgggaagatc
GKASEQLAGKVAEVEKKNRGRI
ggctgggtgtctggcgcgagaccaacagtttgcaatttgggagcatctcttgtgaccatgcccaggg
SLVGLGNDIALSGISHGAR
gtccaccctgtatctttgaggctcatactgtacataacacactccactgt
VHPDLGVWIVWDHTDINTPL
acacaccacacagtggacacactgtatcttttcctccagtgaagagactaaat
TTSSGNNLHGPVSFLLKKELK
egaagatcctGGatgtgccagatattctctttggtgactccctctatatctctggccagatg
GKIPDPVGFSWVTSPISAKD
attgtatatggtcttggagagaagtggcccttgggaaacactacattttgaaactcta
IVYIGLRDVDPGEHYILKTL
ggcattaaatcttttcaatgtacgagcatggaagatggcagacatggaatgcaaggtgctggaa
GIKYFSTMTEVDRLGIKVKME
gaaacactcagtcataactatggaagaagaagacccactctacatattttgtgatgtt
ETLSYLLGRRKRPRITHLPSF
gcagggactgagccaccttttcaacacacagctactggcacaacacagctcttgaggctgaca
DGLDPSTFPATGTPVPVGGLT
tacaagagaaggtcttcataacacgaagaatatcaaaaaacagggctactcctcaggatta
YREGLYITEEIKTGLLSGL
atatgataaggaagatgacccacctcttgggagagacaccaagagaatgatataccacagtt
DIMEVNSLGBKPEEVTRTV
acacacagctgtcaataacatgtgcctttggagacacacttgctcgaggggtataattcacaag
NTAVAITLSFGLAREGNHK
ctattgacatccctaaacccacactaagtt
PITYLNNPPK-
FIG. 3d

atgaagcccaatcataattattacggttcgatggatatcgctagcagaacacacggcggttt
MKPFISIGVPMPLGQTRRGT
qatagggcagcagcaatgctttcgcacgtctgaaacgtttcctcat
DMGPJAMRYAGVIERLRLH
tacgatattgaagattttgagatattccgatgtaggaaagcagagacggttgcacgagcaaa
YDIEDLGIDIFIGXKAERLRLHEQ
ggagattcacgcgtgaaatgaaaggttggagaaagcagagcsaaactttgcggggg
GDSRLRNLNKLAAVASANEKLAAGCGTTGACCAATGCTGTCCAGCGGCGGATTTCGCTTGTTGGCGGCGACCATAGAC
AVDQVARGLPVLGVDGDHSAATGCACTTGCAAGCCTGCCTGGTGGGAGACATTAGACGCGGGTGGAGATGATCTGG
IAIGTLAGYKHYERLGLVIWATGACCGCGCTAGCGACGTCAAACCGCGGAAAGTGGCCTCGCTGGGAAACATTCAATGCG
YDAHGDVNTAETTSPSNINGHATGCCTGCGCGGCGCCCTCGGGTTGGCCATCGGCCGTGACACAAATCGCGGATAC
MPLAAALGFGHPALTTQIGGYTCGCCCAAAATCAAGCGGACATGCTGCTGATCGCTGCGCCGGCTCCCTGGTATGAGGGCG
CPKIKPEHLYGVSLDEEGGAGAAGAGTTATTCGCGGAAAGGAGATCAAACATGAGTCTGGTTGATCGG
EKKPIREKGIKITMHVEVRDCTCGGAATGGAAGATGAGCAGAAGATCAGATATTAAAGAACAAAGATGCGC
LMGETMVEMEITAYLRKERTDGTTCAATGCGCTTGAATGGGAGCTTGGAGGACCAAGCGCGACGGCGAGTCGGAACG
VHSLLDLDGLDPSDAPGCGTCCTGCAATGAGTACGACGGCAAGCAGACTTTGCGGAGATGCGCTGGCAG
PVIGGLTYRESHLAMENLAGECACAATCATTCTCGAGGAAATTGCTGAAAGCAGATCTTGAGTGAACGGGAAAC
AQIITSAEFVEVNPILDERNAAACACGACTACGATGCTGCTGCTGCGAGTTGTTGTCGAGTAATCTGATAGAT
KTASVAVALMGSSLFGKEKLMMH

\[\text{caccatcaccatcaac} \]

\[\text{HHHHH} \]

6xHis-tag encoding codons
FIG. 5a

\[
\text{mPEG-maleimide (Sunbright), } \quad \text{Mw=20 kDa}
\]

\[
\text{6xHis-tagged human arginase I}
\]

\[
\text{Reduction (using TCEP), at pH 7.4}
\]

PEG: protein ratio (PEG: Human arginase I) = 20:1

Native

FIG. 5b

\[
\text{mPEG-maleimide (Sunbright), } \quad \text{Mw=20 kDa}
\]

\[
\text{6xHis-tagged Bacillus caldovelox arginase}
\]

\[
\text{Reduction (using TCEP), at pH 7.4}
\]

PEG: protein ratio (PEG: Bacillus caldovelox arginase) = 20:1

PEGylation, overnight at 4 °C with stirring
FIG. 6b1

History Plot

BC-arg_2xTY_O2 Selection : 9/30/2008 9:06:45 AM - 9/30/2008 7:36:42 PM

- pH.Value,Db 0.20 pH
- PO2.Value,Db 2.0 %Sat
- STIRR.Value,Db 10 rpm
- TEMP.Value,Db 0.50 Degr C

TEMP
Degr C

STIRR
rpm

PO2
%Sat

pH

Time / hours

pH

Degr C

rpm

%Sat
FIG. 6d

6xHis-tagged Bacillus arginase mutant
FIG. 7a

<table>
<thead>
<tr>
<th>Lane</th>
<th>Description</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Low-range protein marker, Bio-Rad</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Before chelating FF sepharose column (2.5 μL)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Flowthrough (2.5 μL)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Fraction A8 (10 μL)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Fraction C2 (10 μL)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Fraction C5 (10 μL)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Fraction C8 (10 μL)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Fraction C11 (10 μL)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Fraction D11 (10 μL)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Fraction D7 (10 μL)</td>
<td></td>
</tr>
</tbody>
</table>

6xHis-tagged human arginase I mutant
FIG. 7b

<table>
<thead>
<tr>
<th>Lane</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lane 1</td>
<td>Low-range marker, Bio-Rad</td>
</tr>
<tr>
<td>Lane 2</td>
<td>Before chelating FF sepharose column (2.5 μL)</td>
</tr>
<tr>
<td>Lane 3</td>
<td>Flowthrough (5 μL)</td>
</tr>
<tr>
<td>Lane 4</td>
<td>Fraction C1 (10 μL)</td>
</tr>
<tr>
<td>Lane 5</td>
<td>Fraction E7 (10 μL)</td>
</tr>
<tr>
<td>Lane 6</td>
<td>Fraction F7 (10 μL)</td>
</tr>
<tr>
<td>Lane 7</td>
<td>Fraction F6 (10 μL)</td>
</tr>
<tr>
<td>Lane 8</td>
<td>Fraction F3 (10 μL)</td>
</tr>
<tr>
<td>Lane 9</td>
<td>Fraction G2 (10 μL)</td>
</tr>
<tr>
<td>Lane 10</td>
<td>Fraction G5 (10 μL)</td>
</tr>
</tbody>
</table>

6xHis-tagged *Bacillus* caldovelox aginase mutant
FIG. 8a

<table>
<thead>
<tr>
<th>Lane 1: Low-range marker, Bio-Rad</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lane 2: Unpegylated human arginase I</td>
</tr>
<tr>
<td>Lane 3: Cys(^{45}) pegylated human arginase I</td>
</tr>
</tbody>
</table>

FIG. 8b

<table>
<thead>
<tr>
<th>Lane 1: Low-range marker, Bio-Rad</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lane 2: Unpegylated \textit{Bacillus} caldovelox arginase</td>
</tr>
<tr>
<td>Lane 3: Cys(^{161}) pegylated \textit{Bacillus} caldovelox arginase</td>
</tr>
</tbody>
</table>
FIG. 12d
FIG. 12e
Fig. 15 Cytotoxicity of Cys$$^ {\text{PEG}}$$ pegylated human arginase I (HAI-PEG20)

![Graph showing cell viability vs. concentration of pegylated human arginase I(Cys$$^ {\text{PEG}}$$).]

Fig. 16

![Graph showing substrate concentration (L-Arg) vs. reaction velocity (V).]
SITE-DIRECTED PEGYLATION OF ARGINASES AND THE USE THEREOF AS ANTI-CANCER AND ANTI-VIRAL AGENTS

CROSS REFERENCE

This application claims benefit from U.S. Provisional Patent Application No. 61/163,863, filed Mar. 26, 2009, the content of which is incorporated herewith in its entirety by reference.

FIELD OF THE INVENTION

The present invention relates to the modification of an arginase for the purpose of increasing the enzyme’s serum or circulating half-life and improving its pharmacokinetic properties, in vivo biological activity, stability, and reducing the immune reaction (immunogenicity) to the enzyme in vivo. More specifically, the invention relates to the site-specific covalent conjugation of monomethylpolylene glycol to the arginase through genetically modifying the gene encoding the enzyme to produce mono- and site-specific, pegylated arginase, which become effective means of a number of arginine-dependent diseases, such as, for example, various cancers and human immunodeficiency virus (HIV) infection.

BACKGROUND OF THE INVENTION

Arginase

Arginase is a manganese metalloenzyme containing a metal-activated hydroxide ion, a critical nucleophile in metalloenzymes that catalyze hydrolysis or hydration reactions. Arginase converts naturally occurring arginine into ornithine and urea. The enzyme exists in many living organisms, including bacteria and humans (Jenkinson et al., 1996, Comp Biochem Physiol B Biochem Mol Biol, 114:107-32). PEGylation of Arginase

Arginase may be used as therapeutic agent and administered parenterally for various indications. However, parenterally administered arginase, which is a protein, may be immunogenic and have a short pharmacological half-life. Consequently, it can be difficult to achieve therapeutically useful blood levels of the proteins in patients. These problems may be overcome by conjugating the proteins to polymers such as polyethylene glycol (PEG).

Covalent attachment of the inert, non-toxic, biodegradable polymer PEG, to molecules has important applications in biotechnology and medicine. PEGylation of biologically and pharmaceutically active proteins has been reported to improve pharmacokinetics, resulting in sustained duration, improve safety (e.g., lower toxicity, immunogenicity and antigenicity), increase efficacy, decrease dosing frequency, improve drug solubility and stability, reduce proteolysis, and facilitate controlled drug release (Roberts et al., 2002, Adv Drug Deliv Rev, 54:459-76; Harris & Chess, 2003, Nat Rev Drug Discov, 2:214-221).

PEG-protein conjugates produced by conventional methods in the art contain heterogeneous species, each being attached with a variable number of PEG molecules, ranging from zero to the number of amino groups that the protein has. Even for species that has the same number of PEG molecule attached, the site of attachment on the protein may vary from species to species. Such non-specific pegylation, however, can result in conjugates that are partially or virtually inactive. Reduction of activity may be caused by shielding the protein’s active receptor binding domain when the PEG is attached at an improper site. Thus, there is a clear need for a better way of producing homogeneously pegylated protein molecules which retain the activity of the parent protein and making possible the administration of correct and consistent dosages necessary for clinical uses.

Cancer Treatment Via Amino Acid Deprivation

Amino acid deprivation therapy is an effective means for the treatment of some cancers. Although normal cells do not require arginine, many cancer cell lines are auxotrophic for this amino acid. Many lines of evidence have shown that in vitro arginine depletion, either with an arginine-degrading enzyme or using arginine-deficient medium, leads to rapid destruction of a wide range of cancer cells (Scott et al., 2000, Br J Cancer, 83:800-10). But direct use of enzymes, which are proteins, has problems of immunogenicity, antigenicity and short circulating half-life.

Inhibition of Virus by Arginine Deprivation

Viral infections are among the leading causes of death with millions of deaths each year being directly attributable to several viruses including hepatitis and human immunodeficiency virus (HIV). However, there are several problems with current anti-viral therapies. First, there are relatively few effective antiviral drugs. Many of the existing anti-virals cause adverse or undesirable side-effects. Most effective therapies (such as vaccination) are highly specific for only a single strain of virus. Frequently the virus undergoes mutation such that it becomes resistant to either the drug or vaccine. There is a need for methods for inhibiting viral replication which do not have the problems associated with the prior art.

Many studies over the last 30 years have demonstrated that extracellular arginine is required for viral replication in vitro. Historically, this has been accomplished by making tissue culture media deficient in arginine and dialyzing the serum used as a supplement in order to achieve arginine free medium. Using this methodology to achieve arginine deprivation results in inhibition of replication of a large number of diverse families of viruses including: adenovirus (Rouse et al., 1963, Virology, 20:357-365), herpes virus (Tankersley, 1964, J Bacteriol, 87:609-13). Human Immunodeficiency Virus (HIV)

Acquired immune deficiency syndrome (AIDS) is a fatal disease, reported cases of which have increased dramatically within the past several years. The AIDS virus was first identified in 1983. It has been known by several names and acronyms. It is the third known T-lymphotropic virus (HTLV-III), and it has the capacity to replicate within cells of the immune system, causing profound cell destruction. The AIDS virus is a retrovirus, a virus that uses reverse transcriptase during replication. Two distinct families of HIV have been described to date, namely HIV-1 and HIV-2. The acronym “HIV” is used herein to refer to human immunodeficiency viruses generically. HIV replication is believed to be arginine-dependent, depletion of which would thus inhibit HIV replication.

SUMMARY OF THE INVENTION

One object of the present invention is to provide novel PEG-arginase conjugates substantially homogeneous and having a PEG moiety covalently bound to a specific site at the arginase molecule. Two preferred embodiments of the present invention are Cys55-human arginase 1 (HAI) and Cys164-Bacillus caldovelos arginase (BCA).

Another object of the present invention is to provide a method of producing site-directed, mono-pegylated arginase conjugates, which have potent anti-cancer and anti-viral effects. One particular embodiment of the present invention comprises three general steps. The first step is a genetically
modification of a gene encoding for an arginase so that the resulting arginase will have a single free cysteine residue at a given position. The second step is expressing the modified gene in a chosen system to produce desired arginase. The expressing system may be human cells or issues, or other organisms including, for example, a bacterial cell, a fungal cell, a plant cell, an animal cell, an insect cell, a yeast cell, or a transgenic animal. The third step is conjugation between the free cysteine residue of the arginase and a maleimide group (MAL) of PEG compound, resulting in a covalent bond between the PEG compound and the free cysteine of the arginase.

Another object of the present invention is to provide a method of treating viral infection via arginase depletion. This treating method employs homogeneous monopegylated arginase to inhibit viruses' replication.

Another object of the present invention is to provide a method of anti-human immunodeficiency virus (HIV). This method employs homogeneous monopegylated arginase to inhibit HIV’s replication.

Another object of the present invention is to provide a method of enhancing arginase’s enzymatic activity by replacing the valine at position 20 of Bacillus caldovelox arginase (or the corresponding position in HAI and other arginases) with another amino acid residue, for example, proline.

Still another object of the present invention is to provide a method of enhancing arginase’s enzymatic activity, which is accomplished by replacing the native metal cofactor manganese with cobalt.

The various features of novelty which characterize the invention are pointed out with particularity in the claims annexed to and forming a part of this disclosure. For a better understanding of the invention, its operating advantages, and specific objects attained by its use, reference should be made to the drawings and the following description in which there are illustrated and described preferred embodiments of the invention.

**BRIEF DESCRIPTION OF THE DRAWINGS**

FIG. 1 shows the nucleotide sequence of human arginase 1 (a) (SEQ ID No: 1), its mutated nucleotide sequence designed for site-directed pegylation (b) (SEQ ID No: 2) according to the present invention, the nucleotide sequence of *Bacillus caldovelox* arginase (c) (SEQ ID No: 3), and its mutated nucleotide sequence designed for site-directed pegylation (d) (SEQ ID No: 4) according to the present invention.

FIG. 2 shows the amino acid sequence of human arginase 1 (a) (SEQ ID No: 5), its modified amino acid sequence designed for Cys 65 site-directed pegylation (b) (SEQ ID No: 6) according to the present invention, the amino acid sequence of *Bacillus caldovelox* arginase (c) (SEQ ID No: 7), and its modified amino acid sequence designed for Cys 65 site-directed pegylation (d) (SEQ ID No: 8) according to the present invention.

FIG. 3 shows the nucleotide and amino acid sequences of the human arginase 1 mutant (C168S/C308S) designed for Cys 65 site-directed pegylation (a) (SEQ ID No: 9 and 10), the alignment of the nucleotide and amino acid sequences of the 6xHis-tagged human arginase 1 mutant (C168S/C308S) designed for Cys 65 site-directed pegylation (b) (SEQ ID No: 11 and 12), the nucleotide and amino acid sequences of the *Bacillus caldovelox* arginase mutant (S161C) designed for Cys 65 site-directed pegylation (c) (SEQ ID No: 13 and 14), and the alignment of the nucleotide and amino acid sequences of the 6xHis-tagged *Bacillus caldovelox* arginase mutant (S161C) designed for Cys 65 site-directed pegylation (d) (SEQ ID No: 15 and 16).

FIG. 4 shows (a) the crystal structure of the wild-type human arginase 1 (downloaded from NCBI website using CN3D 4.1 software), showing that Cys 6 is far away from the active site; (b) the crystal structure of the wild-type *Bacillus caldovelox* arginase, showing that Ser 65 is far away from the active site.

FIG. 5 shows (a) the conjugation procedures for Cys 6-specific mono-pegylation of the 6xHis-tagged human arginase 1 mutant with a single chain mPEG-maleimide (20 kDa), showing that the double bond of a maleimide undergoes an allylation reaction with a sulfhydryl group to form a stable thioether bond, and (b) the corresponding procedures for Cys 6-specific mono-pegylation of the 6xHis-tagged *Bacillus caldovelox* arginase mutant.

FIG. 6 depicts the time-course for fermentation in a 2-liter fermenter by the *E. coli* BL21-DE3 containing the arginase gene, showing the results obtained from the batch fermentation (a1) and the results obtained from the fed-batch fermentation (a2); the history plots of the batch fermentation (b1) and the fed-batch fermentation (b2), showing the changes of pH, dissolved oxygen values; the elution profile of the 6xHis-tagged human arginase 1 mutant from a chelating FF sepharose column (c) with the first peak being protein impurities and the second peak being the purified human arginase 1; and the elution profile of the 6xHis-tagged *Bacillus caldovelox* arginase mutant from a chelating FF sepharose column (d) with the first peak being the protein impurities and the second peak being the purified *Bacillus caldovelox* arginase.

FIG. 7 shows the SDS-PAGE analysis of different fractions involving 6xHis-tagged human arginase 1 mutant (a) and 6xHis-tagged *Bacillus caldovelox* arginase mutant (b).

FIG. 8 shows (a) the SDS-PAGE analysis of the unpegylated human arginase 1 mutant and the Cys 65 pegylated human arginase 1 mutant (Lane 1: protein molecular weight marker, Lane 2: unpegylated human arginase 1 mutant, and Lane 3: Cys 65 pegylated human arginase 1 (HAI-PEG20)); (b) the SDS-PAGE analysis of unpegylated *Bacillus caldovelox* arginase mutant and the Cys 65 pegylated *Bacillus caldovelox* arginase (Lane 1: protein molecular weight marker, Lane 2: the unpegylated *Bacillus caldovelox* arginase mutant; and Lane 3: Cys 65 pegylated *Bacillus caldovelox* arginase (BCA-PEG20)).

FIG. 9 shows (a) the pharmacokinetic profiles of a single dose of non-pegylated and Cys 65 pegylated human arginase 1 (HAI-PEG20) injected intraperitoneally in BALB/c mice, and (b) the pharmacokinetic profiles of a single dose of non-pegylated and Cys 65 pegylated *Bacillus caldovelox* arginase (BCA-PEG20) injected intraperitoneally in BALB/c mice.

FIG. 10 shows (a) the pharmacodynamic profile of a single dose of Cys 65 pegylated human arginase 1 (HAI-PEG20) injected intraperitoneally in BALB/c mice up to Day 14, and (b) the pharmacodynamic profile of a single dose of Cys 65 pegylated *Bacillus caldovelox* arginase (BCA-PEG20) injected intraperitoneally in BALB/c mice up to Day 14.

FIG. 11 shows the average body weight (g) (n=5) of BALB/c nude mice xenografted with Hep3B human liver cancer cells injected with different drugs (a); BALB/c nude mice xenografted with MCF-7 human breast cancer cells injected with Cys 65 pegylated *Bacillus caldovelox* arginase (b); BALB/c nude mice xenografted with A549 lung cancer cells injected with different drugs (c) and BALB/c nude mice xenografted with HCT-15 colorectal cancer cells injected with different drugs (d) during the course of the study.
FIG. 12 shows (a) the in vivo activities (efficacies) of non-polyethylated and Cys\textsuperscript{45} polyethylated human arginase I (HAI-PEG20) in BALB/c nude mice implanted with Hep3B human liver tumor cells subcutaneously; (b) the in vivo activities of Cys\textsuperscript{161} polyethylated Bacillus caldovelox arginase (BCA-PEG20) in BALB/c nude mice xenografted with MCF-7 human breast cancer cells subcutaneously; (c) the in vivo efficacies of Cys\textsuperscript{161} polyethylated Bacillus caldovelox arginase in BALB/c nude mice bearing A549 lung cancer xenograft subcutaneously, (d) the in vivo efficacies of Cys\textsuperscript{161} polyethylated Bacillus caldovelox arginase in BALB/c nude mice bearing HCT-15 colorectal cancer xenograft subcutaneously; and (I) the in vivo efficacies of Cys\textsuperscript{15} polyethylated Bacillus caldovelox arginase in BALB/c nude mice bearing HCT-15 colorectal cancer xenograft subcutaneously (data are expressed as mean number of fold increase in tumor volumes e.m.); (e) the in vivo efficacies of Cys\textsuperscript{161} polyethylated Bacillus caldovelox arginase in BALB/c nude mice bearing A549 lung cancer xenograft subcutaneously (data are expressed as mean number of fold increase in tumor volumes e.m.).

FIG. 13 shows an HIV inhibition assay for Cys\textsuperscript{45} polyethylated human arginase I (HAI-PEG20). FIG. 14 shows the HIV inhibition assay for azido-thymidine (AZT).

FIG. 15 shows the cytotoxicity of Cys\textsuperscript{45} polyethylated human arginase I (HAI-PEG20).

FIG. 16 shows a comparison of steady-state kinetics of human arginase I with different metal cofactors, i.e., Mn\textsuperscript{2+} and Co\textsuperscript{2+}.

FIG. 17 shows a comparison of steady-state kinetics of the V20P mutant of Bacillus caldovelox arginase (BCA) and the wild-type BCA substituted with Mn\textsuperscript{2+} (BCAWTMe\textsuperscript{2+}) or Co\textsuperscript{2+}.

FIG. 18 illustrates a hypothesis and working model for cancer cells that are OTC-sensitive.

**DETAILED DESCRIPTION OF PARTICULAR EMBODIMENTS OF THE INVENTION**

Cloning of Human Arginase I Gene (HAI)

The gene sequence of human arginase I is shown in FIG. 1o (SEQ ID No: 1). The gene for 6×His-tagged human arginase I (HAI) was generated by polymerase chain reaction (PCR) from the pAED4/HAII plasmid using the following oligonucleotides to generate an NdeI site at 5'-end and BamHI site at 3'-end. Primer HuAr07-F: 5’-GATATA.CAT.CAT.GAC.CAC.CAT.CAC.CAC.3’ (SEQ ID NO: 17) and Primer HuAr08-R: 5’-AGT.GCG.AGA.TCC.TTA.CTT.AGG.TGG.GT-TA.A.G.GA.TGC.3’ (SEQ ID NO: 18). The PCR product was cut with NdeI and BamHI and subcloned into pET3a expression plasmid vector (Strategene).

The pET3a E. coli expression plasmid vector contains a T7 promoter. The T7 promoter is positioned upstream from the gene 10 leader fragment. The correct sequence was confirmed by DNA sequencing the entire coding region for human arginase I (FIG. 1o). This plasmid is referred to as pET3a/HAI. Cloning of Bacillus caldovelox Arginase Gene (BCA)

The gene sequence of Bacillus caldovelox arginase is shown in FIG. 1o (SEQ ID No: 3). The gene for 6×His-tagged Bacillus caldovelox arginase (BCA) was cut from the pUC57/ BCA plasmid using NdeI and BamHI restriction enzymes. The insert fragment was subcloned into pET3a expression plasmid vector (Strategene).

The correct sequence was confirmed by sequencing the entire coding region for Bacillus caldovelox arginase (FIG. 1o). This plasmid is referred to as pET3a/BCA.

**Mutagenesis of HAI**

The plasmid pET3a/HAI was used as a template for site-directed mutagenesis according to the QuikChange® site-directed mutagenesis kit (Strategene). The codons for Cys\textsuperscript{161} and Cys\textsuperscript{15} residues were mutated to the codons for Ser\textsuperscript{160} and Ser\textsuperscript{15} respectively using the following mutagenic primers (SEQ ID No: 19, 20, 21, and 22, respectively):

- Codon for Cys\textsuperscript{160} mutated to codon for Ser\textsuperscript{160}:
  - Primer HuAr01-F: 5’-GGG.TCA.CTC.CCT.CTA.TAT.CTG.CCA.AGG.3’
  - Primer HuAr02-R: 5’-GCT.TGC.CAG.ATA.TAG.AGG.GAG.TGA.CCC.3’

- Codon for Cys\textsuperscript{15} mutated to codon for Ser\textsuperscript{15}:
  - Primer HuAr03-F: 5’-GCA.ATA.ACC.TTG.GCT.TCT.TGG.AAA.GTA.GCC.ARG.ATT.GC.3’
  - Primer HuAr04-R: 5’-GCA.ACT.CCG.AAA.GRA.GAA.GCC.ARG.ATT.GC.3’

The mutagenized plasmid was transformed firstly into competent E. coli Top 10 cells. The sequence of mutagenized plasmid was confirmed by DNA sequencing. The gene sequence of HAI mutant designed for site-directed mutagenesis is shown in FIG. 1o (SEQ ID No: 2). The mutagenized plasmid was then transformed into E. coli BL21-DE3 cells for protein expression. The amino acid sequence of the wild-type HAI is shown in FIG. 2a (SEQ ID No: 5). The amino acid sequence of the C168S/C303S mutant is shown in FIG. 2b (SEQ ID No: 6, FIG. 3a (SEQ ID No: 10) and FIG. 3b (SEQ ID No: 12). As shown in FIG. 2b, two cysteine residues in human arginase I were replaced by serine residues. These two serine residues are underlined. The only Cys present is Cys45. This mutant is called C168S/C303S, which only contains one single Cys residue (also underlined). Crystal structure of the wild-type HAI is shown in FIG. 4a. Based on this structure, the rational protein design for constructing the C168S/C303S mutant was made. In FIG. 2d, it is shown that one serine residue in Bacillus caldovelox arginase was replaced by cysteine residue. This cysteine residue is underlined. The 6×His-tag region is also underlined and located at the C terminus. This mutant is called S161C.

**Expression and Purification of 6×His-Tagged Arginases**

E. coli BL21-DE3 harboring the plasmid containing a mutated arginase gene encoding 6×His-tagged human arginase I was grown overnight at 37°C in LB medium containing 80 μg/mL ampicillin. The inoculum was diluted 1:25 and grown to OD600-0.8 in a shake flask or the inoculum was diluted 1:10 and grown to OD600~15 in a fermentor. The cells were then induced with 0.4 mM IPTG for 4 hours. The bacterial cells were collected by centrifugation, resuspended in 50 mM Tris, 0.1 M NaCl, 10 mM MεCl\textsubscript{2}, pH 7.4, and disrupted by high pressure homogenization.

The 6×His-tagged human arginase I was purified by a chelating FF sepharose (GE Healthcare) column (5.0 cm x 9 cm; bed volume of 176 mL) equilibrated with Buffer A (0.02 M sodium phosphate, 0.5 M NaCl, pH 7.4). The 6×His-tagged arginase were eluted with a gradient of 0.15 to 0.25 M imidazole (FIG. 6a & FIG. 6b). The flow rate was 20 mL/min. The fractions (FIG. 7a & FIG. 7b) containing purified arginase were collected. The yields of purified arginase were about 280 mg/L cell cultures.

The exact procedure as described above for 6×His-tagged human arginase I was repeated to obtain purified 6×His-tagged Bacillus caldovelox arginase.
Site-Directed Pegylation of 6xHis-Tagged Arginases

FIG. 5a shows the procedures for conjugating Cys\(^{33}\)-specific mono-pegylation of the 6xHis-tagged human arginase I mutant with a single chain mPEG-maleimide (20 kDa), referred to as "HAI-PEG20". The double bond of a maleimide undergoes an alkylination reaction with a sulphydryl group to form a stable thioether bond. FIG. 5b shows the conjugation procedures for Cys\(^{33}\)-specific mono-pegylation of the 6xHis-tagged *Bacillus caldovelox* arginase mutant with a single chain mPEG-maleimide (20 kDa), referred to as "BCA-PEG20". One gram of 6xHis-tagged arginase was disinfected to 0.02 M sodium phosphate, 0.5 M NaCl, pH 7.4, using Millipore Tangential Flow Filtration system (500 mL) with 10 K (cut-off) membrane (Millipore). The concentration of arginase was finally diluted to ~2 mg/mL. The reducing agent Tris-(2-carboxyethyl)phosphine, TCEP, was added in a molar excess of 10 moles to one mole of arginase for reduction and the solution was gently stirred for 4 hours at room temperature. mPEG-Maleimide or mPEG-MAL (20 kDa) (Sunbright) in a molar excess of 20 moles to one mole of arginase was added to the reduced arginase and stirred for overnight at 4 °C. The progress of site-directed pegylation was monitored by SDS-PAGE (FIGS. 8a & 8b). Under the above described conditions, the free sulphydryl group of cysteine at position 45 on human arginase I was specifically linked via a stable thioether bond to the activated maleimide group of mPEG-MAL (20 kDa). The final products of conjugation comprises predominantly Cys\(^{33}\) pegylated human arginase I, unconjugated human arginase I, and mPEG-MAL (20 kDa). Similarly for *Bacillus caldovelox* arginase, the cysteine residue at position 161 was specifically linked via a stable thioether bond to the activated maleimide group of mPEG-MAL (20 kDa).

The mPEG-MAL (20 kDa) pegylated arginase is advantageous over the mPEG-MAL (5 kDa) pegylated arginase in terms of a lower half-life and advantageous the mPEG-MAL (40 kDa) pegylated arginase in terms of a better solubility.

Batch Fermentation in a 2-L Fermentor

The *E. coli* BL21-DE3 strain containing the arginase gene was stored at ~80 °C. To prepare the seed inoculum for batch and fed-batch fermentation, 100 mL frozen stock of the aforementioned strain was transferred into 250 mL flask containing 80 mL of fermentation medium. The bacterial culture was cultivated at 37 °C and pH 7.0 in an orbital shaker rotating at 250 rpm. The cultivation was terminated when OD\(600\) nm reached 5.5-6.0 at about 8-10 hours. The 12 ml (1%) seed inoculum was introduced into the 2-L fermenter containing 1200 L autoclaved enriched fermentation medium. The batch fermentation was carried out at a temperature of 37 °C. The pH was maintained at 7.0 by adding sodium hydroxide and hydrochloric acid. The dissolved oxygen level was controlled at above 30% air saturation by introducing air at 1.4 L/min and adjusting the stirring rate of the fermenter at 300-1200 rpm. Isopropyl-beta-D-thiogalacto-p (IPTG) 100 mM, inducer of the protein expression of *Bacillus caldovelox* arginase (BCA), was introduced into the fermentation broth to a final concentration of 0.5 mM when the OD\(600\) nm was about 11.0 at 5 hours. After IPTG induction, the fermentation was continued until 9 hours when the OD\(600\) nm was about 16.4. The fermentation cells were harvested for separation and purification of BCA at 4 hours after IPTG induction. The aforementioned strain produced active BCA in an amount of about 105 mg/L of the fermentation medium. The time-course of the fermentation is plotted in FIG. 6a. The history plot of this batch fermentation shows the changes of parameters such as temperature, stirring rate, pH and dissolved oxygen values is depicted in FIG. 6b.

Fed-Batch Fermentation in a 2-L Fermentor

The fed-batch fermentation with high cell density culture was carried out at 37 °C, pH 7.0 and dissolved oxygen was kept above 30% air saturation during the whole fermentation process. The procedure for preparing the seed inoculums was similar to that of the batch fermentation described above. The fermentation was initially started with batch cultivation strategy by introducing 5 mL (1%) seed inoculums in the 2-L fermenter containing 500 mL autoclaved enriched fermentation medium. The dissolved oxygen decreased gradually to around 30% air saturation during the growth phase in batch cultivation period. Once the dissolved oxygen level increased above 80%, representing the depletion of carbon source, the PO\(_3\) stat fed-batch strategy was started with the addition of feeding enriched medium. In this strategy, the feeding rate was adjusted to maintain the dissolved oxygen level below 60%, which provided minimal but adequate amount of carbon source during fermentation process. Isopropyl-beta-D-thiogalacto-p (IPTG) 100 mM was introduced into the fermentation broth to a final concentration of 0.5 mM when the OD\(600\) nm was about 100 at 18 hours. After the IPTG induction, the fermentation was continued until 28 hours when the OD\(600\) nm was about 186.8. The fermentation cells were harvested for separation and purification of BCA at 10 hours after IPTG induction. The aforementioned strain produced active BCA in an amount of about 1480.6 mg per liter of the fermentation medium, which is higher than all the other reported yields of different types of arginase. The time-course of the fermentation is plotted in FIG. 6b. The history plot of this batch fermentation showing the changes of parameters such as temperature, stirring rate, pH and dissolved oxygen values is depicted in FIG. 6b.

Comparison of Batch and Fed-Batch Fermentation

Table 1 below compares the results of batch and fed-batch fermentation. The comparison demonstrates that the fed-batch fermentation is much superior to the batch operation in terms of cell OD\(600\), cell dry weight and yield of BCA per liter culture.

<table>
<thead>
<tr>
<th></th>
<th>Batch fermentation</th>
<th>Fed-batch fermentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum OD(600) reached</td>
<td>16.4</td>
<td>186.8</td>
</tr>
<tr>
<td>Cell dry weight (g)</td>
<td>4.9</td>
<td>76.6</td>
</tr>
<tr>
<td>yield of BCA (mg/L)</td>
<td>105.0</td>
<td>1400.6</td>
</tr>
<tr>
<td>yield of BCA (mg/g-cell)</td>
<td>21.4</td>
<td>19.4</td>
</tr>
</tbody>
</table>

Purification of Site-Directed Pegylated Arginases

Affinity nickel ion column chromatography was used to separate 6xHis-tagged site-directed pegylated arginases from mPEG-MAL (20 kDa) as described as follows. The final products of conjugation were loaded onto a chelating FF sepharose (GE Healthcare) column (5.0 cm x 9 cm; bed volume of 176 mL) equilibrated with Buffer A (0.02 M sodium phosphate, 0.5 M NaCl, pH 7.4). The column was washed with 5 column volumes of Buffer A to remove free mPEG-MAL (20 kDa). The pegylated arginase was eluted using a salt gradient from 30% to 100% of Buffer B (0.02 M sodium phosphate, 0.5 M NaCl, 0.5 M imidazole, pH 7.4) for 5 column volumes. The protein content of the eluent was monitored at 280 nm wave length. The column was eluted at a flow rate of 20 mL/min and the pegylated arginase fractions were collected. The pooled fractions were distilled into PBS buffer (0.01M) and concentrated to 4-6 mg/mL. Before animal study, the endotoxin in the protein drug was removed using a Q-filter (Sartorius).

In Vitro Cytotoxicity of Site-Directed Pegylated Arginases

In vitro cytotoxicity of Cys\(^{33}\) pegylated human arginase I and Cys\(^{33}\) pegylated *Bacillus caldovelox* arginase were stud-
9

ied by standard MTT assay in different human cancer cells (melanoma, hepatocellular carcinoma, gastric adenocarcinoma, colorectal adenocarcinoma, pancreatic carcinoma, pancreatic adenocarcinoma, and T cell leukemia).

The known numbers of cells (5000) were incubated for 68 hr in each well of 96-well plate in a 5% CO₂ incubator at 37°C. In the presence of different concentrations of Cys⁴⁵ pegylated human arginase I and Cys⁴⁶ peggylated *Bacillus caldovelox* arginase. After 68 hr of drug incubation, 50 μg of the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) solution was added in each well and incubated for another 4 hr. The supernatant was discarded and 100 μL of 10% SDS/0.01 M HCl was added in each well and then incubated overnight. The absorbance was recorded at 540 nm by a microplate reader (Bio-Rad). The concentration of each drug required to inhibit the 50% cell growth (IC₅₀) was determined for different cancer cell lines. Experiment was performed in triplicate.

The IC₅₀ values of Cys⁴⁵ peggylated human arginase I and Cys⁴⁶ peggylated *Bacillus caldovelox* arginase for different cell lines were calculated and the results are listed in Table 2. As *Bacillus caldovelox* arginase was never known for anti-cancer response, it is thus the first time to have demonstrated its anti-cancer properties and efficacies. In various melanoma cell lines (SK-MEL-2, SK-MEL-28, A375), the IC₅₀ values of Cys⁴⁵ peggylated human arginase I were lower when compared to those of Cys⁴⁶ peggylated *Bacillus caldovelox* arginase. Among different hepatocellular carcinoma cell lines (HepG2, Hep3B, PLC/PRF/5), HepG2 cells were most sensitive to both Cys⁴⁵ peggylated human arginase I and Cys⁴⁶ peggylated *Bacillus caldovelox* arginase. Taken together, all liver cancer (HCC) and melanoma cell lines tested were inhibited efficiently by BCA-PEG20 and HAI-PEG20.

Cys⁴⁶ peggylated *Bacillus caldovelox* arginase was also tested for the other five cancer cell lines including gastric adenocarcinoma, colorectal adenocarcinoma, pancreatic carcinoma, pancreatic adenocarcinoma, and T cell leukemia. For gastric adenocarcinoma cell lines, the IC₅₀ of Cys⁴⁶ peggylated *Bacillus caldovelox* arginase for MKN-45 cells (0.598 μM/mL) was similar to AGS cells (0.662 μM/mL). Among different colorectal adenocarcinoma cell lines (WiDr, HT-29, SW1116), WiDr cells and HT-29 cells were sensitive to Cys⁴⁶ peggylated *Bacillus caldovelox* arginase. When comparing the pancreatic carcinoma cell line (PANC-1) and the pancreatic adenocarcinoma cell line (BxPC-3), the IC₅₀ of Cys⁴⁶ peggylated *Bacillus caldovelox* arginase was lower in PANC-1 cells by four-fold. For T cell leukemia cell line (Jurkat, Clone E6-1), the IC₅₀ of Cys⁴⁶ peggylated *Bacillus caldovelox* arginase (0.41 U/mL) was also low when compared to the other cancer cell lines. Taken together, all cancer cell lines tested were sensitive to (and inhibited by) HAI-PEG20 and BCA-Peg20 treatments.

### Table 2

<table>
<thead>
<tr>
<th>Tumour</th>
<th>Cell line</th>
<th>Cys⁴⁵ peggylated human arginase I</th>
<th>Cys⁴⁶ peggylated <em>Bacillus caldovelox</em> arginase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanoma</td>
<td>SK-MEL-2</td>
<td>0.079 mg/mL</td>
<td>0.80 mg/mL</td>
</tr>
<tr>
<td></td>
<td>SK-MEL-28</td>
<td>0.064 mg/mL</td>
<td>0.65 mg/mL</td>
</tr>
<tr>
<td></td>
<td>A375</td>
<td>0.088 mg/mL</td>
<td>0.90 mg/mL</td>
</tr>
</tbody>
</table>

Depletion of Arginine by Site-Directed Peggylated Arginases

Pharmacodynamics of Cys⁴⁵ peggylated human arginase I and Cys⁴⁶ peggylated *Bacillus caldovelox* arginase were studied using BALB/c normal mice. The study was carried out in conjunction with pharmacokinetic study (described below). Therefore, the protocol remained the same. Again, the blood samples collected was centrifuged immediately at 13,200 rpm for 5 minutes and the plasma layer were collected for further analysis using the Amino Acid Analyzer (Biochrom 30, Biochrom Ltd., England).

As shown in Fig. 10a, ornithine level started to increase after the injection of Cys⁴⁵ peggylated human arginase I and stayed at a high level (>150 μM) up to Day 3. Arginine was totally depleted starting from 6 hr (Day 0) and started to appear 6-8±2.3 days after arginase administration. This indicated that HAI-PEG20 depleted blood arginine efficiently.

For Cys⁴⁶ peggylated *Bacillus caldovelox* arginase (BCA-PGE20), ornithine level also started to increase and stayed at a high level (>170 μM) up to Day 3 (Fig. 10b). Arginine was totally depleted starting from 6 hr (Day 0) and started to appear 6.7±2.1 days after arginase administration. This indicated that BCA-PGE20 depleted blood arginine efficiently.

Both pegylated arginases (Cys⁴⁵ peggylated human arginase I and Cys⁴⁶ peggylated *Bacillus caldovelox* arginase) displayed a similar pharmacodynamic profile.

In Vivo Anti-Tumour Efficacy on Liver Cancer

In vivo anti-tumour efficacy of non-pegylated (HAI) and Cys⁴⁵ peggylated human arginase I (HAI-PEG20) on liver cancer was then studied.

A number of BALB/c nude mice were injected with hepatocellular carcinoma Hep3B cells intraperitoneally (i.p.) and maintained in vivo. Then each of the 30 BALB/c nude mice was injected with ~1x10⁷ of the in vivo maintained cancer cells to the right axilla subcutaneously. When palpable tumours of 5 mm diameter were developed, the mice were separated into three different groups (see Table 5). Drugs or PBS buffer were administered intraperitoneally weekly starting on day 0 for 8 weeks. Body weights and tumour dimensions (L: length of the longer diameter and W: length of the shorter diameter of the tumour) were measured twice a week. Tumour volume (1/2xLxW²) was calculated and plotted against time. After 60 days or when tumour diameter reached 2.5 cm, the mice were euthanized. Survival rates of the mice were recorded at the end of the study.
As shown in FIG. 11a, the average body weights of the PBS control group, the Cys\(^{48}\) pegylated human arginase I group, and the non-pegylated human arginase I group were 25.9±0.2 g, 25.0±0.2 g, and 25.5±0.2 g respectively, with no significant change throughout the experiment for each group.

In terms of the tumour volume, Cys\(^{48}\) pegylated human arginase I (HAI-PEG20) significantly reduced the rate of tumour growth starting from Day 47 compared to the PBS control group (p<0.01); while non-pegylated human arginase I (HAI) did not show any significant effect (p>0.05) (FIG. 11a).

In Vivo Anti-Tumour Efficacy on Breast Cancer

In vivo anti-tumour efficacy of Cys\(^{48}\) pegylated Bacillus caldovelox arginase (BCA-PEG20) on breast cancer was determined next.

Athymic nude BALB/c mice (age of 6-8 weeks) were housed under sterile conditions with 12 hour light-dark cycle and provided with autoclaved feed ad libitum. The mice were acclimated for at least 1 week before the start of experiments. Each nude mouse was injected with 5x10\(^6\) A549 human lung cancer cells to the right axilla subcutaneously with matrigel growth supplement. When palpable tumours of ~5 mm diameter were developed, the mice were randomly separated into three different groups (Table 5). Drugs or control vehicle (PBS) were injected intraperitoneally once per week starting from Day 0. Tumour dimensions (L: longest diameter and W: its perpendicular diameter) and body weights were measured on every Mondays, Wednesdays and Fridays with Vernier caliper. Tumour volume was calculated with the formula \(\frac{1}{2}\times L\times W^2\) and no. of fold increase in tumour volume (relative tumour volume) was calculated with reference to Day 0.

<table>
<thead>
<tr>
<th>Group</th>
<th>Testing drug</th>
<th>Units/mouse route</th>
<th>Mouse NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PBS</td>
<td>NA</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>Unpegylated Bacillus caldovelox arginase</td>
<td>250 i.p.</td>
<td>35</td>
</tr>
<tr>
<td>3</td>
<td>Cys(^{48}) pegylated Bacillus caldovelox arginase</td>
<td>250 i.p.</td>
<td>40</td>
</tr>
</tbody>
</table>

No significant difference in average body weights between different groups was observed throughout the experiment and last recorded as 23.98±2.68 g for the control group, 23.68±1.50 g for the unpegylated Bacillus caldovelox arginase and 23.16±2.08 g for the Cys\(^{48}\)-pegylated Bacillus caldovelox arginase at the end of experiment (FIG. 11c).

Cys\(^{48}\)-pegylated Bacillus caldovelox arginase (BCA-PEG20) however suppressed tumour growth significantly and statistically in comparison to vehicle control group in terms of progressive changes of tumour volume (FIG. 11c) and no. of folds of tumour volume (FIG. 12d). Two-way ANOVA showed p values at <0.0001 for both parameters while Bonferroni post-test indicated the difference to start from Day 28 (p<0.05) to Day 35 (p<0.001) for tumour volume and from Day 30 to Day 35 (p<0.01 for all points) for relative tumour volume. The unpegylated Bacillus caldovelox arginase (BCA) at the same dose regime also showed anti-lung cancer effects in a similar extent with statistical significance for both parameters (two-way ANOVA, both with p<0.0001).

In Vivo Anti-Tumour Efficacy on Colorectal Cancer

In vivo anti-tumour efficacy of non-pegylated (BCA) and Cys\(^{48}\) pegylated Bacillus caldovelox arginase (BCA-PEG20) on colorectal cancer was determined as follows.

Athymic nude BALB/c mice (age of 6-8 weeks) were housed under sterile conditions with 12 hour light-dark cycle and provided with autoclaved feed ad libitum. The mice were acclimated for at least 1 week before the start of experiments. Each nude mouse was implanted with ~3 mm\(^3\) of in vivo maintained HCT-15 human colorectal cancer cells to the right axilla subcutaneously. When stable palpable tumours of ~5 mm diameter were developed, the mice were randomly separated into five different groups (Table 6). Intraperitoneal administration of arginase drugs or control vehicle (PBS) were given twice per week while 5-fluorouracil was given once per week starting from Day 0. Tumour dimensions (L: longest diameter and W: its perpendicular diameter) and body weights were measured on every Mondays, Wednesdays and Fridays with Vernier caliper. Tumour volume was calculated with the formula \(\frac{1}{2}\times L\times W^2\) and no. of fold increase in tumour volume (relative tumour volume) was calculated with reference to Day 0. The results were plotted against time.

<table>
<thead>
<tr>
<th>Group</th>
<th>Testing drug</th>
<th>Units/mouse route</th>
<th>Mouse NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PBS (control)</td>
<td>NA</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>Unpegylated Bacillus caldovelox arginase</td>
<td>250 i.p.</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>Cys(^{48})-pegylated Bacillus caldovelox arginase</td>
<td>250 i.p.</td>
<td>20</td>
</tr>
</tbody>
</table>
mice were euthanized at the end of experiment or when tumour diameter reached 2.5 cm.

<table>
<thead>
<tr>
<th>Group</th>
<th>Testing dmg</th>
<th>Unit/mouse route</th>
<th>Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 PBS (control)</td>
<td>N/A</td>
<td>i.p.</td>
<td>4M 4F</td>
</tr>
<tr>
<td>2 Unpegylated Bacillus caldovelox arginase</td>
<td>500</td>
<td>i.p.</td>
<td>4M 3F</td>
</tr>
<tr>
<td>3 Cys&lt;sup&gt;61&lt;/sup&gt;-pegylated Bacillus caldovelox arginase</td>
<td>250</td>
<td>i.p.</td>
<td>4M 3F</td>
</tr>
<tr>
<td>4 Cys&lt;sup&gt;61&lt;/sup&gt;-pegylated Bacillus caldovelox arginase + 5-fluorouracil</td>
<td>250</td>
<td>i.p.</td>
<td>4M 3F</td>
</tr>
<tr>
<td>5 5-fluorouracil</td>
<td>10 mg/kg</td>
<td>i.p.</td>
<td>2M 2F</td>
</tr>
</tbody>
</table>

No significant difference in average body weights between different groups was observed throughout the experiment and last recorded as 24.3±0.9 g for the control group, 22.1±1.0 g for the unpegylated Bacillus caldovelox arginase group, 24.2±0.7 g for the Cys<sup>61</sup>-pegylated Bacillus caldovelox arginase group, 23.5±1.2 g for the Cys<sup>61</sup>-pegylated Bacillus caldovelox arginase + 5-fluorouracil group and 24.5±1.4 g for the 5-fluorouracil group at the end of experiment (FIG. 11D).

Both Cys<sup>61</sup>-pegylated Bacillus caldovelox arginase (BCA-PEG20) and unpegylated Bacillus caldovelox arginase (BCA) in all three arginase drugs treated groups suppressed tumour growth with statistical significance (FIG. 12C and FIG. 12F). For the drug combination group (Cys<sup>61</sup>-pegylated Bacillus caldovelox arginase plus 5-fluorouracil), two-way ANOVA showed significance for no. of folds of tumour volume and tumour volume with p<0.0001 in both cases. Bonferroni post-test further pinpointed the significant difference for no. of folds of tumour volume to be from Day 36 to Day 40. For Cys<sup>61</sup>-pegylated Bacillus caldovelox arginase alone group, two-way ANOVA showed significance for no. of folds of tumour volume and tumour volume with p=0.0005 and p=0.0011, respectively. Bonferroni post-test indicated the difference to be from Day 38 to Day 40 for no. of folds of tumour volume and Day 40 for tumour volume. For unpegylated Bacillus caldovelox arginase group, the p values for no. of folds of tumour volume and tumour volume were 0.0020 and <0.0001, respectively. The 5-fluorouracil group did not show significant tumour suppression in terms of no. of folds of tumour volume (FIG. 12B). The drug combination group resulted in statistically significant lower tumour volume and no. of folds of tumour volume than both the Cys<sup>61</sup>-pegylated Bacillus caldovelox arginase alone group (p<0.0001) and p=0.0120, respectively) and the 5-fluorouracil alone group (p=0.0158 and p=0.0434, respectively). The results indicated a synergistic therapeutic effect for the Cys<sup>61</sup>-pegylated Bacillus caldovelox arginase and 5-fluorouracil.

In Vivo Inhibitory Efficacy on Breast Cancer Metastasis

1x10<sup>5</sup> cells of a mouse metastatic breast cancer cell line (4T1) were injected orthotopically into the 4th inguinal mammary fat pad of wild-type BALB/c mice at the age of 6-8 weeks. When the tumors reached an average diameter of 5 mm, the mice were divided into two different treatment groups (Table 7). BCA-PEG20 (250 U/mouse) or control vehicle (PBS) were injected intraperitoneally twice per week starting from Day 0. Body weight was measured every week. After three weeks, the mice were sacrificed and analyzed for tumor metastasis. The number of lung metastases was counted under a dissecting microscope after rinsing with PBS.

No significant difference in average body weight between different groups was observed throughout the experiment and last recorded as 21.8 g for control group and 21.5 g for the BCA-PEG20 group at the end of experiment. Results demonstrate that BCA-PEG20 reduced the spontaneous lung tumor nodules formation compared with the PBS vehicle group. The spontaneous lung metastases were too numerous to count in PBS group but only 4 nodules on the average were found in the BCA-PEG20 treatment group (Table 8). The result demonstrates that arginine depletion by BCA-PEG20 inhibits breast tumor metastasis.

<table>
<thead>
<tr>
<th>Group</th>
<th>Testing dmg</th>
<th>Unit/mouse route</th>
<th>Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 PBS (control)</td>
<td>N/A</td>
<td>i.p.</td>
<td>1M</td>
</tr>
<tr>
<td>2 BCA-PEG20</td>
<td>250</td>
<td>i.p.</td>
<td>2M</td>
</tr>
</tbody>
</table>

Effect on HIV (HAI-PEG20)

The 50% inhibition concentration (IC<sub>50</sub>) of the Cys<sup>61</sup>-pegylated human arginase 1 (HAI-PEG20) on human immunodeficiency virus (HIV) was determined as a measure of its effect on HIV.

The efficiency of an antiviral drug can be estimated using cell culture models for viral replication. The HIV replication assay utilizes H9 cells and HIV-1 strain RF. H9 cells, derived from human T lymphocytes, are highly susceptible to infection by CXCR4-using HIV-1 isolates, and show clear signs of cytotoxic effects a few days post infection. HIV-1 strain RF is a CXCR4-using class B isolate that replicates to high levels in H9 cells.

H9 cells were seeded in four 96-well plates at 5x10<sup>4</sup> viable cells/mL and the cultures incubated at 37° C. The following day, two 96-well plates were inoculated with HIV-1 at 0.005 multiplicity of infection (50 µl per well). Twenty-four hours after infection, the cells of one infected 96-well plate were treated with the Cys<sup>61</sup>-pegylated human arginase 1 (HAI-PEG20) diluted to a final concentration of 1 U/mL, 10 U/mL and 50 U/mL in tissue culture medium (10% RPMI). Eight replicates were tested for each drug concentration and 100 µL were added per well.

Azido-thymidine (AZT) was used as a benchmark drug for this assay to ensure that a dose response was obtained. This was diluted appropriately (0.01, 0.1 and 1 µg/mL) in 10% RPMI and added to the second infected plate. Eight replicates were tested for each drug concentration and 100 µL were added per well.

A cytotoxicity control was set up in parallel; this consisted of one 96-well plate of uninfected cells treated with three drug concentrations (1 U/mL, 10 U/mL and 50 U/mL). Six replicates per drug concentration. This should allow the cytotxic concentration to be determined (CC<sub>50</sub>).

The remaining 96-well plate was inoculated with tissue culture medium alone to serve as the negative control.

Five days post infection plates were examined for cytopathic effect and the IC<sub>50</sub> of the drug determined by comparing syncytial cell number in drug treated and non-treated cells.
The results show that H9 cells inoculated with HIV strain RF had viral infection, whereas H9 cells inoculated with tissue culture medium alone remained healthy throughout the study. Cytotoxic effect was observed in the H9 cultures infected with HIV and treated with the Cys<sup>55</sup> pegylated human arginase 1 (HAI-PEG20) at all concentrations. Eight out of eight (8/8) infected wells treated with the pegylated enzyme at a final concentration of 1 U/mL displayed cytotoxic effect. For infected wells treated with the enzyme at a final concentration of 10 U/mL, 6/8 wells displayed cytotoxic effect. When the drug was tested at the highest final concentration of 50 U/mL, 3/8 wells displayed cytotoxic effect. These results are shown in Table 9 and Fig. 13. The IC<sub>50</sub> of the drug was found to be approximately 37 U/mL.

When the benchmark drug AZT was added to infected wells at 0.01 μg/mL, 7/8 wells displayed cytotoxic effect. For infected wells treated with AZT at 0.1 μg/mL, 6/8 wells displayed cytotoxic effect and when tested at 1 μg/mL, 2/8 wells displayed cytotoxic effect. These results are illustrated in Fig. 14. The IC<sub>50</sub> of the AZT was found to be 0.58 μg/mL.

TABLE 9

<table>
<thead>
<tr>
<th>Sample</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV without Cys&lt;sup&gt;55&lt;/sup&gt; pegylated human arginase 1 treatment</td>
<td>24/24</td>
</tr>
<tr>
<td>HIV without Cys&lt;sup&gt;55&lt;/sup&gt; pegylated human arginase 1 treatment</td>
<td>22/24</td>
</tr>
<tr>
<td>HIV treated with Cys&lt;sup&gt;55&lt;/sup&gt; pegylated human arginase 1 (50 U/mL)</td>
<td>3/8</td>
</tr>
<tr>
<td>HIV treated with Cys&lt;sup&gt;55&lt;/sup&gt; pegylated human arginase 1 (10 U/mL)</td>
<td>6/8</td>
</tr>
<tr>
<td>HIV treated with Cys&lt;sup&gt;55&lt;/sup&gt; pegylated human arginase 1 (1 U/mL)</td>
<td>8/8</td>
</tr>
<tr>
<td>HIV treated with AZT (0.01 μg/mL)</td>
<td>7/8</td>
</tr>
<tr>
<td>HIV treated with AZT (0.1 μg/mL)</td>
<td>6/8</td>
</tr>
<tr>
<td>HIV treated with AZT (1 μg/mL)</td>
<td>2/8</td>
</tr>
<tr>
<td>Negative control</td>
<td>0/96</td>
</tr>
<tr>
<td>Cytotoxicity control - uninfected cells treated with Cys&lt;sup&gt;55&lt;/sup&gt; pegylated human arginase 1 (50 U/mL)</td>
<td>8/8*</td>
</tr>
<tr>
<td>Cytotoxicity control - uninfected cells treated with Cys&lt;sup&gt;55&lt;/sup&gt; pegylated human arginase 1 (10 U/mL)</td>
<td>8/8*</td>
</tr>
<tr>
<td>Cytotoxicity control - uninfected cells treated with Cys&lt;sup&gt;55&lt;/sup&gt; pegylated human arginase 1 (1 U/mL)</td>
<td>8/8*</td>
</tr>
</tbody>
</table>

Each well was inoculated with 50 μL of HIV at 0.05 multiplicity of infection.

* Cytotoxicity observed in each well, therefore viability counts performed for 1 well for each concentration. The results are recorded as a ratio, e.g. 1/5, where 1 is the no. of positive wells and 5 is the no. of wells inoculated.

Table 10 presents the viability counts for the cytotoxicity control. In the cytotoxicity test, all wells displayed symptoms of cytotoxicity, therefore viability counts were performed on one well for each concentration of Cys<sup>55</sup> pegylated human arginase 1. The highest concentrations resulted in cell viabilities of 30% and 39% for 50 U/mL and 10 U/mL respectively. For 1 U/mL, cell viability was 58%. Based on this cell viability assessed for all 8 wells and the average determined to be 48.9%. This approximates a 50% reduction in cell viability based on the cell viability of (96.8%) when cells were seeded onto the 96 well plates. These results are displayed in Table 10 and Fig. 15, clearly demonstrating that HAI-PEG20 has inhibitory effects on HIV replication.

TABLE 10

<table>
<thead>
<tr>
<th>Sample</th>
<th>Live cells</th>
<th>Total cells</th>
<th>% viability</th>
<th>Average %</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 U/mL - 1 well</td>
<td>9</td>
<td>33</td>
<td>30</td>
<td>N/A</td>
</tr>
<tr>
<td>10 U/mL - 1 well</td>
<td>11</td>
<td>33</td>
<td>39</td>
<td>N/A</td>
</tr>
</tbody>
</table>

In Vitro Anti-Cancer Effects

In vitro cancer cell culture studies on the anti-cancer efficacy of different arginine-depleting enzymes were conducted for various cancer types.

Cell Proliferation Assay: For each cancer cell line, cells (5x10<sup>4</sup>) in 100 μL culture medium were seeded to the wells of a 96-well plate and incubated for 24 hours by standard method. The culture medium was replaced with medium containing different concentrations of one of the arginases or arginine deaminase (ADI). The plates were incubated for an additional 3 days at 37°C in an atmosphere of 95% air/5% CO<sub>2</sub>. The metabolically viable cell fraction was determined by the MTT assay, which was performed to estimate the number of viable cells in the culture. Non-linear regression with Prism 4 (Graphpad Software) was used to fit a sigmoidal dose response curve, and the amount of each of the arginine-degrading enzymes (in terms of U/mL or μg/mL) needed to achieve 50% inhibition of cell growth was defined as IC<sub>50</sub>.

RT-PCR studies: Total RNA was extracted from cancer cell lines grown in culture using the Qiagen RNasey kit. For reverse transcription-polymerase chain reaction (RT-PCR), the RNA was first reverse-transcribed into cDNA by iScript cDNA Synthesis kit (Bio-Rad, CA) according to the manufacturer’s instruction. Briefly, 5 μL of total RNA was subjected to RT at 42°C for 30 min. A 2 μL portion of cDNA was then amplified using 50 μL of reaction mixture containing 0.5 units of iTag DNA polymerase (Bio-Rad, CA). PCR was performed in a DNA thermal Mycycler (Bio-Rad, CA). The following flanking primers were used:

(a) Human ASS (448 bp product):

\[
\text{Sense: } 5'-GGGTTCCCTCTTGAAGTGACC-3';
\]
\[
\text{Anti-sense: } 5'-GCTGCTGTCTCACCTGC-3';
\]

(b) Human ASL (218 bp product):

\[
\text{Sense: } 5'-CTCTCGTAGACCCCTGAGGGA-3';
\]
\[
\text{Anti-sense: } 5'-CATCTCTCTGCGGAGGACCGTGA-3';
\]

(c) Human OTC (221 bp product):

\[
\text{Sense: } 5'-GGACTGAGCCTCCTGAAAGTG-3';
\]
\[
\text{Anti-sense: } 5'-GATTTTGCCACCCCTGCTAA-3';
\]
Human GAPDH (306 by product):

**Sense:**

5' AGGCAATGCTCAGGACG-3'

**Anti-sense**:

5' GCGAAATCGACAAATC-3'

The reaction products were subjected to 1% agarose gel electrophoresis. After electrophoresis and staining with ethidium bromide, all PCR product band intensities were analyzed by Lumi-Imager (Boehringer Mannheim, Inc.), and the relative mRNA expression levels were estimated by normalization with the house keeping gene GAPDH.

As the results indicate, arginases and ADI are all efficient arginine-degrading enzymes. Unexpectedly, we found that all the cancer cell lines tested here are sensitive to arginases but many cancer cell lines are actually resistant to ADI treatment. It was discovered in the present invention that this difference is due to the fact that arginase converts arginine to ornithine and urea while ADI converts it to citrulline and ammonia. Citrulline can be recycled back to arginine if the cancer cells are ASS-positive and ASL-positive, leading to drug resistance. Most strikingly, if the cancer cells are OTC-negative, they cannot recycle ornithine back to arginine in the cells even if they are ASS-positive and ASL-positive. This guideline provided by the present invention has been found to be consistent with all our data as well as data from other research groups. Under this guideline, for instance, if the cancer cells are either ASS-negative or ASL-negative or both, they would be arginase-sensitive and ADI-sensitive. On the other hand, if the cancer cells are both ASS-positive and ASL-positive but OTC-negative, they would be arginase-sensitive and ADI-resistant. Therefore, it is believed that arginases have broader anti-cancer applications than ADI. Furthermore, ammonia (product from ADI reaction) is more toxic than urea (product from arginase reaction). Thus, the arginase anti-cancer agents of the present invention are believed to be more safe than ADI.

In vitro anti-cancer efficacy results are summarized in Tables 11a-11g. As indicated in Table 11a, all the melanoma cell lines tested were sensitive to arginase treatments. When arginase was added to culture medium, arginase was converted to ornithine and urea. All these cells were OTC-negative and according to the guideline discussed above, these cells cannot recycle the arginase reaction product ornithine back to arginine in the cells and therefore the cells are inhibited due to the lack of arginine. According to the IC50 values, all the arginases tested were very effective on the inhibition of cancer cell growth.

Although all the melanoma cell lines tested were all ASS-positive and ASL-positive, the expression levels of ASS were low, which can be confirmed by performing an ASS activity assay. The low ASS expression level explains why these cell lines were all sensitive to ADI treatments. B16 is a mouse melanoma cell line and it is also sensitive to both arginases and ADI. Thus, it is believed that ADI kills the melanoma cells due to the low level of ASS expression while arginases kill the melanoma cells because they are OTC-negative.

In Table 11b, it is shown that all the leukemia cell lines tested were sensitive to arginase treatments. Some of these cancer cells tested were OTC-negative and according to the guideline discussed above, these cells cannot recycle the arginase reaction product ornithine back to arginine in the cells and therefore the cells are inhibited due to the lack of arginine. According to the IC50 values, all the arginases tested were very effective on inhibition of leukemia cancer cell growth. For ADI treatments, all the 4 leukemia cell lines tested were sensitive except the RPMI8226 cell line, which is resistant to ADI treatment, most likely due to the fact that it is both ASS-positive and ASL-positive. Therefore, for inhibiting leukemia cells, arginases are advantageous over ADI.

Table 11c shows that all the colorectal cancer cell lines tested were sensitive to arginase treatments. All these cancer cell lines tested were OTC-negative. In consistent with the guideline discussed above, these cells cannot recycle the arginase reaction product ornithine back to arginine in the cells and therefore the cells are inhibited due to the lack of arginine. According to the IC50 values, all the arginases tested were very effective on the inhibition of colorectal cancer cell growth. For ADI treatments, only 2 colorectal cancer cell lines (WLDL and HT29) were tested and the other 2 (SW1116 and HCT15) were resistant to ADI treatment, most likely due to the fact that they are both ASS-positive and ASL-positive. For HT29, although it was ASS-positive and ASL-positive according to the RT-PCR data, the expression level of ASS was low, as confirmed by performing an ASS activity assay, which explains why this cell line was sensitive to ADI treatment.

Also shown in Table 11c, most strikingly, all the pancreatic cancer cell lines tested were sensitive to arginase treatments. All these cancer cells tested were OTC-negative. As discussed above, these cells cannot recycle the arginase reaction product ornithine back to arginine in the cells and therefore the cells are inhibited due to the lack of arginine. According to the IC50 values, all the arginases tested were very effective on the inhibition of pancreatic cancer cell growth. For ADI treatments, only one pancreatic cancer cell line (Pancl) was tested and the other 2 (BxPC3 and HPAFII) were resistant to ADI treatment. Clearly, for inhibiting pancreatic cancer cells, arginases are better than ADI.

Table 11d shows that all the gastric cancer cell lines tested were sensitive to arginase treatments. All these cancer cells tested were OTC-negative and thus, as discussed above, these cells cannot recycle the arginase reaction product ornithine back to arginine in the cells and therefore the cells are inhibited due to the lack of arginine. As the IC50 values indicate, all the arginases tested were very effective on the inhibition of gastric cancer cell growth. In a sharp contrast, all the gastric cancer cell lines tested were resistant to ADI treatment, most likely due to the fact that they are both ASS-positive and ASL-positive. This similar result was obtained for the liver cancer (or HCC) cell lines tested as shown in Table 11e.

Table 11e also shows that the retinoblastoma cancer cell line Y79 tested was sensitive to arginase treatments but resistant to ADI treatment, most likely due to the fact that they are both ASS-positive and ASL-positive. Table 11f shows that the lung cancer cell line A549 tested was sensitive to arginase treatments. These cancer cells tested were OTC-negative. It is also sensitive to ADI treatment, most likely due to the fact that they are either ASS-negative or ASL-negative. In contrast, also shown in Table 11f, all the cervical cancer cell lines tested were sensitive to arginase treatments (they were all OTC-negative), but only 2 cervical cancer cell line (SiHa and C-33A) tested were sensitive and the other 3 (HeLa, ME139, HCC3) were resistant to ADI treatment, most likely due to the fact that they are both ASS-positive and ASL-positive.

The results for breast cancer cells are shown in Table 11g. As it shows, all the breast cancer cell lines tested were sensitive to arginase treatments (they were all OTC-negative). Strikingly, only one breast cancer cell line (MDA-MB-231) tested was sensitive and the other 3 (MCF-7, ZR-75-1, Ha5787) were resistant to ADI treatments.

Also shown in Table 11g are results for the prostate cancer cell line, which was found to be sensitive to both arginase and ADI treatments. As discussed above, such results can be explained by the fact that this cell line is both OTC-negative and ASS-negative.
### Table 11a

<table>
<thead>
<tr>
<th>Type of cancer</th>
<th>Cell line name (medium, source)</th>
<th>BCA U/mL (µg/mL)</th>
<th>HAI U/mL (µg/mL)</th>
<th>rhArg U/mL (µg/mL)</th>
<th>ADI U/mL (µg/mL)</th>
<th>ARG</th>
<th>OTC</th>
<th>ASS</th>
<th>ASL</th>
</tr>
</thead>
<tbody>
<tr>
<td>melanoma</td>
<td>SK-mel-2 (DMEM, 10% FBS, 1% PS ATCC)</td>
<td>0.612 (11.25)</td>
<td>0.079 (0.86)</td>
<td>0.0556 (1.31)</td>
<td>0.0022 (0.082)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>L</td>
</tr>
<tr>
<td></td>
<td>SK-mel-24 (DMEM, 10% FBS, 1% PS NCI)</td>
<td></td>
<td></td>
<td>0.204 (4.82)</td>
<td>0.012 (0.45)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>L</td>
</tr>
<tr>
<td></td>
<td>SK-mel-28 (DMEM, 10% FBS, 1% PS ATCC)</td>
<td>0.91 (16.72)</td>
<td>0.064 (0.65)</td>
<td>0.0523 (1.233)</td>
<td>0.00064 (0.011)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>L</td>
</tr>
<tr>
<td></td>
<td>A375 (DMEM, 10% FBS, 1% PS ATCC)</td>
<td></td>
<td></td>
<td>0.15 (2.76)</td>
<td>0.061 (0.679)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>L</td>
</tr>
<tr>
<td></td>
<td>B16 (DMEM, 10% FBS, 1% PS ATCC)</td>
<td></td>
<td></td>
<td>0.02 (0.48)</td>
<td>0.004 (0.11)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>L</td>
</tr>
</tbody>
</table>

### Table 11b

<table>
<thead>
<tr>
<th>Type of cancer</th>
<th>Cell line name (medium, source)</th>
<th>BCA U/mL (µg/mL)</th>
<th>HAI U/mL (µg/mL)</th>
<th>rhArg U/mL (µg/mL)</th>
<th>ADI U/mL (µg/mL)</th>
<th>ARG</th>
<th>OTC</th>
<th>ASS</th>
<th>ASL</th>
</tr>
</thead>
<tbody>
<tr>
<td>leukemia</td>
<td>HL60 (RPMI 10% FBS, 1% PS ATCC)</td>
<td>0.03 (0.679)</td>
<td>0.016 (0.591)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>K562 (RPMI 10% FBS, 1% PS ATCC)</td>
<td>0.06 (3.357)</td>
<td>0.003 (0.085)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>RPMI8226 (RPMI 10% FBS, 1% PS ATCC)</td>
<td>0.09 (2.036)</td>
<td>R</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Jurkat (RPMI 10% FBS, 1% PS ATCC)</td>
<td>0.41 (7.54)</td>
<td>0.037 (0.86)</td>
<td>0.092 (0.074)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

### Table 11c

<table>
<thead>
<tr>
<th>Type of cancer</th>
<th>Cell line name (medium, source)</th>
<th>BCA U/mL (µg/mL)</th>
<th>HAI U/mL (µg/mL)</th>
<th>rhArg U/mL (µg/mL)</th>
<th>ADI U/mL (µg/mL)</th>
<th>ARG</th>
<th>OTC</th>
<th>ASS</th>
<th>ASL</th>
</tr>
</thead>
<tbody>
<tr>
<td>colorectal</td>
<td>WiDr (DMEM, 10% FBS, 1% PS ATCC)</td>
<td>0.215 (3.06)</td>
<td>0.075 (0.76)</td>
<td>0.038 (0.84)</td>
<td>0.035 (0.9)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SW1116 (RPMI 10% FBS, 1% PS ATCC)</td>
<td>1.417 (20.98)</td>
<td>0.41 (4.18)</td>
<td>0.15 (3.394)</td>
<td>R</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>J12 (DMEM, 10% FBS, 1% PS ATCC)</td>
<td>0.231 (4.24)</td>
<td>0.03 (0.679)</td>
<td>0.032 (0.83)</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>JCT15 (RPMI 10% FBS, 1% PS ATCC)</td>
<td>0.63 (6.44)</td>
<td>0.083 (1.043)</td>
<td>R</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pancreatic</td>
<td>Panc1 (DMEM, 10% FBS, 1% PS ATCC)</td>
<td>0.263 (4.84)</td>
<td>0.09 (2.036)</td>
<td>0.049 (1.39)</td>
<td>R</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>BXPC3 (DMEM, 10% FBS, 1% PS ATCC)</td>
<td>0.846 (24.34)</td>
<td>0.08 (2.036)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Type of cancer</td>
<td>Cell line name (medium, source)</td>
<td>BCA U/mL (µg/mL)</td>
<td>HAI U/mL (µg/mL)</td>
<td>rhArg U/mL (µg/mL)</td>
<td>ADI U/mL (µg/mL)</td>
<td>ARG OTC ASS ASL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------</td>
<td>---------------------------------</td>
<td>------------------</td>
<td>------------------</td>
<td>-------------------</td>
<td>-----------------</td>
<td>-----------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(EMEM 10% FBS, 1% PS ATCC)</td>
<td>JPAFl</td>
<td>(15.54)</td>
<td>(1.809)</td>
<td>R</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(DMEM 10% FBS, 1% PS ATCC)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### TABLE 11d

<table>
<thead>
<tr>
<th>Type of cancer</th>
<th>Cell line name (medium, source)</th>
<th>BCA U/mL (µg/mL)</th>
<th>HAI U/mL (µg/mL)</th>
<th>rhArg U/mL (µg/mL)</th>
<th>ADI U/mL (µg/mL)</th>
<th>ARG OTC ASS ASL</th>
</tr>
</thead>
<tbody>
<tr>
<td>gastric</td>
<td>AGS (RPMI 10% FBS, 1% PS ATCC)</td>
<td>0.662 (12.17)</td>
<td>0.10 (2.262)</td>
<td>R</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>MKN45 (RPMI 10% FBS, 1% PS Rikes Cell Bank, Japan)</td>
<td>0.798 (14.67)</td>
<td>0.79 (17.873)</td>
<td>R</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>BCG-823 (RPMI 10% FBS, 1% PS Beijing Institute of Cancer Research)</td>
<td>0.11 (2.457)</td>
<td>R</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

### TABLE 11e

<table>
<thead>
<tr>
<th>Type of cancer</th>
<th>Cell line name (medium, source)</th>
<th>BCA U/mL (µg/mL)</th>
<th>HAI U/mL (µg/mL)</th>
<th>rhArg U/mL (µg/mL)</th>
<th>ADI U/mL (µg/mL)</th>
<th>ARG OTC ASS ASL</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCC (liver cancer)</td>
<td>PLC/PRF/5 (DMEM 10% FBS, 1% PS ATCC)</td>
<td>2.376 (43.67)</td>
<td>0.84 (9.56)</td>
<td>0.312 (3.07)</td>
<td>R</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>HepG2 (DMEM 10% FBS, 1% PS ATCC)</td>
<td>9.1 (57.88)</td>
<td>0.29 (2.93)</td>
<td>0.65 (6.50)</td>
<td>R</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>SK-HEP-1 (DMEM 10% FBS, 1% PS ATCC)</td>
<td>2.002 (36.79)</td>
<td>0.697 (9.99)</td>
<td>0.177 (1.77)</td>
<td>R</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>HepG2 (DMEM 10% FBS, 1% PS ATCC)</td>
<td>1.61 (43)</td>
<td>R</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Muhl7 (DMEM 10% FBS, 1% PS ATCC)</td>
<td>1.59 (43)</td>
<td>R</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>retinoblastoma</td>
<td>Y79 (RPMI 10% FBS, 1% PS ATCC)</td>
<td>0.5 (11.3)</td>
<td>R</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

### TABLE 11f

<table>
<thead>
<tr>
<th>Type of cancer</th>
<th>Cell line name (medium, source)</th>
<th>BCA U/mL (µg/mL)</th>
<th>HAI U/mL (µg/mL)</th>
<th>rhArg U/mL (µg/mL)</th>
<th>ADI U/mL (µg/mL)</th>
<th>ARG OTC ASS ASL</th>
</tr>
</thead>
<tbody>
<tr>
<td>lung</td>
<td>A549 (DMEM 10% FBS, 1% PS ATCC)</td>
<td>0.325 (2.09)</td>
<td>0.035 (0.44)</td>
<td>0.011 (0.29)</td>
<td>R</td>
<td>-</td>
</tr>
</tbody>
</table>
### TABLE 11f-continued

<table>
<thead>
<tr>
<th>Type of cancer</th>
<th>Cell line name (medium, source)</th>
<th>BCA (µg/mL)</th>
<th>HAI (µg/mL)</th>
<th>rhArg (µg/mL)</th>
<th>ADI (µg/mL)</th>
<th>ARG</th>
<th>OTC</th>
<th>ASS</th>
<th>ASL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cervical</td>
<td>HeLa (DMEM 10% FBS, 1% PS ATCC)</td>
<td>0.719</td>
<td>0.366</td>
<td>0.065 R</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>ME180 (DMEM 10% FBS, 1% PS ATCC)</td>
<td>1.42</td>
<td>0.214</td>
<td>0.153 R</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CC3 (DMEM 10% FBS, 1% PS ATCC)</td>
<td>0.84</td>
<td>0.42</td>
<td>R</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>S135 (DMEM 10% FBS, 1% PS ATCC)</td>
<td>0.32</td>
<td>0.024</td>
<td>0.03</td>
<td>0.0025</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>C-33A (DMEM 10% FBS, 1% PS ATCC)</td>
<td>0.18</td>
<td>0.033</td>
<td>0.058</td>
<td>0.0014</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

### TABLE 11g

<table>
<thead>
<tr>
<th>Type of cancer</th>
<th>Cell line name (medium, source)</th>
<th>BCA (µg/mL)</th>
<th>HAI (µg/mL)</th>
<th>rhArg (µg/mL)</th>
<th>ADI (µg/mL)</th>
<th>ARG</th>
<th>OTC</th>
<th>ASS</th>
<th>ASL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast</td>
<td>MCF-7 (EMEM 10% FBS, 1% PS ATCC)</td>
<td>0.05</td>
<td>0.28</td>
<td>R</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>ZR-75 (DMEM 10% FBS, 1% PS ATCC)</td>
<td>0.14</td>
<td>0.14</td>
<td>R</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Hs778T (DMEM 10% FBS, 1% PS ATCC)</td>
<td>3.75</td>
<td></td>
<td></td>
<td>3.75</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>MDA-MB-231 (DMEM 10% FBS, 1% PS NCI)</td>
<td>0.22</td>
<td>0.273</td>
<td>0.44</td>
<td>0.16</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4T1 (EMEM 10% FBS, 1% PS NCI)</td>
<td>0.68</td>
<td>0.58</td>
<td>0.023</td>
<td>0.0007</td>
<td>0.29</td>
<td>(0.017)</td>
<td>L</td>
<td></td>
</tr>
<tr>
<td>Prostate</td>
<td>PC3 (DMEM 10% FBS, 1% PS ATCC)</td>
<td>0.263</td>
<td>0.40 (4.07)</td>
<td>0.08</td>
<td>0.0025</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>1NCap (EMEM 10% FBS, 1% PS ATCC)</td>
<td>2.119</td>
<td>0.47 (4.78)</td>
<td>0.41</td>
<td>0.13</td>
<td>(3.94)</td>
<td>(5.16)</td>
<td>(3.34)</td>
<td></td>
</tr>
</tbody>
</table>

For Table 11, ‘*’=mRNA was detected by RT-PCR, indicating the corresponding gene is expressed; ‘**’=mRNA was not detected by RT-PCR, indicating the gene is not expressed; ‘R’ indicates that the cell line is ADI-resistant and the IC<sub>50</sub> value cannot be estimated; and ‘L’ indicates that the cell line has a relatively low level of ASS expression and therefore the cell line is still ADI-sensitive.

While not wish to be bound by the following hypothesis and working models, applicants believe the following hypothesis and working models are consistent with the experimental data of the present invention and thus are useful guides for further utilization of the inventions disclosed here-with (also see FIG. 18).

Hypothesis and working models explaining why OTC-negative cancer cells are arginine-sensitive but can be ADI-resistant. When arginine is added in the culture medium or pegylated arginine is injected in the blood (in the body), arginine is converted into ornithine and urea by the arginine enzymatic reaction. Ornithine formed then passes into the cancer cells. Unlike normal cells, cancer cells grow rapidly and require much more arginine than normal cells for protein synthesis and other cellular processes. If the cancer cells are OTC-positive, ASS-positive and ASL-positive, ornithine can be recycled back into arginine. Therefore, cancer cells still have arginine and they are not arginine-deficient and cancer growth is not inhibited. On the other hand, cancer cells that are OTC-negative or ASS-negative or ASL-negative or any combination of these deficiencies or low expression level of any of these genes, the synthesis (or recycle) pathway from ornithine to arginine is blocked and therefore cancer cells are lack of arginine and cancer cell growth is thus inhibited and cancer cell death may occur.

Hypothesis and working models for liver cancer cells that are OTC-negative. Model relating urea cycle gene expression and resistance towards pegylated arginine deiminase (ADI-PEG) and pegylated Bacillus caldovolox arginase (BCA-PEG20). Liver cancer cells express the urea cycle enzymes argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL) and arginase (ARC), but lack ornithine transcarbamylase (OTC). BCA-PEG20 in the bloodstream depletes arginine and produces ornithine, which enters the cell but fails to be recycled via the urea cycle owing to the absence of OTC. ADI-PEG converts arginine to citrulline, which can be readily converted back to arginine by ASS and ASL, after uptake into liver cancer cells. Therefore, in this model, the liver cancer cells are sensitive to BCA-PEG20 treatment (inhibited by BCA-PEG20) but resistant to ADI-PEG treatment.
Hypothesis and working models for cancer cells that are OTC-negative. Model relating gene expression in cancer cells and resistance towards pegylated arginine deiminase (ADI-PEG) and pegylated _Bacillus caldovelox_ arginase (BCA-PEG20). For cancer cells that do not express arginase (ARG), cancer cells express the enzymes argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL), but lack ornithine transcarbamylase (OTC). BCA-PEG20 in the bloodstream depletes arginine and produces ornithine, which enters the cell but fails to be recycled owing to the absence of OTC. ADI-PEG converts arginine to citrulline, which can be readily converted back to arginine by ASS and ASL after uptake into the cancer cells. Therefore, in this model, the cancer cells are sensitive to BCA-PEG20 treatment (inhibited by ADI-PEG20) but resistant to ADI-PEG treatment. This model can be applied to cancer cells in general.

Method of Further Enhance Arginase Activity by Using Cobalt as Metal Cofactor

The native metal cofactor of arginase is manganese (Mn^{2+}). It is surprisingly discovered by the present invention that replacing the manganese with cobalt dramatically enhances the enzyme’s activity. Either _Bacillus caldovelox_ arginase (BCA) or the human arginase I (HAI) was expressed as described previously. The purification method was the same as described before except except 10 mM of metal ion (CoSO_4_ or MnSO_4_) was added into the purified protein elution from Nickel affinity chromatography instead of added before Nickel affinity chromatography. Eluted factions containing the arginase enzyme were incubated with 10 mM metal for 15 min at 50–55°C, followed by filtration through a 0.45 μm syringe filter. Then the solution was exchanged with storage buffer by ultrafiltration.

Disaccharidase (DAMO) assay was used to determine the kinetic parameters of human arginase with different metal cofactors. All enzymatic reactions were carried out at pH 7.4. The results are shown in Fig. 16. The steady-state kinetics of recombinant human arginase I (HAI) or hArg substituted with Mn^{2+} or Co^{2+} were measured in sodium phosphate buffer pH 7.4, 25°C. The Km of HAI with Mn^{2+} (HAI Mn^{2+}) or hArg Mn^{2+} and HAI with Co^{2+} (HAI Co^{2+}) or hArg Co^{2+} are 1.83 mM and 0.19 mM respectively. Since the Km value is improved about 10-fold in HAI Co^{2+} or hArg Co^{2+}, its specific activity is improved 10-fold and is a much more efficient drug to deplete arginine than HAI Mn^{2+} or hArg Mn^{2+}.

Enhancing Arginase Activity by Further Modifying Genetic Modification

It is surprisingly discovered by the present invention that the position 20 of BCA can be substituted with other amino acids to improve enzyme activity. The 20th amino acid residue valine in the wild-type sequence was substituted with proline (or any other amino acids for example serine or glycine, which improves the specific activity of BCA) by site-directed mutagenesis (for example codon GTT [valine] to CGG [proline]). The mutant genes were cloned, expressed and purified for detailed studies. An exemplary such mutant enzyme was made by replacing valine with proline, referred to as “BCA mutant V20P” or “hArg V20P mutant”. Steady-state kinetics of the BCA mutant V20P and BCA with Mn^{2+} or Co^{2+} were measured in sodium phosphate buffer pH 7.4, 25°C. and were shown in Fig. 17. The Km values of BCA mutant V20P with Mn^{2+} (BCA mutant V20P Mn^{2+}) and BCA mutant V20P with Co^{2+} (BCA mutant V20P Co^{2+}) are about 1.29 mM and 0.18 mM respectively. The Km of BCA with Mn^{2+} (BCA-WTMn^{2+}) is about 3.23 mM. Therefore, the BCA mutant V20P with Co^{2+} as cofactor (Km=0.18 mM) is a much more efficient drug to deplete arginine than the BCA (BCA-WTMn^{2+}) (Km=3.2 mM).

In Vitro Cancer Cell Line Studies Using BCA Mutant V20P

Cell proliferation assay was conducted as follows. 2.5×10^5 Sk-mel-28 (EMEM), 5×10^5 HEK293 (EMEM), MCF-7 (EMEM), HCT-15 (RPIM), Hep3B (DMEM), PANC-1 (DMEM), Hela (DMEM) and A549 (DMEM) cells were seeded to each well of a 96-well plate in 100 μl culture medium and were allowed to adhere to the plate overnight. On the next day, the culture medium was replaced with medium containing different concentrations of BCA and BCA mutant V20P protein drug, 2×10^5 Jurkat (RPIM) floating cells were seeded to each well of a 96-well plate in 50 μl culture medium at the day of adding protein drug and different concentrations of protein drug in 50 μl were added directly to each well. The cells were allowed to incubate for an additional 5 days at 37°C in an atmosphere of 95% air/5% CO2. MITT cell proliferation assay (Invitrogen) was then performed to estimate the number of viable cells in the culture. In brief, 10 μl of 5 mg/mL of water-soluble MTT regents was added to 100 μl culture medium and incubated at 37°C for 4 h. MTT is chemically reduced by cells into purple formazan, which is then dissolved by acidified SDS (0.01 N HCl in 10% SDS) in tissue culture medium. Concentration of the cleavage product formazan was then measured by reading its absorbance with a spectrophotometer with a 570 nm filter. Cell proliferation data were expressed as a percentage of control. Non-linear regression was used to fit a sigmoidal dose response curve with Prism 4.0 (Graphpad Software), and the amount of protein drug needed to achieve 50% cell growth inhibition was defined as IC_{50}. The results are shown in Table 12. The corresponding enzymatic activities are shown in Table 13.

### TABLE 12

| IC_{50} of BCA and BCA mutant V20P in different kinds of cancer cells |
|-------------------------|-----------------|-----------------|-----------------|
| IC_{50} Value            | Fold of Difference |
| (BCA)                    | (BCA mutant V20P) |
| (BCA)                    | (BCA mutant V20P) |
| mm (mg/mL)               | mm (mg/mL)       | mm (mg/mL)      | mm (mg/mL)      |
| HCT-15                   | Colon            | 15.62           | 0.0916          |
|                         |                 | 7.34            | 0.0132          | 2.13             | 6.96             |
| Jurkat                   | Leukemia         | 6.84            | 0.0401          |
|                         |                 | 0.90            | 0.0016          | 7.60             | 24.85            |
| MCF-7                    | Breast           | 5.51            | 0.0323          |
|                         |                 | 2.87            | 0.0651          | 1.92             | 6.28             |
| sk-mel-28                | Melanoma         | 3.35            | 0.0197          |
|                         |                 | 1.52            | 0.0027          | 2.20             | 7.21             |
| HEK293                   | Kidney           | 3.86            | 0.0226          |
|                         |                 | 3.40            | 0.0061          | 1.14             | 3.71             |
| A549                     | Lung             | 2.67            | 0.0157          |
|                         |                 | 1.64            | 0.0029          | 1.63             | 5.32             |
| Hep3B                    | Liver            | 9.42            | 0.0552          |
|                         |                 | 9.43            | 0.0169          | 1.00             | 3.27             |
| Hela                     | Cervical         | 2.83            | 0.0166          |
|                         |                 | 1.37            | 0.0025          | 2.07             | 6.75             |
| PANC-1                   | Pancreatic       | 1.20            | 0.0070          |
|                         |                 | 0.87            | 0.0016          | 1.38             | 4.51             |
The results show that BCA mutant V20P is much more efficient in killing various types of cancer cells in in vitro drug efficacy studies. While there have been described and pointed out fundamental novel features of the invention as applied to a preferred embodiment thereof, it will be understood that various omissions and substitutions and changes, in the form and details of the embodiments illustrated, may be made by those skilled in the art without departing from the spirit of the invention. The invention is not limited by the embodiments described above which are presented as examples only but can be modified in various ways within the scope of protection defined by the appended patent claims.

<table>
<thead>
<tr>
<th>Protein concentration (ng/ml)</th>
<th>Specific activity (U/mg)</th>
<th>Enzyme activity (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCA</td>
<td>3.064</td>
<td>170.47</td>
</tr>
<tr>
<td>BCA mutant</td>
<td>2.63</td>
<td>557.3</td>
</tr>
<tr>
<td>V20P</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 13**

**SEQUENCE LISTING**

| <160> NUMBER OF SEQ ID NOS: 22 |
| <210> SEQ ID NO 1               |
| <211> LENGTH: 969              |
| <212> TYPE: DNA                |
| <213> ORGANISM: Homo sapiens   |
| <400> SEQUENCE: 1              |
| aggacgcgcca agtccgagaac cataggagatt attggagctc ttttctcaaa gggacagcga 60 |
| cggagggggt tgacgaaggt cctcagctat tcggtgtcgt tcgaaactt 120 |
| aagagacag agtgtagtg gaaaggatt tggggcgtc tttttgctga catcctcaat 180 |
| gcagctcctc ttcacatttt gcgaatccca aagtctgtgg gaaagacagc cggacgctg 240 |
| gttggcaggg tcggagaggt cagaaaagac gcagcagttg gggggagac 300 |
| cacaggtgg catcagtgga cctcctgctg catgccgagcg ttcacactggat tttcgggtgct 360 |
| actgggtgg cgttcccaac tcgatcactgc acctcaacg caacagcaag gttgaacttg 420 |
| catggacacac cttgatttctt ccctcgatga gaaactaag gaaagatccc cagatgctca 480 |
| gatctcctct gccctagcc ttcgattatc gcgaagttatc cgggtgtacat tggcttgagta 540 |
| gcgggtgagc ccgagcggac cttacactgg agaacttatg gcctacaatg ttttcctag 600 |
| actcagatggt acagagatgg aatgcagccc ggtatggaag aacaatcgct ctctcataa 660 |
| ggaagagga aagagccagtc ttggctggtg aggcagcttg ccatcccttc 720 |
| asgacgactg ctcgagcagc actcgtgatct acacgagagc atctcatacct 780 |
| aacaagcact cctcagcactg agtgctgttc cggagattg atacaactg gaagtacacc 840 |
| tccttgtggga agacggcaga gaaactcaatc cgaaactgt aagactaagg 900 |
| tggctttgtg tggaggtgct cggagcagc atcagccagc ctattgacta cottaacc 960 |
| cttcagtaa 969                     |

| <210> SEQ ID NO 2               |
| <211> LENGTH: 969              |
| <212> TYPE: DNA                |
| <213> ORGANISM: artificial sequence |
| <220> FEATURE:                 |
| <223> OTHER INFORMATION: Human arginase 1 designed for site-directed  |
| pegylation                      |
| <400> SEQUENCE: 2              |
| aggacgcgcca agtccgagaac cataggagatt attggagctc ttttctcaaa gggacagcga 60 |
| cggagggggt tgacgaaggt cctcagctat tcggtgtcgt tcgaaactt 120 |
| aagagacag agtgtagtg gaaaggatt tggggcgtc tttttgctga catcctcaat 180 |
| gcagctcctc ttcacatttt gcgaatccca aagtctgtgg gaaagacagc cggacgctg 240 |
<210> SEQ ID NO: 3
<211> LENGTH: 990
<212> TYPE: DNA
<213> ORGANISM: Bacillus caldovelox

<400> SEQUENCE: 3

gtcggcaggg ttgctagaag ctasaagaac ggasgacact gcgcgtggtc gcggcggctc 300
ccagagtttg caattgaaga ccactctggc cagcgcaggg tccactcggta tccttggacgc 360
actctggtgg atgcctcaac ttgatcaac acctoctgtg caacccaaac taagaaacttg 420
catggcacaac ctgatcttct cttctgaaga gacaatggta tggctgata tgcctgaga 480
ggttaactct gggtgtaaccc ctctcatatct gcaacggaat tttgtgata tgcctgaga 540
gcgctgagcc ctggaggaac atcaccttgg gaaatcttct gattaaaaa ctttcttcgct 600
actgaaggttg acagaattgg aaagcccaag gttgatggag aacaatccag caatctacta 660
ggagaaaagaa cagggcacaat tctatcaagt ttttagttcg cgccgtcgag ccaattcttc 720
cacccgctta ctggccacac ccgctgctgg ggtctgagat acagagaagc tttctactac 780
acagaaagaa ccctatcaac acggtctaac tccaggtaga atataatgga ggtgacccca 840
tcctggttgga agaacccga agataatact cggacgtacg caacccgctt cttttctccgat 900
tgcgcttctt gtcgacttcg tcggagggct aatacacaag ctatatgaat ctttacccca 960
cttaaagga 969

<210> SEQ ID NO: 4
<211> LENGTH: 918
<212> TYPE: DNA
<213> ORGANISM: artificial sequence

<400> SEQUENCE: 4

gtcggcaggg ttgctagaag ctasaagaac ggasgacact gcgcgtggtc gcggcggctc 300
ccagagtttg caattgaaga ccactctggc cagcgcaggg tccactcggta tccttggacgc 360
actctggtgg atgcctcaac ttgatcaac acctoctgtg caacccaaac taagaaacttg 420
catggcacaac ctgatcttct cttctgaaga gacaatggta tggctgata tgcctgaga 480
ggttaactct gggtgtaaccc ctctcatatct gcaacggaat tttgtgata tgcctgaga 540
gcgctgagcc ctggaggaac atcaccttgg gaaatcttct gattaaaaa ctttcttcgct 600
actgaaggttg acagaattgg aaagcccaag gttgatggag aacaatccag caatctacta 660
ggagaaaagaa cagggcacaat tctatcaagt ttttagttcg cgccgtcgag ccaattcttc 720
cacccgctta ctggccacac ccgctgctgg ggtctgagat acagagaagc tttctactac 780
acagaaagaa ccctatcaac acggtctaac tccaggtaga atataatgga ggtgacccca 840
tcctggttgga agaacccga agataatact cggacgtacg caacccgctt cttttctccgat 900

<210> SEQ ID NO: 5
<211> LENGTH: 920
<212> TYPE: DNA
<213> ORGANISM: Bacillus caldovelox arginase designed for site-directed mutagenesis

<400> SEQUENCE: 5

gtcggcaggg ttgctagaag ctasaagaac ggasgacact gcgcgtggtc gcggcggctc 300
ccagagtttg caattgaaga ccactctggc cagcgcaggg tccactcggta tccttggacgc 360
actctggtgg atgcctcaac ttgatcaac acctoctgtg caacccaaac taagaaacttg 420
catggcacaac ctgatcttct cttctgaaga gacaatggta tggctgata tgcctgaga 480
ggttaactct gggtgtaaccc ctctcatatct gcaacggaat tttgtgata tgcctgaga 540
gcgctgagcc ctggaggaac atcaccttgg gaaatcttct gattaaaaa ctttcttcgct 600
actgaaggttg acagaattgg aaagcccaag gttgatggag aacaatccag caatctacta 660
ggagaaaagaa cagggcacaat tctatcaagt ttttagttcg cgccgtcgag ccaattcttc 720
cacccgctta ctggccacac ccgctgctgg ggtctgagat acagagaagc tttctactac 780
acagaaagaa ccctatcaac acggtctaac tccaggtaga atataatgga ggtgacccca 840
tcctggttgga agaacccga agataatact cggacgtacg caacccgctt cttttctccgat 900

<210> SEQ ID NO: 6
<211> LENGTH: 920
<212> TYPE: DNA
<213> ORGANISM: Bacillus caldovelox arginase designed for site-directed mutagenesis

<400> SEQUENCE: 6

gtcggcaggg ttgctagaag ctasaagaac ggasgacact gcgcgtggtc gcggcggctc 300
ccagagtttg caattgaaga ccactctggc cagcgcaggg tccactcggta tccttggacgc 360
actctggtgg atgcctcaac ttgatcaac acctoctgtg caacccaaac taagaaacttg 420
catggcacaac ctgatcttct cttctgaaga gacaatggta tggctgata tgcctgaga 480
ggttaactct gggtgtaaccc ctctcatatct gcaacggaat tttgtgata tgcctgaga 540
gcgctgagcc ctggaggaac atcaccttgg gaaatcttct gattaaaaa ctttcttcgct 600
actgaaggttg acagaattgg aaagcccaag gttgatggag aacaatccag caatctacta 660
ggagaaaagaa cagggcacaat tctatcaagt ttttagttcg cgccgtcgag ccaattcttc 720
cacccgctta ctggccacac ccgctgctgg ggtctgagat acagagaagc tttctactac 780
acagaaagaa ccctatcaac acggtctaac tccaggtaga atataatgga ggtgacccca 840
tcctggttgga agaacccga agataatact cggacgtacg caacccgctt cttttctccgat 900

<210> SEQ ID NO: 7
<211> LENGTH: 920
<212> TYPE: DNA
<213> ORGANISM: Bacillus caldovelox arginase designed for site-directed mutagenesis

<400> SEQUENCE: 7

gtcggcaggg ttgctagaag ctasaagaac ggasgacact gcgcgtggtc gcggcggctc 300
ccagagtttg caattgaaga ccactctggc cagcgcaggg tccactcggta tccttggacgc 360
actctggtgg atgcctcaac ttgatcaac acctoctgtg caacccaaac taagaaacttg 420
catggcacaac ctgatcttct cttctgaaga gacaatggta tggctgata tgcctgaga 480
ggttaactct gggtgtaaccc ctctcatatct gcaacggaat tttgtgata tgcctgaga 540
gcgctgagcc ctggaggaac atcaccttgg gaaatcttct gattaaaaa ctttcttcgct 600
actgaaggttg acagaattgg aaagcccaag gttgatggag aacaatccag caatctacta 660
ggagaaaagaa cagggcacaat tctatcaagt ttttagttcg cgccgtcgag ccaattcttc 720
cacccgctta ctggccacac ccgctgctgg ggtctgagat acagagaagc tttctactac 780
acagaaagaa ccctatcaac acggtctaac tccaggtaga atataatgga ggtgacccca 840
tcctggttgga agaacccga agataatact cggacgtacg caacccgctt cttttctccgat 900

<210> SEQ ID NO: 8
<211> LENGTH: 920
<212> TYPE: DNA
<213> ORGANISM: Bacillus caldovelox arginase designed for site-directed mutagenesis

<400> SEQUENCE: 8

gtcggcaggg ttgctagaag ctasaagaac ggasgacact gcgcgtggtc gcggcggctc 300
ccagagtttg caattgaaga ccactctggc cagcgcaggg tccactcggta tccttggacgc 360
actctggtgg atgcctcaac ttgatcaac acctoctgtg caacccaaac taagaaacttg 420
catggcacaac ctgatcttct cttctgaaga gacaatggta tggctgata tgcctgaga 480
ggttaactct gggtgtaaccc ctctcatatct gcaacggaat tttgtgata tgcctgaga 540
gcgctgagcc ctggaggaac atcaccttgg gaaatcttct gattaaaaa ctttcttcgct 600
actgaaggttg acagaattgg aaagcccaag gttgatggag aacaatccag caatctacta 660
ggagaaaagaa cagggcacaat tctatcaagt ttttagttcg cgccgtcgag ccaattcttc 720
cacccgctta ctggccacac ccgctgctgg ggtctgagat acagagaagc tttctactac 780
acagaaagaa ccctatcaac acggtctaac tccaggtaga atataatgga ggtgacccca 840
tcctggttgga agaacccga agataatact cggacgtacg caacccgctt cttttctccgat 900
<210> SEQ ID NO 5
<211> LENGTH: 322
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 5

Met Ser Ala Lys Ser Arg Thr Ile Gly Ile Ile Gly Ala Pro Phe Ser
                           1  5   10  15
Lys Gly Gln Pro Arg Gly Gly Val Glu Gly Gly Pro Thr Val Leu Arg
                           20  25  30
Lys Ala Gly Leu Leu Glu Lys Leu Lys Gly Glu Cys Asp Val Lys
                           35  40  45
Asp Tyr Gly Asp Leu Pro Phe Ala Asp Ile Pro Asn Asp Ser Pro Phe
                           50  55  60
Gln Ile Val Lys Asn Pro Arg Ser Val Gly Lys Ala Ser Glu Glu Leu
                           65  70  75  80
Ala Gly Lys Val Ala Glu Val Lys Asn Gly Arg Ile Ser Leu Val
                           85  90  95
Leu Gly Gly Asp His Ser Leu Ala Ile Gly Ser Ile Ser Gly His Ala
                          100 105 110
Arg Val His Pro Asp Leu Gly Val Ile Trp Val Asp Ala His Thr Asp
                          115 120 125
Ile Asn Thr Pro Leu Thr Thr Ser Gly Asn Leu His Gly Glu Pro
                          130 135 140
Val Ser Phe Leu Leu Lys Glu Leu Lys Gly Ile Pro Asp Val Pro
                          145 150 155 160
Gly Phe Ser Trp Val Thr Pro Cys Ile Ser Ala Lys Asp Ile Val Tyr
                          165 170 175
Ile Gly Leu Arg Asp Val Asp Gly Gly His Tyr Ile Leu Lys Thr
                          180 185 190
Leu Gly Ile Gly Tyr Phe Ser Met Thr Glu Val Val Arg Leu Gly Ile
                          195 200 205
Gly Lys Val Met Glu Gly Thr Leu Ser Tyr Leu Leu Gly Arg Lys Lys
                          210 215 220
Arg Pro Ile His Leu Ser Phe Asp Val Asp Gly Leu Asp Pro Ser Phe
                          225 230 235 240
<table>
<thead>
<tr>
<th>Thr</th>
<th>Pro</th>
<th>Ala</th>
<th>Thr</th>
<th>Gly</th>
<th>Thr</th>
<th>Pro</th>
<th>Val</th>
<th>Val</th>
<th>Gly</th>
<th>Gly</th>
<th>Leu</th>
<th>Thr</th>
<th>Thr</th>
<th>Tyr</th>
<th>Arg</th>
<th>Glu</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>248</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>285</td>
</tr>
<tr>
<td>Gly</td>
<td>Leu</td>
<td>Tyr</td>
<td>Ile</td>
<td>Thr</td>
<td>Gly</td>
<td>Glu</td>
<td>Ile</td>
<td>Tyr</td>
<td>Lys</td>
<td>Thr</td>
<td>Gly</td>
<td>Leu</td>
<td>Leu</td>
<td>Ser</td>
<td>Gly</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>260</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>270</td>
</tr>
<tr>
<td>Leu</td>
<td>Asp</td>
<td>Ile</td>
<td>Met</td>
<td>Glu</td>
<td>Val</td>
<td>Asn</td>
<td>Pro</td>
<td>Ser</td>
<td>Leu</td>
<td>Gly</td>
<td>Lys</td>
<td>Thr</td>
<td>Pro</td>
<td>Glu</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>275</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>285</td>
</tr>
<tr>
<td>Val</td>
<td>Thr</td>
<td>Arg</td>
<td>Thr</td>
<td>Val</td>
<td>Asn</td>
<td>Thr</td>
<td>Ala</td>
<td>Thr</td>
<td>Ala</td>
<td>Ile</td>
<td>Thr</td>
<td>Thr</td>
<td>Ala</td>
<td>Cys</td>
<td>Phe</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>290</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>300</td>
</tr>
<tr>
<td>Gly</td>
<td>Leu</td>
<td>Ala</td>
<td>Arg</td>
<td>Gly</td>
<td>Asn</td>
<td>His</td>
<td>Lys</td>
<td>Pro</td>
<td>Ile</td>
<td>Asp</td>
<td>Tyr</td>
<td>Leu</td>
<td>Asn</td>
<td>Pro</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>310</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>320</td>
</tr>
<tr>
<td></td>
<td>Pro</td>
<td>Lys</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**SEQ ID NO:** 6  
**LENGTH:** 322  
**TYPE:** PRT  
**ORGANISM:** artificial sequence  
**FEATURE:**  
**OTHER INFORMATION:** amino acid sequence of human arginase I with Cys168 and Cys303 replaced by Ser  
**SEQUENCE:** 6  

<table>
<thead>
<tr>
<th>Met</th>
<th>Ser</th>
<th>Ala</th>
<th>Lys</th>
<th>Ser</th>
<th>Arg</th>
<th>Thr</th>
<th>Ile</th>
<th>Gly</th>
<th>Ile</th>
<th>Gly</th>
<th>Ala</th>
<th>Pro</th>
<th>Phe</th>
<th>Ser</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>10</td>
<td>16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lys</th>
<th>Gly</th>
<th>Gin</th>
<th>Pro</th>
<th>Arg</th>
<th>Gly</th>
<th>Val</th>
<th>Glu</th>
<th>Gly</th>
<th>Pro</th>
<th>Thr</th>
<th>Val</th>
<th>Leu</th>
<th>Arg</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td></td>
<td></td>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lys</th>
<th>Ala</th>
<th>Gly</th>
<th>Leu</th>
<th>Leu</th>
<th>Lys</th>
<th>Leu</th>
<th>Lys</th>
<th>Glu</th>
<th>Glu</th>
<th>Gin</th>
<th>Cys</th>
<th>Val</th>
<th>Lys</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td></td>
<td></td>
<td>40</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Asp</th>
<th>Tyr</th>
<th>Gly</th>
<th>Asp</th>
<th>Leu</th>
<th>Pro</th>
<th>Phe</th>
<th>Ala</th>
<th>Asp</th>
<th>Ile</th>
<th>Pro</th>
<th>Asn</th>
<th>Asp</th>
<th>Ser</th>
<th>Pro</th>
<th>Phe</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td></td>
<td></td>
<td>55</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gln</th>
<th>Ile</th>
<th>Val</th>
<th>Lys</th>
<th>Asn</th>
<th>Pro</th>
<th>Arg</th>
<th>Ser</th>
<th>Val</th>
<th>Gly</th>
<th>Lys</th>
<th>Ala</th>
<th>Ser</th>
<th>Glu</th>
<th>Gin</th>
<th>Leu</th>
</tr>
</thead>
<tbody>
<tr>
<td>65</td>
<td></td>
<td></td>
<td>70</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ala</th>
<th>Gly</th>
<th>Lys</th>
<th>Val</th>
<th>Ala</th>
<th>Val</th>
<th>Lys</th>
<th>Asn</th>
<th>Gly</th>
<th>Arg</th>
<th>Ile</th>
<th>Ser</th>
<th>Leu</th>
<th>Val</th>
</tr>
</thead>
<tbody>
<tr>
<td>85</td>
<td></td>
<td></td>
<td>90</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Leu</th>
<th>Gly</th>
<th>Asp</th>
<th>His</th>
<th>Ser</th>
<th>Leu</th>
<th>Ala</th>
<th>Ile</th>
<th>Gly</th>
<th>Ser</th>
<th>Ile</th>
<th>Ser</th>
<th>Gly</th>
<th>His</th>
<th>Ala</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td></td>
<td></td>
<td>105</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Arg</th>
<th>Val</th>
<th>His</th>
<th>Pro</th>
<th>Asp</th>
<th>Leu</th>
<th>Gly</th>
<th>Val</th>
<th>Ile</th>
<th>Trp</th>
<th>Val</th>
<th>Asp</th>
<th>Ala</th>
<th>His</th>
<th>Thr</th>
<th>Asp</th>
</tr>
</thead>
<tbody>
<tr>
<td>115</td>
<td></td>
<td></td>
<td>120</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ile</th>
<th>Asn</th>
<th>Thr</th>
<th>Pro</th>
<th>Leu</th>
<th>Thr</th>
<th>Thr</th>
<th>Ser</th>
<th>Gly</th>
<th>Asn</th>
<th>Leu</th>
<th>His</th>
<th>Gly</th>
<th>Gin</th>
<th>Pro</th>
</tr>
</thead>
<tbody>
<tr>
<td>130</td>
<td></td>
<td></td>
<td>135</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Val</th>
<th>Ser</th>
<th>Phe</th>
<th>Leu</th>
<th>Leu</th>
<th>Lys</th>
<th>Glu</th>
<th>Leu</th>
<th>Lys</th>
<th>Gly</th>
<th>Ile</th>
<th>Pro</th>
<th>Asp</th>
<th>Val</th>
<th>Pro</th>
</tr>
</thead>
<tbody>
<tr>
<td>145</td>
<td></td>
<td></td>
<td>150</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gly</th>
<th>Phe</th>
<th>Ser</th>
<th>Trp</th>
<th>Val</th>
<th>Thr</th>
<th>Pro</th>
<th>Ser</th>
<th>Ile</th>
<th>Ser</th>
<th>Ala</th>
<th>Lys</th>
<th>Asp</th>
<th>Ile</th>
<th>Val</th>
<th>Tyr</th>
</tr>
</thead>
<tbody>
<tr>
<td>165</td>
<td></td>
<td></td>
<td>170</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ile</th>
<th>Gly</th>
<th>Leu</th>
<th>Arg</th>
<th>Asp</th>
<th>Val</th>
<th>Asp</th>
<th>Pro</th>
<th>Gly</th>
<th>Glu</th>
<th>His</th>
<th>Tyr</th>
<th>Ile</th>
<th>Leu</th>
<th>Lys</th>
<th>Thr</th>
</tr>
</thead>
<tbody>
<tr>
<td>180</td>
<td></td>
<td></td>
<td>185</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Leu</th>
<th>Gly</th>
<th>Ile</th>
<th>Lys</th>
<th>Tyr</th>
<th>Phe</th>
<th>Ser</th>
<th>Met</th>
<th>Thr</th>
<th>Glu</th>
<th>Val</th>
<th>Asp</th>
<th>Arg</th>
<th>Leu</th>
<th>Gly</th>
<th>Ile</th>
</tr>
</thead>
<tbody>
<tr>
<td>195</td>
<td></td>
<td></td>
<td>200</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gly</th>
<th>Lys</th>
<th>Val</th>
<th>Met</th>
<th>Glu</th>
<th>Thr</th>
<th>Leu</th>
<th>Ser</th>
<th>Tyr</th>
<th>Leu</th>
<th>Leu</th>
<th>Gly</th>
<th>Arg</th>
<th>Lys</th>
<th>Lys</th>
</tr>
</thead>
<tbody>
<tr>
<td>210</td>
<td></td>
<td></td>
<td>215</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Arg</th>
<th>Pro</th>
<th>Ile</th>
<th>His</th>
<th>Leu</th>
<th>Ser</th>
<th>Phe</th>
<th>Asp</th>
<th>Val</th>
<th>Asp</th>
<th>Gly</th>
<th>Leu</th>
<th>Asp</th>
<th>Pro</th>
<th>Ser</th>
<th>Phe</th>
</tr>
</thead>
<tbody>
<tr>
<td>225</td>
<td></td>
<td></td>
<td>230</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Thr</th>
<th>Pro</th>
<th>Ala</th>
<th>Thr</th>
<th>Gly</th>
<th>Thr</th>
<th>Pro</th>
<th>Val</th>
<th>Val</th>
<th>Gly</th>
<th>Gly</th>
<th>Leu</th>
<th>Thr</th>
<th>Tyr</th>
<th>Arg</th>
<th>Glu</th>
</tr>
</thead>
<tbody>
<tr>
<td>245</td>
<td></td>
<td></td>
<td>250</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gly</th>
<th>Leu</th>
<th>Tyr</th>
<th>Ile</th>
<th>Thr</th>
<th>Gly</th>
<th>Glu</th>
<th>Ile</th>
<th>Tyr</th>
<th>Lys</th>
<th>Thr</th>
<th>Gly</th>
<th>Leu</th>
<th>Leu</th>
<th>Ser</th>
<th>Gly</th>
</tr>
</thead>
<tbody>
<tr>
<td>260</td>
<td></td>
<td></td>
<td>265</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Leu Asp Ile Met Glu Val Asn Pro Ser Leu Gly Lys Thr Pro Glu Glu
275 280 285
Val Thr Arg Thr Val Asn Thr Ala Ala Ile Thr Leu Ala Ser Phe
290 295 300
Gly Leu Ala Arg Glu Gly Asn His Lys Pro Ile Asp Tyr Leu Asn Pro
305 310 315 320
Pro Lys

<210> SEQ ID NO 7
<211> LENGTH: 299
<212> TYPE: PRT
<213> ORGANISM: Bacillus caldovelox

<400> SEQUENCE: 7
Met Lys Pro Ile Ser Ile Ile Gly Val Pro Met Asp Leu Gly Gln Thr
1  5  10  15
Arg Arg Gly Val Asp Met Gly Pro Ser Ala Met Arg Tyr Ala Gly Val
20 25  30
Ile Glu Arg Leu Glu Arg Leu His Tyr Asp Ile Glu Asp Leu Gly Asp
35 40  45
Ile Pro Ile Gly Lys Ala Glu Arg Leu His Glu Gln Gly Asp Ser Arg
50 55  60
Leu Arg Asn Leu Lys Ala Val Ala Ala Asn Glu Lys Leu Ala Ala
65 70  75  80
Ala Val Asp Gln Val Val Gln Arg Gly Arg Phe Pro Leu Val Leu Gly
85 90  95
Gly Asp His Ser Ile Ala Ile Gly Thr Leu Ala Gly Val Ala Lys His
100 105 110
Tyr Glu Arg Leu Gly Val Ile Thr Tyr Asp Ala His Gly Asp Val Asn
115 120 125
Thr Ala Glu Thr Ser Pro Ser Gly Asn Ile His Gly Met Pro Leu Ala
130 135 140
Ala Ser Leu Gly Phe Gly His Pro Ala Leu Thr Gln Ile Gly Lys Tyr
145 150 155 160
Ser Pro Lys Ile Lys Pro Glu His Val Leu Ile Gly Val Arg Ser
165 170 175
Leu Asp Gly Glu Gly Lys Lys Phe Ile Arg Glu Gly Lys Ile Lys Ile
180 185 190
Tyr Thr Met His Gly Glu Val Asp Arg Leu Gly Met Thr Arg Val Met Glu
195 200 205
Glu Thr Ile Ala Tyr Leu Lys Glu Arg Thr Asp Gly Val His Leu Ser
210 215 220
Leu Asp Leu Asp Leu Asp Pro Ser Asp Ala Pro Gly Val Gly Thr
225 230 235 240
Pro Val Ile Gly Leu Thr Tyr Arg Glu Ser His Leu Ala Met Glu
245 250 255
Met Leu Ala Glu Ala Gln Ile Thr Ser Ala Glu Phe Val Glu Val
260 265 270
Asn Pro Ile Leu Asp Glu Arg Asn Lys Thr Ala Ser Val Ala Ala
275 280 285
Leu Met Gly Ser Leu Phe Gly Glu Lys Leu Met
290 295

<210> SEQ ID NO 8
<211> LENGTH: 305
<212> TYPE: PRT
<213> ORGANISM: artificial sequence

<220> FEATURE: 

<223> OTHER INFORMATION: amino acid sequence of 6xHis-tagged Bacillus caldiovelox arginase with Ser13 replaced by Cys.

<400> SEQUENCE: 8

Met Lys Pro Ile Ser Ile Gly Val Pro Met Asp Leu Gly Gln Thr
1 5 10 15
Arg Arg Gly Val Asp Met Gly Pro Ser Ala Met Arg Tyr Ala Gly Val
20 25 30
Ile Glu Arg Leu Glu Arg Leu His Tyr Asp Ile Glu Asp Leu Gly Asp
35 40 45
Ile Pro Ile Gly Lys Ala Glu Arg Leu His Glu Gin Gly Asp Ser Arg
50 55 60
Leu Arg Asn Leu Asn Ala Val Ala Glu Asn Leu Ala Ala
65 70 75 80
Ala Val Asp Gin Val Val Gin Gly Arg Phe Pro Leu Val Leu Gly
85 90 95
Gly Asp His Ser Ile Ala Ile Gly Thr Leu Ala Gly Val Ala Lys His
100 105 110
Tyr Glu Arg Leu Gly Val Ile Tyr Thr Asp Ala His Gly Asp Val Asn
115 120 125
Thr Ala Glu Thr Ser Pro Ser Gly Asn Ile His Gly Met Pro Leu Ala
130 135 140
Ala Ser Leu Gly Phe Gly His Pro Ala Leu Thr Gin Ile Glu Gly Tyr
145 150 155 160
Cys Pro Lys Ile Lys Pro Glu His Pro Val Leu Ile Gly Val Arg Ser
165 170 175
Leu Asp Glu Gly Lys Lys Phe Ile Arg Glu Gly Lys Ile
180 185 190
Tyr Thr Met His Gly Val Asp Arg Leu Gly Met Thr Arg Val Met Glu
195 200 205
Glu Thr Ile Ala Tyr Leu Lys Glu Arg Thr Asp Gly Val His Leu Ser
210 215 220
Leu Asp Leu Arg Asp Gly Leu Asp Pro Ser Asp Ala Pro Gly Val Gly Thr
225 230 235 240
Pro Val Ile Gly Leu Thr Tyr Glu Ser His Leu Ala Met Glu
245 250 255
Met Leu Ala Glu Ala Gin Ile Ile Thr Ser Ala Glu Phe Val Glu Val
260 265 270
Asn Pro Ile Leu Asp Glu Arg Asn Lys Thr Ala Ser Val Ala Val Ala
275 280 285 290
Leu Met Gly Ser Leu Phe Gly Glu Lys Leu Met His His His His
290 295 300
His 305

<210> SEQ ID NO 9
<211> LENGTH: 969
<212> TYPE: DNA
<213> ORGANISM: artificial sequence

<220> FEATURE: 

<223> OTHER INFORMATION: human arginase I mutant (C1685/C1635)

<400> SEQUENCE: 9

atgagcgcga aagtcgaacag cattaggttt ataggagcgc atttctcaaaa gggacagc 60
cggaggaggg tggagaaggg cctcagaga ttgagaaag ctggtcgtgc tggaaaactt 120
<table>
<thead>
<tr>
<th>39</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>39</td>
<td>-continued</td>
</tr>
<tr>
<td>aaagaacaag agtggtacgt gaaaggttat ggggacctgc ctttgtgctga cattcctaat</td>
<td>100</td>
</tr>
<tr>
<td>gcacgctcct tccaatttgt tgaagatctct cgggtcgcttg gagaaccaag cagacgctg</td>
<td>240</td>
</tr>
<tr>
<td>gctggacaggt tygctgaagta caagaagaag ggaagaactca gctctgtgctg ggtggcaaggg</td>
<td>300</td>
</tr>
<tr>
<td>cactcgttctt catcttggaag catctgtcag cactgctgct gcaagctgtag gatggactgc</td>
<td>360</td>
</tr>
<tr>
<td>atctgggggtctt gggaactaccc tctatatct gcaaggata ttttgtatat tggcctgaga</td>
<td>420</td>
</tr>
<tr>
<td>gcagttgaccc cttgggcaag ataacttttg aasactctag gcttaaata acctttccag</td>
<td>480</td>
</tr>
<tr>
<td>actgagttgg acacactagg aatggcagga gcttgagggag aagactcaag cactctacta</td>
<td>540</td>
</tr>
<tr>
<td>ggagaagaaga aagggcctacctagaat ttttgagggt gacagacagc gcaacctttc</td>
<td>600</td>
</tr>
<tr>
<td>acacagctta cttggcaacgg acctctgtcgg ggtgtgacttt gcagcagcag tcctttacac</td>
<td>660</td>
</tr>
<tr>
<td>acagaagaga cctttctcacc ggagttactc tctgtgtagtt atatcatgg gattgagcc</td>
<td>720</td>
</tr>
<tr>
<td>ttcgctggga agaccaagac tgaagaact ccagactgag cagcaactg tgaaataacc</td>
<td>780</td>
</tr>
<tr>
<td>tgygtctctt ctggactttgc cggaggggtaac cccactag ctattcacta ccctaaacca</td>
<td>840</td>
</tr>
<tr>
<td>cctagaagta</td>
<td>900</td>
</tr>
</tbody>
</table>

<210> SEQ ID NO 10
<211> LENGTH: 222
<212> TYPE: PRT
<213> ORGANISM: artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: human arginase I mutant (C1685/C3035)

<400> SEQUENCE: 10

Met Ser Ala Lys Ser Arg Thr Ile Gly Ile Ile Gly Ala Pro Phe Ser
1  5  10  15
Lys Gly Gin Pro Arg Gly Gly Val Glu Glu Gly Pro Thr Val Leu Arg
20 25 30
Lys Ala Gly Leu Leu Glu Leu Lys Leu Gly Gin Gly Cys Asp Val Lys
35 40 45
Arg Tyr Gly Asp Leu Pro Phe Ala Asp Pro Aam Asp Ser Pro Phe
50 55 60
Gln Ile Val Lys Aam Pro Arg Ser Val Gly Lys Ala Ser Glu Glu Leu
65 70 75 80
Ala Gly Lys Val Ala Glu Val Lys Aam Gly Arg Ile Ser Leu Val
85 90 95
Leu Gly Gly Asp His Ser Leu Ala Ile Gly Ser Ile Ser Gly His Ala
100 105 110
Arg Val His Pro Asp Leu Gly Val Ile Trp Val Asp Ala His Thr Asp
115 120 125
Ile Aam Thr Pro Leu Thr Thr Ser Gly Aam Leu His Gly Gin Pro
130 135 140
Val Ser Phe Leu Leu Lys Leu Lys Gly Lys Ile Pro Asp Val Pro
145 150 155 160
Gly Phe Ser Trp Val Thr Pro Ser Ile Ser Ala Lys Asp Ile Val Tyr
165 170 175
Ile Gly Leu Arg Asp Val Asp Pro Gly Glu His Tyr Ile Leu Lys Thr
180 185 190
Leu Gly Ile Lys Tyr Phe Ser Met Thr Glu Val Asp Arg Leu Gly Ile
195 200 205
Gly Lys Val Met Glu Glu Thr Leu Ser Tyr Leu Leu Gly Arg Lys Lys

Arg Pro Ile His Leu Ser Phe Asp Val Asp Gly Leu Asp Pro Ser Phe

Thr Pro Ala Thr Gly Thr Pro Val Gly Gly Leu Thr Tyr Arg Glu

Gly Leu Tyr Ile Thr Glu Glu Ile Tyr Lys Thr Gly Leu Leu Ser Gly

Leu Asp Ile Met Glu Val Asn Pro Ser Leu Gly Thr Pro Glu Glu

Val Thr Arg Thr Val Asn Thr Ala Val Ile Thr Leu Ala Ser Phe

Gly Leu Ala Arg Glu Gly Asn His Lys Pro Ile Asp Tyr Leu Aen Pro

Pro Lys

<210> SEQ ID NO 11
<211> LENGTH: 990
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: 6xHis-tagged human arginase I mutant (C1688/C3038)

<400> SEQUENCE: 11

atgcatacacc atcaatcata ctagagcc cagtcagaga ccataggagt tattggagtct

cccttctcac agggacagcc agcagaggag gttggaagag gcctacagct ttgagaagaag

gctgtgcc tcggagactct taagaaacca gatgtgtag tgaagaggtga gttggacogct

cccttagctg acatactcct tcacggtcct cttcaaatgt ggaagatcag aaggtatcctg

gggaagagtt ccttctctgc agattctctc tggggtgactc caatgtagcat gcacgatcagt

atctgtgata ttcggctgag aagcctggac cctgggggac actacatctt gaaactctta

ggcgattaaat actcttcgat gacagactag gaattgtggct ggtgtctggag

ggacatacata ctagatctct aggagagaag agaaggagca ttcatacagat ttttgagtttc

gagcagctgg aaccatctct cccacacgct actggagacc actctctgag ggtcgatcgca

tacagcaagtg cgttatatat caacgagacac atctacaaca caggggtact ctcgagattca

gatataggg aaggtgacac cctccctggg ggcagcacag aacaagact acagctctct

acctggat cccatcagat cccagcagag cctcctggg ggcgcagcag aacaagagct

<210> SEQ ID NO 12
<211> LENGTH: 329
<212> TYPE: PRT
<213> ORGANISM: artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: 6xHis-tagged human arginase I mutant (C1688/C3038)

<400> SEQUENCE: 12

Met His His His His His Met Ser Ala Lys Ser Arg Thr Ile Gly
<table>
<thead>
<tr>
<th>1</th>
<th>5</th>
<th>10</th>
<th>15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ile Ile Gly Ala Pro Phe Ser Lys Gly Gln Pro Arg Gly Gly Val Glu</td>
<td>20</td>
<td>25</td>
<td>30</td>
</tr>
<tr>
<td>Glu Gly Pro Thr Val Leu Arg Lys Ala Gly Leu Leu Glu Lys Leu Lys</td>
<td>35</td>
<td>40</td>
<td>45</td>
</tr>
<tr>
<td>Glu Gln Glu Cys Asp Val Lys Asp Tyr Gly Asp Leu Pro Phe Ala Asp</td>
<td>50</td>
<td>55</td>
<td>60</td>
</tr>
<tr>
<td>Ile Pro Asn Asp Ser Pro Phe Gln Ile Val Lys Asn Pro Arg Ser Val</td>
<td>65</td>
<td>70</td>
<td>75</td>
</tr>
<tr>
<td>Gly Lys Ala Ser Glu Gln Leu Ala Gly Lys Val Ala Glu Val Lys Lys</td>
<td>85</td>
<td>90</td>
<td>95</td>
</tr>
<tr>
<td>Asn Gly Arg Ile Ser Leu Val Leu Gly Gly Asp His Ser Leu Ala Ile</td>
<td>100</td>
<td>105</td>
<td>110</td>
</tr>
<tr>
<td>Gly Ser Ile Ser Gly His Ala Arg Val His Pro Asp Leu Gly Val Ile</td>
<td>115</td>
<td>120</td>
<td>125</td>
</tr>
<tr>
<td>Trp Val Asp Ala His Thr Asp Ile Asn Thr Pro Leu Thr Thr Thr Ser</td>
<td>130</td>
<td>135</td>
<td>140</td>
</tr>
<tr>
<td>Gly Asn Leu His Gly Gln Pro Val Ser Phe Leu Leu Lys Glu Leu Lys</td>
<td>145</td>
<td>150</td>
<td>155</td>
</tr>
<tr>
<td>Gly Lys Ile Pro Asp Val Pro Gly Phe Ser Thr Val Thr Pro Ser Ile</td>
<td>165</td>
<td>170</td>
<td>175</td>
</tr>
<tr>
<td>Ser Ala Lys Asp Ile Val Tyr Ile Gly Leu Arg Asp Val Asp Pro Gly</td>
<td>180</td>
<td>185</td>
<td>190</td>
</tr>
<tr>
<td>Glu His Tyr Ile Leu Lys Thr Leu Gly Ile Lys Tyr Phe Ser Met Thr</td>
<td>195</td>
<td>200</td>
<td>205</td>
</tr>
<tr>
<td>Glu Val Asp Arg Leu Gly Ile Gly Lys Val Met Glu Thr Leu Ser</td>
<td>210</td>
<td>215</td>
<td>220</td>
</tr>
<tr>
<td>Tyr Leu Leu Gly Arg Lys Arg Pro Ile His Leu Ser Phe Asp Val</td>
<td>225</td>
<td>230</td>
<td>235</td>
</tr>
<tr>
<td>Asp Gly Leu Asp Pro Ser Phe Thr Pro Ala Thr Gly Thr Pro Val Val</td>
<td>245</td>
<td>250</td>
<td>255</td>
</tr>
<tr>
<td>Gly Gly Leu Thr Tyr Arg Glu Gly Leu Tyr Ile Thr Glu Ile Ile Gly</td>
<td>260</td>
<td>265</td>
<td>270</td>
</tr>
<tr>
<td>Lys Thr Gly Leu Leu Ser Gly Leu Asp Met Glu Val Asn Pro Ser</td>
<td>275</td>
<td>280</td>
<td>285</td>
</tr>
<tr>
<td>Leu Gly Lys Thr Pro Glu Glu Val Thr Arg Thr Val Asn Thr Ala Val</td>
<td>290</td>
<td>295</td>
<td>300</td>
</tr>
<tr>
<td>Ala Ile Thr Leu Ala Ser Phe Gly Leu Ala Arg Gly Gly His Lys</td>
<td>305</td>
<td>310</td>
<td>315</td>
</tr>
<tr>
<td>Pro Ile Asp Tyr Leu Asn Pro Pro Lys</td>
<td>325</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<210> SEQ ID NO 13
<211> LENGTH: 900
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Bacillus caldovelox arginine mutant (S161C)

<400> SEQUENCE: 13
taggaagccaa tttcaattat cggggttcag atggatttag gcgcagacaag cgcocgcttcgtt
| 60 |
gatagcgggc cgcgcaacat gctttatgcg gcgcgtcacgc aacgtctggga acgtctttcat
| 120 |
tagcatttag agagattgag agatgctccag atgggaaagc cgacagcgggt gcacagcgaag
| 180 |
ggagatttac ggtggccaa tttgaagcg gttggcgaag cgcacgagaa actgtctgagcg
| 240 |
gctggctgacc aagcggctca gcgggacg ttctccgctt ttggtggcgc gcaggatatg 300
cggcggt gacgctgc gcgggggctg aaacattatg aggccttttg aggatgacg 360
tatgacgctg ctggcagcgt cactcagcgg gaaacagcgc cgtgctgaaa cattctgacg 420
tagcgcggct cagcctgcgt ccggtgcgct ctacggcggc tggcacaat cgggcgtatc 480
tgcacccaaa tcaagcggcc aatgctgcttg tttagctgtgct cattctgacgtctgaaagg 540
gagaaaggt ttcgggacag cagcgaatc cccatcttcacc cattctgacgtc ggttgactg 600
ttcgctcatg ccggaagaat aagaaacag aatggtttcttt aaaaagatc aacgggtacg 660
gctctgattgt ggcttgctct gtatggctt cagcgaagc gcccgagccg aagcgcgacg 720
tcgcgtcgct atccggtgc cccccgacag cggcggcacg cggcggcacg 780
gccgacccca tcgtgatgct cgcgtctctt gcacgtatcgct cggcggcacg 840
<210> SEQ ID NO 14
<211> LENGTH: 199
<212> TYPE: PRT
<213> ORGANISM: artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Bacillus caldovelox arginase mutant [S161C]
<400> SEQUENCE: 14
Met Lys Pro Ile Ser Ile Ile Gly Val Pro Met Asp Leu Gly Gln Thr
1  5 10 15
Arg Arg Gly Val Asp Met Gly Pro Ser Ala Met Arg Tyr Ala Gly Val
20 25 30
Ile Glu Arg Leu Glu Arg Leu His Tyr Asp Ile Glu Asp Leu Gly Asp
35 40 45
Ile Pro Ile Gly Lys Ala Glu Arg Leu His Gly Glu Asp Ser Arg
50 55 60
Leu Arg Asn Leu Lys Ala Val Ala Glu Ala Asn Lys Tyr Ala
65 70 75 80
Ala Val Asp Gln Val Val Gln Arg Gly Arg Phe Pro Leu Val Leu Gly
85 90
Gly Asp His Ser Ile Ala Ile Gly Thr Leu Ala Gly Val Ala Lys His
100 105 110
Tyr Glu Arg Leu Gly Val Ile Trp Tyr Asp Ala His Gly Asp Val Asn
115 120 125
Thr Ala Glu Thr Ser Pro Ser Gly Asn Ile His Glu Met Pro Leu Ala
130 135 140
Ala Ser Leu Gly Phe Gly His Pro Ala Leu Thr Gln Ile Gly Gly Tyr
145 150 155 160
Cys Pro Lys Ile Lys Pro Glu His Val Val Leu Gly Val Arg Ser
165 170 175
Leu Asp Glu Gly Lys Lys Lys Phe Ile Arg Glu Gly Lys Ile Ile Lys
180 185 190
Tyr Thr Met His Glu Val Asp Arg Leu Gly Met Thr Arg Val Met Glu
195 200 205
Glu Thr Ile Ala Tyr Leu Lys Glu Arg Thr Asp Gly Val His Leu Ser
210 215 220
Leu Asp Leu Asp Gly Leu Asp Pro Ser Asp Ala Pro Gly Val Gly Thr
225 230 235 240
Pro Val Ile Gly Leu Thr Tyr Arg Glu Ser His Leu Ala Met Glu
245 250 255
<210> SEQ ID NO 15
<211> LENGTH: 918
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: 6xHis-tagged Bacillus caldovelox arginase mutant (6161C)

<400> SEQUENCE: 15
atgaagccaa tttcaattat cggggttccgg atgatttag ggcagacagc ccgcgggcgtt 60
gataggggcc cagggccatg cggtttatcga ggcgttcatg aacgtgtgaa aagctttcact 120
tcgattatag aagatttggg agatattccag atgggaagag cagagaggtt ggcagacagaa 180
ggaggcattc ggtggcgcac tttgaacagc gttgccggaag ccagcagcagaa actttggccc 240
gcggtgacag cctcgttctca cggggtggtcc tttccgccttgg tgggggctgg ccacccacagc 300
atccgctag cgtcattcgcg cgggtttggaag aacatttag agcggctttgg atgcatttgg 360
tattgacctgc atgtgctttg caggcttggc aacgtttttg ccgttttattgc ctcgtttttgg 420
atggggccgt cgaccggtcg ccgggtgtttgg ccgggtgtttgg caggctttttc ctcgtttttgg 480
tgccacaaaa ctagctggcg ttgatccgga tccgctttcc tgcagatgag 540
gagagaccgc tattgcggct gaaaggtacct aaaaatgtatca ctagatgagc gttgatgagc 600
cctggattsca ggaagccagg acacgtctttt cccagaactg acaggttggac 660
gttgctcggg ggtggttcgg taccaagccag cagcagcagag ggtgaagcagc 720
cggctcgggg gggatgactc gccggtgtcc cagcagcagag ggtgaagcagc 780
gcggcgtctac tggcttgcgg gaaatgtgcc cagcagcagag ggtgaagcagc 840
aagccagagc ctagctggcg ttgatccgga tccgctttcc tgcagatgagc 900
caccctgcctg acactaas 918

<210> SEQ ID NO 16
<211> LENGTH: 395
<212> TYPE: PRT
<213> ORGANISM: artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: 6xHis-tagged Bacillus caldovelox arginase mutant (6161C)

<400> SEQUENCE: 16
Met Lys Pro Ile Ser Ile Ile Gly Val Pro Met Asp Leu Gly Gln Thr 1 5 10 15
Arg Arg Gly Val Asp Met Gly Pro Ser Ala Met Arg Tyr Ala Gly Val 20 25 30
Ile Glu Arg Leu Arg Leu His Tyr Asp Ile Glu Asp Leu Gly Asp 35 40 45
Ile Pro Ile Gly Lys Ala Glu Arg Leu His Glu Gly Asp Ser Arg 50 55 60
Leu Arg Asn Leu Lys Ala Val Ala Glu Ala Asp Leu Ala Ala 65 70 75 80
Ala Val Asp Gln Val Val Gln Arg Gly Arg Phe Pro Leu Val Leu Gly 85 90 95
Gly Asp His Ser Ile Ala Ile Gly Thr Leu Ala Gly Val Ala Lys His
100   105
110
Tyr Glu Arg Leu Gly Val Ile Thr Tyr Asp Ala His Gly Asp Val Asn
115   120   125
Thr Ala Glu Thr Ser Pro Ser Gly Asn Ile His Gly Met Pro Leu Ala
130   135   140
145
Ala Ser Leu Gly Phe Gly His Pro Ala Leu Thr Glu Ile Gly Gly Tyr
150   155   160
165
Cys Pro Lys Ile Lys Pro Glu His Val Val Leu Ile Gly Val Arg Ser
170   175
180
Leu Asp Glu Gly Lys Lys Phe Ile Arg Glu Gly Lys Lys Ile
185   190
195
200
205
Tyr Thr Met His Glu Val Asp Arg Leu Gly Met Thr Arg Val Met Glu
210   215   220
225
230
235
240
245
250
255
Glu Thr Ile Ala Tyr Leu Lys Glu Arg Thr Asp Gly Val His Leu Ser
260   265
270
Leu Asp Leu Asp Gly Leu Asp Pro Ser Asp Ala Pro Gly Val Gly Thr
275   280
285
Met Leu Ala Glu Ala Glu Ile Thr Ser Ala Glu Phe Val Glu Val
290   295
300
His
305

<210> SEQ ID NO 17
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer
<400> SEQUENCE: 17

gatatacata tgcacacca tcac

<210> SEQ ID NO 18
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer
<400> SEQUENCE: 18

agttcaggat cttaacttag gtggttaag gtgttc

<210> SEQ ID NO 19
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Primer
<400> SEQUENCE: 19

ggggtgtcct ccttatct gccaag
What is claimed is:

1. A pharmaceutical composition for treating an arginine-dependent disease comprising a polyethylene glycol-arginase conjugate having a polyethylene glycol moiety covalently attached to a genetically-modified human arginase, wherein said genetically-modified human arginase has a single amino acid position for covalently attaching to the polyethylene glycol moiety, wherein said polyethylene glycol-arginase conjugate has a serum circulation half-life higher than the serum circulation half-life of pure, unmodified human arginase, wherein said polyethylene glycol-arginase conjugate has decreased immunogenicity compared to the immunogenicity of pure, unmodified human arginase, wherein said single amino acid position is sufficiently far from the active site of the genetically-modified human arginase such that the polyethylene glycol attachment does not interfere with the active site, wherein the genetically-modified Bacillus caldovelox arginase comprises SEQ ID NO: 8 and the single amino acid position for the attachment of polyethylene glycol is position 161 of SEQ ID NO: 8 (Cys). [17]

3. The pharmaceutical composition of claim 1 wherein the ratio of said polyethylene glycol moiety to the genetically-modified human arginase is substantially one.

4. The pharmaceutical composition of claim 2 wherein the ratio of said polyethylene glycol moiety to the genetically-modified Bacillus caldovelox arginase is substantially one.

5. The pharmaceutical composition of claim 1 wherein the polyethylene glycol is a single chain or branched chain polyethylene glycol.

6. The pharmaceutical composition of claim 2 wherein the polyethylene glycol is a single chain or branched chain polyethylene glycol.

7. The pharmaceutical composition of claim 1 further comprising a pharmaceutically acceptable carrier, excipient, or auxiliary agent.

8. The pharmaceutical composition of claim 2 further comprising a pharmaceutically acceptable carrier, excipient, or auxiliary agent.

9. The pharmaceutical composition of claim 1 wherein the arginine-dependent disease is an arginine-dependent cancer or a viral infection by a virus selected from HIV, hepatitis B, and hepatitis C.

10. The pharmaceutical composition of claim 2 wherein the arginine-dependent disease is an arginine-dependent cancer or a viral infection by a virus selected from HIV, hepatitis B, and hepatitis C.

* * * *