PHARMACEUTICAL COMPOSITION COMPRISING ALBUMIN-BINDING ARGININE DEIMINASE FOR CANCER TARGETING TREATMENT

Cross-reference to Related Application

[0001] The present application claims benefit from US provisional patent application serial number 61/773,214 filed March 6, 2013 and US non-provisional patent application serial number 14/197,236 filed March 5, 2014, and the disclosures of which are incorporated herein by reference in its entirety.

Technical Field

[0002] The present invention describes albumin-binding arginine deiminase (AAD) fusion protein that has been genetically modified to create a material having high activity and long *in vivo* half-life. The present invention further describes the designs for DNA and protein engineering for creating different AAD fusion proteins. The AAD fusion proteins can be isolated and purified from soluble fraction and insoluble fraction (inclusion bodies) of the crude proteins. The present invention further relates to albumin-binding arginine deiminase-containing pharmaceutical compositions for cancer targeting treatment and curing arginine-dependent diseases in humans and other animals.

Background of the Invention

[0003] The incidence of pancreatic cancer, colon cancer, liver cancer, melanoma and cervical cancer in the worldwide population is increasing. Effective treatments for these diseases are urgently needed. In many types of cancer including leukemia, melanoma, pancreatic, colon, renal cell carcinoma, lung, prostate, breast, brain, cervical and liver cancers, the cancer cells are auxotrophic for arginine since they lack of expression of argininosuccinate synthetase (ASS), making these cancers excellent targets for arginine depletion therapy.

[0004] Arginine is a semi-essential amino acid for humans and other mammals. It can be synthesized from citrulline *via* a two step process catalyzed by the urea cycle enzymes

argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL). Arginine can be metabolized to ornithine by the enzyme arginase, and ornithine can be converted to citrulline by ornithine carbamoyltransferase (OTC) in the mitochondria. The citrulline can be utilized to synthesize arginine again. Normal cells usually do not require an exogenous supply of arginine for growth because of the abundant catalytic activity of ASS and ASL. In contrast, many types of cancers do not express ASS and therefore are auxotrophic for arginine. Their growth is dependent on arginine solely obtained from blood circulation. Therefore, targeting circulating arginine by using arginine-degrading enzymes is a feasible strategy to inhibit ASS-negative tumor growth [Feun et al., Curr. Pharm. Des. 14:1049-1057 (2008); Kuo et al., Oncotarget. 1:246-251 (2010)]

[0005] Arginine can be degraded by arginase, arginine decarboxylase, and arginine deiminase (ADI). Among them, arginine deiminase (ADI) appears to have the highest affinity for arginine (a low K_m value). ADI converts arginine to citrulline and ammonia, the metabolites of the urea cycle. Unfortunately, ADI can only be found in prokaryotes e.g. *Mycoplasma sp*. There are some problems associated with the isolation and purification of ADI from prokaryotes. ADI isolated from *Pseudomonas pudita* fails to exhibit efficacy *in vivo* because of its low enzymatic activity in neutral pH. ADI produced from *Escherichia coli* is enzymatically inactive and subsequently requires multiple denaturation and renaturation process which raises the subsequent cost of production.

[0006] As the native ADI is found in microorganisms, it is antigenic and rapidly cleared from circulation in a patient. The native form of ADI is immunogenic upon injection into human circulation with a short half-life (~4 hours) and elicits neutralizing antibodies [Ensor et al., Cancer Res. 62:5443-5450 (2002); Izzo et al., J. Clin. Oncol. 22:1815-1822 (2004)]. These shortcomings can be remedied by pegylation. Among various forms of pegylated ADI, ADI bound with PEG (molecular weight 20,000) *via* succinimidyl succinate (ADI-PEG 20) has been found to be an efficacious formulation. However, the activity of ADI after pegylation is greatly decreased on the order of 50% [Ensor et al., Cancer Res. 62:5443-5450 (2002)]. The previous attempts to create pegylated ADI resulted in materials that are not homogenous (due to the random attachment of PEG on protein surface Lys residues) and also difficult to characterize and perform quality control during the manufacturing process. Also, PEG is very expensive, greatly increasing the production cost. After the intravenous injection of pegylated ADI *in vivo*, leakage

or detachment of free PEG is observed and the ADI (without PEG) can elicit the immunogenicity problem. Therefore, there is a need for improved cancer-treatment compositions, particularly, improved cancer-treatment compositions that have enhanced activity and *in vivo* half-life.

Summary of the Invention

[0007] In the present invention, albumin-binding arginine deiminase (AAD) fusion protein has increased its activity and plasma half-life in order to efficiently deplete arginine in cancer cells. Native ADI may be found in microorganisms and is antigenic and rapidly cleared from circulation in a patient. The present invention constructs different AAD fusion proteins with one or two albumin-binding proteins to maintain high activity with longer *in vivo* half-life (at least 5 days of arginine depletion after one injection). In the present invention, the albumin binding protein in the AAD fusion protein product does not appear to influence its specific enzyme activity but instead appears to increase the circulating half-life. The specific activities of wild-type ADI and AAD fusion protein in the present invention are 8.4 and 9.2 U/mg (at physiological pH 7.4), respectively.

[0008] In its broadest sense, the present invention provides an albumin-binding arginine deiminase fusion protein comprising a first portion comprising one or two components selected from an albumin-binding domain, an albumin-binding peptide or an albumin-binding protein(s) fused to a second portion comprising arginine deiminase to form the albumin-binding arginine deiminase fusion protein such that the albumin-binding arginine deiminase fusion protein retains the activity of arginine deiminase and is also able to bind serum albumin.

[0009] The present invention further relates to albumin-binding arginine deiminase (AAD) fusion protein –containing pharmaceutical compositions for targeted cancer treatment in humans and other animals. The first aspect of the present invention is to construct the modified AAD fusion protein with high activity against cancer cells. The second aspect of the present invention is to purify AAD fusion protein with high purity from both soluble and insoluble fractions of the crude proteins. The third aspect of the present invention is to lengthen the half-life of AAD fusion protein as it can bind to albumin very well in the circulation. The fourth aspect of the present invention is to provide a method of using the AAD-containing pharmaceutical composition of the present invention for treating cancer by administering said composition to a

subject in need thereof suffering from various tumors, cancers or diseases associated with tumors or cancers or other arginine-dependent diseases.

[0010] The AAD fusion protein of the present invention is also modified to avoid dissociation into albumin-binding protein and ADI such that it becomes more stable and has a longer half-life in circulation. ADI is fused to an albumin-binding domain/peptide/protein in AAD fusion product to extend the plasma half-life and reduce the immunogenicity of the fusion product. Albumin binding domain (ABD) is a peptide that binds albumin in the blood. There are different variants of ABD showing different or improved human serum albumin (HSA) affinities. Different variants of ABD can be constructed and can be fused to ADI. Unlike the naturally occurring ADI, this longer half-life property facilitates the depletion of arginine efficiently in cancerous cells, cancer stem cells and/or cancer progenitor cells.

[0011] The pharmaceutical composition containing AAD fusion protein can be used for intravenous (i.v.) injection (for rapid-acting dosage of medication) and intramuscular (i.m.) injection (for fairly rapid-acting and long-lasting dosage of medication). The application of AAD fusion protein in the present invention can be used in the treatment of various cancers such as pancreatic cancer, leukemia, head and neck cancer, colorectal cancer, lung cancer, breast cancer, prostate cancer, cervical cancer, liver cancer, nasopharyngeal cancer, esophageal cancer and brain cancer. The present invention is directed to AAD fusion proteins, to methods of treating cancer, to methods of treating and/or inhibiting metastasis of cancerous tissue, and to methods of curing arginine-dependent diseases.

[0012] The method of the present invention also includes using a combination of different chemotherapeutic drugs and/or radiotherapy with the AAD fusion protein of the present invention to give a synergistic effect on cancer treatment.

Brief Description of the Drawings

[0013] **FIG. 1** shows the design approach for construction of different AAD fusion proteins with one or two albumin-binding domain/peptide/protein(s) in three-dimensional structure. One or two albumin-binding domain/peptide/protein(s) can be fused to ADI to form the AAD fusion protein. The position of albumin-binding domain/peptide/protein is far from the ADI active site.

The albumin-binding domain/peptide/protein can be fused to the N-terminus or/and C-terminus of ADI. The structure in this figure is based on the *Mycoplasma arginini* ADI structure (Protein Data Bank: 1LXY). (A) Native ADI; (B) AAD fusion protein with two ABD or ABD1; (C) AAD fusion protein with one ABD or ABD1 at N-terminus; (D) AAD fusion protein with one ABD or ABD1 at C-terminus.

[0014] **FIG. 2** shows the sequence alignment for ADI in some bacterial species including *Mycoplasma arginini* (SEQ ID No. 23), *Lactococcus lactis* (SEQ ID No. 24), *Bacillus cereus* (SEQ ID No. 25) and *Bacillus licheniformis* (SEQ ID No. 26).

[0015] **FIG. 3** shows the designs and amino acid sequences for different AAD fusion proteins originated from *Mycoplasma arginini* (A to E) and AAD fusion protein originated from *Bacillus cereus* (F).

[0016] **FIG. 4** shows the creation of AAD fusion protein in two embodiments (A) and (B) by the use of intein-fusion proteins and expressed protein ligation (CBD, chitin binding domain) under the following schemes; (C) C-terminal fusion; (D) N-terminal fusion; (E) Intein-mediated protein ligation.

[0017] **FIG. 5** shows the plasmid map of the expression vector constructed for producing AAD fusion protein.

[0018] **FIG. 6** shows the (A) gene map, (B) nucleotide sequence (SEQ ID No. 44) and (C) amino acid sequence (SEQ ID No. 40) of His-ABD-PolyN–ADI. (ADI: the *Mycoplasma arginini* ADI)

[0019] **FIG. 7** shows the (A) gene map, (B) nucleotide sequence (SEQ ID No. 45) and (C) amino acid sequence (SEQ ID No. 41) of His-ABD-PolyN–bcADI. (bcADI, the *Bacillus cereus* ADI)

[0020] **FIG. 8** shows the expression and purification of AAD fusion protein: (A) AAD is ~90% soluble when expressed at 20^0C (lanes 2 and 3) and ~90% insoluble (inclusion body) when expressed at 37^0C (lanes 4 and 5); (B) The purified AAD fusion protein in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel: lane 1, purified AAD fusion protein (52.8 kDa); lane 2, molecular weight marker.

[0021] **FIG. 9** illustrates that AAD fusion protein depletes arginine efficiently and inhibits the growth of various types of human cancer cell lines in *in vitro* tissue culture studies, including human melanoma (A375), human colon carcinoma (HCT116), and human pancreatic cancer (PancI).

[0022] **FIG. 10** shows the albumin binding results of AAD fusion protein: (A) A non-denaturing native polyacrylamide gel (12%) showing the increase in the amount of HSA+AAD complex when the amount of AAD fusion protein (the amino acid sequence is shown in SEQ ID NO: 36; FIG. 3A) added increases. The mole ratios of human serum albumin (HSA): AAD in lanes 3-6 are 1:1, 1:2, 1:5, and 1:15, respectively. Lanes 1 and 2 represent HSA and AAD at 6 and 30 pmole, respectively; (B) In another experiment based on AAD fusion protein (SEQ ID NO: 40; FIG. 3E), an albumin: AAD ratio of 1:8 is sufficient to bind all the albumin present (lane 5).

[0023] **FIG. 11** is a graph showing the dose response of AAD fusion protein on plasma arginine levels in mice. A dose of 100 μg of AAD is sufficient to deplete plasma arginine for at least 5 days.

Definitions

[0024] The term "cancer stem cell" refers to the biologically distinct cell within the neoplastic clone that is capable of initiating and sustaining tumor growth *in vivo* (i.e. the cancer-initiating cell).

Detailed Description of the Invention

[0025] Arginine is a semi-essential amino acid for humans and other mammals. It can be synthesized from citrulline *via* a two step process catalyzed by urea cycle enzymes argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL). Arginine can be metabolized to ornithine by the enzyme arginase, and ornithine can be converted to citrulline by ornithine carbamoyltransferase (OTC) in the mitochondria. The citrulline can be utilized to synthesize arginine again. Normal cells do not typically require an exogenous supply of arginine for growth because of the abundant catalytic activity of ASS and ASL. In contrast, many types of cancers do not express ASS and are therefore auxotrophic for arginine. Their growth is solely

dependent on arginine from circulation. Therefore, targeting circulating arginine by using arginine-degrading enzymes is a feasible strategy to inhibit ASS-negative tumor growth.

[0026] Arginine can be degraded by arginine deiminase (ADI). ADI converts arginine to citrulline and ammonia, the metabolites of the urea cycle. Unfortunately, ADI can only be found in prokaryotes e.g. *Mycoplasma sp*. There are many problems associated with the isolation and purification of arginine deiminase from prokaryotes. ADI isolated from *Pseudomonas pudita* failed to exhibit efficacy *in vivo* because of its low enzymatic activity in neutral pH. ADI produced from *Escherichia coli* is enzymatically inactive and subsequently requires multiple denaturation and renaturation process which raised the subsequent cost of production. The plasma half-life of the native form of ADI is short (~4 hours) upon injection into human circulation [Ensor et al., Cancer Res. 62:5443-5450 (2002); Izzo et al., J. Clin. Oncol. 22:1815-1822 (2004)]. These shortcomings can be partially remedied by pegylation. Among various forms of pegylated ADI, ADI bound with PEG (molecular weight 20,000) *via* succinimidyl succinate (ADI-PEG 20) has been found to be an efficacious formulation. However, the activity of ADI after pegylation is greatly decreased (by ~50%) [Ensor et al., Cancer Res. 62:5443-5450 (2002); Wang et al., Bioconjug. Chem. 17:1447-1459 (2006)]. Also, the succinimidyl succinate PEG linker can easily be hydrolyzed and detached from the protein, causing immunogenic problems after a short period of use in the body. Therefore, there is a need for improved cancer-treatment compositions, particularly, improved cancer-treatment compositions with enhanced activity.

[0027] ADI isolated from *P. pudita* failed to exhibit efficacy *in vivo* because it had little enzyme activity at a neutral pH and was rapidly cleared from the circulation of experimental animals. ADI derived from *Mycoplasma arginini* is described, for example, by Takaku et al, Int. J. Cancer, 51:244-249 (1992), and U.S. Pat. No. 5,474,928. However, a problem associated with the therapeutic use of such a heterologous protein is its antigenicity. The chemical modification of ADI from *Mycoplasma arginini*, *via* a cyanuric chloride linking group, with polyethylene glycol (PEG) was described by Takaku et al., Jpn. J. Cancer Res., 84:1195-1200 (1993). However, the modified protein was toxic when metabolized due to the release of cyanide from the cyanuric chloride linking group. In contrast, even for the ADI-PEG20, the PEG linker can easily be hydrolyzed and detached from the protein, causing immunogenic problems after a short period of

use in the body. Therefore, there is a need for compositions which degrade non-essential amino acids and which do not have the problems associated with the prior art.

[0028] In many types of cancer including melanoma, pancreatic, colon, leukemia, breast, prostate, renal cell carcinoma and liver cancers, cancer cells are auxotrophic for arginine since they lack of expression of argininosuccinate synthetase (ASS), making them excellent targets for arginine depletion therapy. In this invention, albumin-binding arginine deiminase (AAD) fusion proteins have high activity with long half-lives for efficient depletion of arginine in cancer cells.

[0029] The size of the monomer for ADI is on the order of 45 kDa and it exists as dimer (on the order of 90 kDa) [Das et al., Structure. 12:657-667 (2004)]. A design for construction of an AAD fusion protein is shown in FIG. 1. One or two albumin-binding domain/peptide/protein(s) with or without linker(s), SEQ ID NO: 46-49, are fused to ADI to form the AAD fusion protein. It is noteworthy that the selection of one or two particular albumin-binding domain/peptide/protein(s) can be made depending upon the type of cancer tissue to be targeted, the desired size and half-life of the resulting fusion protein, and whether a domain or entire protein is selected. Further, the selected albumin-binding material may be the same or different. That is, a protein and a peptide can be fused, two proteins, two domains, a domain and a protein, etc., as long as the resultant molecule retains the activity of the ADI and is also able to bind serum albumin with neither function of one portion of the fusion protein being interfered with by the other portion of the fusion protein. The position of the albumin-binding domain/peptide/protein is far from the active site. The albumin-binding domain/peptide/protein can be fused to the N-terminus or/and C-terminus of ADI. There are different variants of ABD showing different or improved human serum albumin (HSA) affinities. Different variants of ABD can be constructed and can be fused to ADI. Some micro-organisms endowed with ADI (for example *Pseudomonas* sp) cannot be used, due to their potential pathogenicity and pyrogenicity. The source of ADI can be from, but not limited to, different microorganisms, e.g. *Mycoplasma* (e.g. *Mycoplasma arginini, Mycoplasma arthritidis, Mycoplasma hominis*), *Lactococcus* (e.g. *Lactococcus lactis*), *Pseudomonas* (e.g. *Pseudomonas plecoglossicida, Pseudomonas putida, Pseudomonas aeruginosa*), *Steptococcus* (e.g. *Streptococcus pyogenes, Streptococcus pneumonia, Streptococcus pneumoniae), Escherichia*, *Mycobacterium* (e.g. *Mycobacterium tuberculosis*) and *Bacillus* (e.g. *Bacillus licheniformis, Bacillus cereus*). It is preferred that ADI is cloned from

Mycoplasma arginini, *Lactococcus lactis*, *Bacillus licheniformis, Bacillus cereus*, or any combination thereof. Their amino acid sequences with SEQ ID (SEQ ID NO: 23-35) and the sequence alignment for some of the amino acid sequences in FIG. 2 are disclosed herein and also in the literature [Das et al., Structure. 12:657-667 (2004); Wang et al., Bioconjug. Chem. 17:1447-1459 (2006); Ni et al., Appl. Microbiol. Biotechnol. 90:193-201 (2011)].

[0030] The design and amino acid sequence for (A) native *Mycoplasma arginini* ADI protein (SEQ ID NO: 23), (B) different AAD fusion proteins originated from the *Mycoplasma arginini* ADI (SEQ ID NO: 36-40) and (C) AAD fusion protein originated from the *Bacillus cereus* ADI (SEQ ID NO: 41) are shown in FIG. 3. Different AAD fusion proteins are successfully constructed. A linker is inserted between the albumin-binding protein and ADI in the AAD fusion protein in these embodiments.

[0031] On the other hand, a novel AAD fusion protein is also created by the use of intein-fusion proteins and expressed protein ligation (FIG. 4). The novel AAD fusion protein can be formed (1) by reacting the ADI having a N-terminal cysteine residue with a reactive thioester at C-terminus of the ABD, or (2) by reacting the ABD having a N-terminal cysteine residue with a reactive thioester at C-terminus of the ADI so that the ADI and the ABD are linked by a covalent bond. In FIG. 4E, ADI with N-terminal cysteine residue reacts with reactive thioester at the C-terminus of ABD. The thioester tag at the C-terminus of ABD, and an α-cysteine at the N-terminus of ADI are required to facilitate protein ligation. These fragments are produced using a pTWIN1 vector (New England Biolabs) according to the manufacturer's manual. In particular, the gene coding for the ABD-Intein-CBD fusion protein is synthesized and it is cloned into the vector under the control of T7 promoter for expression in *E. coli* (FIG. 4C). The ABD-Intein-CBD fusion protein produced binds to chitin in a column. The amino acid sequence of ABD-Intein-CBD (SEQ ID NO: 42) is shown in FIG. 4A. After thiol-inducible cleavage and elution from the column, the ABD with reactive thioester at its C-terminus is obtained (FIG. 4C). On the other hand, the gene coding for the CBD-Intein-ADI fusion protein is synthesized and cloned into the vector under the control of the T7 promoter for expression in *E. coli* (FIG. 4D). The CBD-Intein-ADI fusion protein produced binds to chitin in a column. The amino acid sequence of the CBD-Intein-ADI (SEQ ID NO: 43) is shown in FIG. 4B. After cleavage at pH 7 and 25°C, and elution from the column, the ADI with α-cysteine at its N-terminus is obtained (FIG. 4D). Finally, the AAD fusion protein is produced by the protein ligation reaction as shown in FIG. 4E.

[0032] Importantly, AAD fusion proteins can be produced and purified in a convenient manner. For example, an AAD fusion protein is successfully expressed and purified from *E. coli* both in soluble fraction and insoluble fraction, and this result is shown in FIG. 8. Furthermore, FIG. 8 shows the purified AAD fusion protein analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The size of the purified AAD fusion protein is determined as 52.8 kDa.

[0033] The pharmaceutical composition of the present invention contains AAD fusion protein with high activity for depleting arginine in tumor cells for cancer treatment. The specific activity of the purified AAD fusion protein is found to be similar to that of the wild-type ADI. IC_{50} is the half maximal inhibitory concentration, that is, it represents the concentration of AAD fusion protein that is required for 50% inhibition of a cancer cell line. The IC_{50} is a measure of the effectiveness of a drug. The IC_{50} of AAD fusion protein (amino acid sequence is shown in SEQ ID NO: 40, FIG. 3E) for different cancer cell lines (human melanoma, A375 & SK-mel-28; human colon carcinoma, HCT116; human pancreatic cancer, PancI; human liver cancer, Sk-hep1; human cervical cancer, C-33A) is shown in TABLE 1. The *in vitro* efficacy of AAD fusion protein on different cancer cell lines is demonstrated in FIG. 9. It illustrates that AAD fusion protein can kill many cancer types, including human melanoma, human colon carcinoma and pancreatic cancer cell lines.

[0034] **TABLE 1**

Cancer cell line	**IC_{50} of AAD (μg/ml)**
A375 (human melanoma)	0.104
SK-mel-28 (human melanoma)	1.92
PancI (human pancreatic cancer)	1
Sk-hep1 (human liver cancer)	10

C-33A (human cervical cancer)	0.063
HCT116 (human colon carcinoma)	1.30

[0035] For the albumin binding study, we have demonstrated successfully that the engineered AAD fusion protein can bind to human serum albumin (HSA). FIG. 10 shows that the AAD fusion protein (amino acid sequence is shown in SEQ ID NO: 40, FIG. 3E) binds to HSA readily. At a mole ratio of 1:5 or 1:15, the formation of the HSA-AAD complex forms according to the construct of FIG. 1 using the linker molecule design. It is expected that the circulating half-life of AAD fusion protein in the blood is increased by the non-covalent HSA-AAD complex formation. Therefore, a long-lasting version of AAD fusion protein has been successfully created.

[0036] No commercial products show high efficacy when compared to the AAD fusion protein-containing pharmaceutical composition prepared in this invention. For uses in cancer treatment, the AAD fusion protein-containing pharmaceutical composition of the present invention serves as an anti-cancer agent to deplete the arginine in tumor tissues. AAD fusion protein is a good candidate to be used in combination with other molecular targeting or cytotoxic agents.

Examples

[0037] The following examples are provided by way of describing specific embodiments of this invention without intending to limit the scope of this invention in any way.

[0038] Several of the Examples below relate to methods of making an albumin-binding arginine deiminase fusion protein. Various techniques can be used including cloning and intein-mediated protein ligation. As used herein, the term "cloning" is broadly used and comprises constructing a fusion gene coding for the albumin-binding arginine deiminase fusion protein, inserting the fusion gene into a vector, inserting the vector into a host organism and expressing a protein that includes an albumin-binding arginine deiminase fusion protein. Numerous variants on this technique can be performed and still fall within the cloning contemplated by the present invention.

[0039] **Example 1**

[0040] **Construction of the gene coding for albumin-binding domain/peptide/protein (ABD)**

[0041] The gene coding for ABD is constructed by two rounds of PCR. In the first round, the PCR reaction mixture (total volume of 25 μl) contains the following materials:

1 x iProof PCR buffer (Bio-Rad)

50 μM dNTP mixture

0.5 unit of iProof DNA Polymerase (Bio-Rad)

10 nM of each of the following oligos

ABD-F1 forward primer (SEQ ID NO: 01):

5′-CATGATGCGAATTCCTTAGCTGAAGCTAAAGTCTTAGCTAACAGAGAACT-3′

ABD-R2 reverse primer (SEQ ID NO: 02):

5′-TAGTCACTTACTCCATATTTGTCAAGTTCTCTGTTAGCTAAGACTTTAGC-3′

ABD-F3 forward primer (SEQ ID NO: 03):

5′-GAACTTGACAAATATGGAGTAAGTGACTATTACAAGAACCTAATCAACAA-3′

ABD-R4 reverse primer (SEQ ID NO: 04):

5′-TACACCTTCAACAGTTTTGGCATTGTTGATTAGGTTCTTGTAATAGTCAC-3′

ABD-F5 forward primer (SEQ ID NO: 05):

5′-GCCAAAACTGTTGAAGGTGTAAAAGCACTGATAGATGAAATTTTAGCTGC-3′

ABD-R6 reverse primer (SEQ ID NO: 06):

5′-AGCTACGATAAGCTTAAGGTAATGCAGCTAAAATTTCATCTATCAGTG-3′

The following PCR program is used:

98°C 30 s; 20 cycles of {98°C 10 s, 50°C 20 s, 72°C 20 s}

[0042] In the second round of PCR, the PCR mixture (total volume of 50 μl) contains the following materials:

1 x iProof PCR buffer (Bio-Rad);

50 μM dNTP mixture;

1 μl of PCR reactant as DNA template from the first round;

1 unit of iProof DNA Polymerase (Bio-Rad);

200 nM of each of the following oligos:

ABD-F7 forward primer (SEQ ID NO: 07):

5′-CATGATGCGAATTCCTTAGCTGAAGCTAAAGTCTTAGCTAACAGAGAACT-3′

ABD-R8 reverse primer (SEQ ID NO: 08):

5′-AGCTACGATAAGCTTAAGGTAATGCAGCTAAAATTTCATCTATCAGTG-3′

The following PCR program is used:

98°C 30 s; 35 cycles of {98°C 10 s, 60°C 20 s, 72°C 20 s}; 72°C 5 min

[0043] A PCR product containing the DNA sequence of ABD (169 bp) is obtained and purified by Qiagen DNA Gel Extraction Kit for cloning purpose.

[0044] **Example 2A**

[0045] **Construction of the fusion gene coding for the AAD fusion protein**

[0046] In the first PCR, the PCR mixture (total volume of 50 μl) contains the following materials:

1 x iProof PCR buffer (Bio-Rad);

50 μM dNTP mixture;

25 ng of *Mycoplasma arginini* genomic DNA;

1 unit of iProof DNA Polymerase (Bio-Rad);

200 nM of each of the following oligos:

ADINde-F forward primer (SEQ ID NO: 09):

5′-ATCGATCGATGTCTGTATTTGACAGTAAATTTAAAGG-3′

ADIhis-R reverse primer (SEQ ID NO: 10):

5′-AGCTAAGGAATTCGCATCATGATGGTGATGGTGGTGGCTACCCCACTTAAC-3′

The following PCR program is used:

98°C 1 min; 35 cycles of {98°C 10s, 50°C 20s, 72°C 40s}; 72°C 5 min

A PCR product of 1280 bp long is obtained and purified by Qiagen DNA Gel Extraction Kit. After that, the second PCR is performed. The PCR mixture (total volume of 50 μl) contains the following materials:

1 x iProof PCR buffer (Bio-Rad);

50 μM dNTP mixture;

10 ng of the 1280 bp PCR product;

10 ng of the 169 bp PCR product;

1 unit of iProof DNA Polymerase (Bio-Rad);

200 nM of each of the following oligos:

ADINde-F forward primer (SEQ ID NO: 11):

5′-ATCGATCGATGTCTGTATTTGACAGTAAATTTAAAGG-3′

ABD-R10 reverse primer (SEQ ID NO: 12):

5′-AGCTACGATAAGCTTAAGGTAATGCAGCTAAAATTTCATCTATCAGTG-3′

The following PCR program is used:

98°C 1 min; 35 cycles of {98°C 10s, 50°C 20s, 72°C 45s}; 72°C 5 min

[0047] A PCR product of 1428 bp is obtained and purified by Qiagen DNA Gel Extraction Kit. Then it is digested with restriction enzymes NdeI and HindIII, and ligated to plasmid pREST A (Invitrogen) that is predigested with the same enzymes. The ligation product is then transformed into *E. coli* BL21 (DE3) cells. The sequence of the constructed fusion gene is confirmed by DNA sequencing.

[0048] **Example 2B**

[0049] **Cloning of His-ABD-PolyN-ADI**

[0050] The construction of **His-ABD-PolyN–ADI** (SEQ ID NO: 40, in FIG. 3E) is done by two steps of overlapping PCR, the PCR fragment obtained from the last step is inserted into the vector pET3a between the NdeI and BamHI sites. The gene map, nucleotide sequence and amino acid sequence of **His-ABD-PolyN–ADI** are shown in FIG. 6.

Primers involved in construction of **His-ABD-PolyN–ADI**:

hisABDNde-F forward primer (SEQ ID NO: 13):

5′-GGAGATATACATATGCATCATCACCATCACCATGATGAAGCCGTGGATG-3′

ABDnn-R1 reverse primer (SEQ ID NO: 14):

5′-TTGTTATTATTGTTGTTACTACCCGAAGGTAATGCAGCTAAAATTTCATC-3′

ABDn-R2 reverse primer (SEQ ID NO: 15):

5′-AGAACCGCCGCTACCATTGTTATTATTGTTGTTACTACCCGA-3′

ADln-F forward primer (SEQ ID NO: 16):

5′-AATAATAACAATGGTAGCGGCGGTTCTGTATTTGACAGTAAATTTAAAGG-3′

ADIBam-R reverse primer (SEQ ID NO: 17):

5′-TAGATCAATGGATCCTTACCACTTAACATCTTTACGTGATAAAG-3′

[0051] In the first round of PCR, 50 µl of reaction volume containing the known concentration of components are prepared in two PCR tubes. In each of the tubes, dNTP, iProof buffer (BIO-RAD), iProof DNA polymerase (BIO-RAD), primers and DNA template are mixed and added up to 50 µl by ddH_2O. The DNA template used in the reaction is a pET3a vector containing the gene of ADI from *Mycoplasma arginini* with a removal of an internal NdeI site mutation without altering the protein sequence of the ADI gene.

[0052] The two reaction tubes contain the primer mixtures of (A) 10 pmol *hisABDNde-F* (SEQ ID NO: 13), 0.5 pmol *ABDnn-R1* (SEQ ID NO: 14) and 10 pmol *ABDn-R2* (SEQ ID NO: 15); and (B) 10 pmol *ADIn-F* (SEQ ID NO: 16) and 10 pmol *ADIBam-R* (SEQ ID NO: 17), respectively.

[0053] The PCR program is set according to the recommended steps in the manual with an annealing and extension temperature (time) at 50 °C (20 s) and 72 °C (40 s), respectively. The

two products generated by PCR with the size of 237 bp and 1278 bp. The products are extracted and applied as template for the next round of PCR.

[0054] In the second overlapping step, the reaction mixture is prepared in a similar way to the first round except the template used was the mixture of 1 pmol of the 237 bp PCR product and 1 pmol of the 1278 bp PCR product from the first round PCR. Primers used are changed to 10 pmol *hisABDNde-F* (SEQ ID NO: 13) and 10 pmol *ADIBam-R* (SEQ ID NO: 17).

[0055] The annealing and extension temperature (time) are 50 °C (20 s) and 72 °C (60 s), respectively. A PCR product with the size of 1484 bp is generated from the reaction. The PCR product is purified and digested with NdeI and BamHI and then ligated into the pre-digested pET3a plasmid. The ligated product is then transformed into *E. coli* BL21 (DE3) for the production of recombinant protein.

[0056] **Example 2C**

[0057] **Cloning of His-ABD-PolyN-bcADI**

[0058] The construction of **His-ABD-PolyN–bcADI** (SEQ ID NO: 41, in FIG. 3F) is done by two steps of overlapping PCR, the PCR fragment obtained from the last step is inserted into the vector pET3a between the NdeI and BamHI sites. The gene map, nucleotide sequence and amino acid sequence of **His-ABD-PolyN–bcADI** are shown in FIG. 7.

Primers involved in construction of **His-ABD-PolyN–bcADI**:

hisABDNde-F2 forward primer (SEQ ID NO: 18):

5′-GGAGATATACATATGCATCATCACCATCACCATGATGAAGCCGTGGATG-3′

bcABDnn-R1 reverse primer (SEQ ID NO: 19):

5′-TTGTTATTATTGTTGTTACTACCCGAAGGTAATGCAGCTAAAATTTCATC-3′

bcABDn-R2 reverse primer (SEQ ID NO: 20):

5′-TTTACCGCCGCTACCATTGTTATTATTGTTGTTACTACCCGA-3′

bcADIn-F forward primer (SEQ ID NO: 21):

5′-AATAATAACAATGGTAGCGGCGGTAAACATCCGATACATGTTACTTCAGA-3′

bcADIBam-R reverse primer (SEQ ID NO: 22):

5′-TAGATCAATGGATCCCTAAATATCTTTACGAACAATTGGCATAC-3′

[0059] In the first round of PCR, 50 µl of reaction volume containing the known concentration of components are prepared in two PCR tubes. In each of the tubes, dNTP, iProof buffer (BIO-RAD), iProof DNA polymerase (BIO-RAD), primers and DNA template are mixed and added up to 50 µl by ddH_2O. The DNA template used in the reaction is a pET3a vector containing the gene of ADI from *Bacillius cereus* with a removal of an internal NdeI site mutation without altering the protein sequence of the ADI gene.

[0060] The two reaction tubes contain the primer mixtures of (A) 10 pmol *hisABDNde-F2* (SEQ ID NO: 18), 0.5 pmol *bcABDnn-R1* (SEQ ID NO: 19) and 10 pmol *bcABDn-R2* (SEQ ID NO: 20); and (B) 10 pmol *bcADIn-F* (SEQ ID NO: 21) and 10 pmol *bcADIBam-R* (SEQ ID NO: 22), respectively. The PCR program is set according to the recommended steps in the manual with an annealing and extension temperature (time) at 50 °C (20 s) and 72 °C (40 s), respectively. The two products are generated by PCR with the size of 237 bp and 1250 bp. The products are extracted and applied as template for the next round of PCR.

[0061] In the second overlapping step, the reaction mixture is prepared in a similar way to the first round except the template used is the mixture of 1 pmol of the 237 bp PCR product and 1 pmol of the 1250 bp PCR product from the first round PCR. Primers used are changed to 10 pmol *hisABDNde-F2* (SEQ ID NO: 18) and 10 pmol *bcADIBam-R* (SEQ ID NO: 22).

[0062] The annealing and extension temperature (time) are 50 °C (20 s) and 72 °C (60 s), respectively. A PCR product with the size of 1512 bp is generated from the reaction. The PCR product is purified and digested with NdeI and BamHI and then ligated into the pre-digested pET3a plasmid. The ligated product is then transformed into *E. coli* BL21 (DE3) for the production of recombinant protein.

[0063] **Example 3**

[0064] **Expression and purification of the AAD fusion protein**

[0065] For preparing the seed culture, the strain *E. coli* BL21(DE3) carrying the plasmid encoding the AAD fusion protein (FIG. 5) is cultured in 5 ml of 2xTY medium, 30°C, 250 rpm, overnight. The overnight seed culture (2.5 ml) is added to 250 ml of 2xTY, 37°C, 250 rpm, 2.5 h (until $OD_{600} \approx 0.6$-0.7). When the OD_{600} reached, IPTG is added to the culture (0.2 mM final concentration). The growth is continued for 22 more hours at 20°C and then the cells are collected by centrifugation. The cell pellet is resuspended in 25 ml of 10 mM sodium phosphate buffer, pH 7.4. The cells are lysed by sonication. The soluble portion is collected after centrifugation. The fusion protein (containing a His tag) is then purified by nickel affinity chromatography. TABLE 2 shows that cultivation temperature is an important factor in affecting the solubility of AAD fusion protein (amino acid sequence is shown in SEQ ID NO: 40, FIG. 3E) obtained from the expression host.

[0066] For isolating the soluble fraction of AAD fusion protein, the cell pellet is resuspended in 25 ml of 10 mM sodium phosphate buffer, pH 7.4. The cells are lysed by sonication. The soluble portion is collected after centrifugation. The AAD fusion protein (contains a His tag) is then purified by nickel affinity chromatography.

[0067] For isolating the insoluble fraction of AAD fusion protein, the cell pellet is resuspended in 25 ml of 20 mM Tris-HCl, pH 7.4, 1% Triton X-100. The cells are lysed by sonication. The insoluble portion (inclusion bodies) is collected by centrifugation. The protein is unfolded by resuspending in 10 ml of 20 mM Tris-HCl, pH 7.4, 6 M Guanidine HCl, and vortexed until it becomes soluble. The protein is refolded by adding the unfolded protein solution drop by drop into a fast stirring solution of 100 ml of 20 mM Sodium phosphate buffer, pH 7.4. The insoluble

materials are removed by centrifugation. Salting out of the protein is performed by adding solid ammonium sulphate powder into the supernatant to achieve 70% saturation. The insoluble portion is collected by centrifugation and it is resuspended in 10 ml of 20 mM sodium phosphate buffer. The AAD fusion protein (contains a His tag) is then purified by nickel affinity chromatography.

[0068] **TABLE 2**

AAD	1	2	3
Cultivation temperature (°C)	30	20	37
Yield (mg) / 250ml culture	~0.66	~12.0	~7.0
solubility	50% soluble	90% soluble	90% inclusion body
IC_{50} (μg/ml) on A375 cells	0.10	0.68	0.23

[0069] **Example 4**

[0070] **Enzyme activity assay and Enzyme kinetics for AAD fusion protein**

[0071] To determine the enzyme activity for wild-type ADI and AAD fusion protein in the present invention, the diacetyl monoxime (DAM) - thiosemicarbazide (TSC) assay for citrulline detection is used. The reaction is shown below.

[0072] L-Arginine $\xrightarrow{\text{arginine deiminase (ADI) or AAD fusion protein}}$ L-Citrulline + Ammonia

[0073] This assay is run by adding sample to a color reagent, which is made by mixing acidic ferric chloride solution with DAM-TSC solution. Briefly, enzyme is incubated with 20 mM arginine, 10 mM sodium phosphate pH 7.4 for 5 min at 37°C. The reaction mixture is heated at 100°C for 5 min to develop the color and read at 540 nm (light path = 1 cm). A standard curve is constructed using various concentrations of citrulline. One unit of the ADI native enzyme is the amount of enzyme activity that converts 1 μmol of arginine to 1 μmol of citrulline per minute at

37°C under the assay conditions. The specific activities of wild-type ADI and AAD fusion protein in the present invention are 8.4 and 9.2 U/mg (at pH 7.4, physiological pH) respectively. The specific activities for wild-type ADI and AAD fusion protein at different pH range (from pH 5.5 to 9.5) are also determined, and the optimum pH is at 6.5. Therefore, the results indicate that AAD fusion protein depletes arginine efficiently, as the fusion with albumin-binding protein does not affect enzyme activity of ADI.

[0074] The Michaelis constant K_m is the substrate concentration at which the reaction rate is at half-maximum, and is an inverse measure of the substrate's affinity for the enzyme. A small K_m indicates high affinity for the substrate, and it means that the rate will approach the maximum reaction rate more quickly. For determination of the enzyme kinetics or K_m value, the activity of wild-type ADI and AAD fusion protein are measured under different concentration of substrate arginine (2000 μM, 1000 μM, 500 μM, 250 μM, 125 μM, 62.5 μM) at pH 7.4. The measured K_m values of the AAD fusion protein shown in FIG. 3E (SEQ ID NO: 40, ADI protein is originated from *Mycoplasma arginini*) and AAD fusion protein shown in FIG. 3F (SEQ ID NO: 41, ADI protein is originated from *Bacillus cereus*) are 0.0041 mM and 0.132 mM respectively. The results suggest that the fusion to ABD did not affect the binding affinity of the different AAD fusion proteins to arginine.

[0075] **Example 5**

[0076] **Cell proliferation assay and *in vitro* efficacy of AAD fusion protein on cancer cell lines**

[0077] Culture medium DMEM is used to grow the human melanoma A375 & SK-mel-28, human pancreatic cancer PancI and human cervical cancer C-33A cell lines. The EMEM medium is used to culture the SK-hep 1 liver cancer and C-33A cervical cancer cell line. Cancer cells ($2\text{-}5\times10^3$) in 100 μl culture medium are seeded to the wells of 96-well plates and incubated for 24 h. The culture medium is replaced with medium containing different concentrations of AAD fusion protein. The plates are incubated for an additional 3 days at 37°C in an atmosphere of 95% air/5% CO_2. MTT assay is performed to estimate the number of viable cells in the culture according to manufacturer's instructions. The amount of enzyme needed to achieve 50% inhibition of cell growth is defined as IC_{50}.

[0078] As shown in TABLE 1 and FIG. 9, the results indicate that AAD fusion protein depletes arginine efficiently and inhibits the growth of various types of human cancer cell lines in *in vitro* tissue culture studies. For example, human melanoma, human colon carcinoma, human pancreatic cancer, human liver cancer and human cervical cancer, all have low values of IC_{50} (see TABLE 1), as these cancer types are all inhibited by AAD fusion protein readily. As predicted, AAD fusion protein would inhibit all cancer types that are arginine-dependent (for example, the ASS-negative cancers).

[0079] **Example 6**

[0080] ***In vivo*** **half-life determination of AAD fusion protein**

[0081] Balb/c mice (5-7 weeks) are used in this study and they are allowed to acclimatize for a week before the experiment. Mice (n=3) are separated into four groups and injected with 0, 100, 500 or 1000 μg of AAD fusion protein (SEQ ID NO: 40, FIG. 3E) in 100 μl PBS intraperitoneally, respectively. Blood of each mouse is collected at 0 h and Day 1-7. Sera are obtained after centrifugation. The sera are then deproteinised and analyzed by amino acid analyzer for arginine.

[0082] As shown in FIG. 11, AAD fusion protein (SEQ ID NO: 40, FIG. 3E), even at the lowest dosage of 100 μg, depletes plasma arginine efficiently at Day 1, 3 and 5, suggesting that AAD can deplete arginine *in vivo* efficiently for at least 5 days. The arginine level returns to normal gradually at Day 6 and Day 7 in all treatment groups.

[0083] **Example 7**

[0084] ***In vivo*** **efficacy of AAD fusion protein on cancer cell xenografts**

[0085] Nude balb/c mice (5-7 weeks) are used in this study and they are allowed to acclimatize for a week before the experiment. Mice are inoculated subcutaneously with $2x10^6$ cancer cells in 100 μl of fresh culture medium. Ten days later, the mice are randomly separated into control and treatment group. Control group receives 100 μl PBS and treatment group receives 100 μl AAD fusion protein intraperitoneally weekly. Tumor size is measured by caliper and tumor volume is

calculated using formula: (length x width^2)/2. Blood draw are obtained at Day 5 after each treatment for plasma measurement of arginine.

WHAT IS CLAIMED IS:

1. An albumin-binding arginine deiminase fusion protein comprising a first portion comprising one or two components selected from an albumin-binding domain, an albumin-binding peptide or an albumin-binding protein(s) fused to a second portion comprising arginine deiminase to form the albumin-binding arginine deiminase fusion protein, and one or more linker molecules; the first portion being positioned far from active site of the second portion by said linker molecule such that the albumin-binding arginine deiminase fusion protein retains the activity of arginine deiminase and binds serum albumin with neither function of one portion of the fusion protein being interfered with by the other portion of the fusion protein, wherein pegylation of said arginine deiminase is avoided, and wherein the albumin-binding arginine deiminase fusion protein comprises a sequence selected from SEQ ID NO: 36, 37, 38, 39, 40, or 41.

2. The albumin-binding arginine deiminase fusion protein of claim 1 wherein the two components of the first portion are the same.

3. The albumin-binding arginine deiminase fusion protein of claim 1 wherein the two components of the first portion are different.

4. The albumin-binding arginine deiminase fusion protein of claim 1 wherein the albumin-binding domain is SEQ ID NO: 46, 47, 48, or 49.

5. The albumin-binding arginine deiminase fusion protein of claim 1 wherein the albumin binding peptide is SEQ ID NO: 46, 47, 48, or 49.

6. The albumin-binding arginine deiminase fusion protein of claim 1 wherein the albumin binding protein is SEQ ID NO: 46, 47, 48, or 49.

7. The albumin-binding arginine deiminase fusion protein of claim 1 wherein the linker molecule comprises a sequence selected from SEQ ID NO: 50, 51, 52, 53, or serine-glycine-serine (SGS) amino acid sequence.

8. The albumin-binding arginine deiminase fusion protein of claim 1 further comprising at least one of Poly-N or a His tag.

9. The albumin-binding arginine deiminase fusion protein of claim 1 wherein the fusion comprises a remaining portion of an intein-mediated protein ligation between the first portion and the second portion.

10. The albumin-binding arginine deiminase fusion protein of claim 9 wherein the intein-mediated protein comprises a chitin binding domain.

11. The albumin-binding arginine deiminase fusion protein of claim 1 wherein the arginine deiminase is selected from arginine deiminase produced from a *Mycoplasma*, *Lactococcus*, *Pseudomonas, Streptococcus, Escherichia, Mycobacterium* or *Bacillus* microorganism.

12. The albumin-binding arginine deiminase fusion protein of claim 11 wherein the arginine deiminase is produced from *Mycoplasma arginini, Lactococcus lactis, Bacillus licheniformis, Bacillus cereus, Mycoplasma arthritidis, Mycoplasma hominis, Streptococcus pyogenes,*

Streptococcus pneumoniae, Mycobacterium tuberculosis, Pseudomonas plecoglossicida, Pseudomonas putida, Pseudomonas aeruginosa or a combination thereof.

13. The albumin-binding arginine deiminase fusion protein of claim 1 wherein the fusion protein is formed by reacting the arginine deiminase having a N-terminal cysteine residue with a reactive thioester at C-terminus of the albumin-binding domain so that the arginine deiminase and the albumin-binding domain are linked by a covalent bond.

14. The albumin-binding arginine deiminase fusion protein of claim 1 wherein the fusion protein is formed by reacting the albumin-binding domain having a N-terminal cysteine residue with a reactive thioester at C-terminus of the arginine deiminase so that the arginine deiminase and the albumin-binding domain are linked by a covalent bond.

15. The albumin-binding arginine deiminase fusion protein of claim 1 wherein the fusion protein is formed by using SEQ ID NO: 42 and 43 and by reacting the arginine deiminase having a N-terminal cysteine residue with a reactive thioester at C-terminus of the albumin-binding domain so that the arginine deiminase and the albumin-binding domain are linked by a covalent bond.

16. A pharmaceutical composition comprising the albumin-binding arginine deiminase fusion protein of claim 1 in a pharmaceutically-acceptable carrier.

17. The pharmaceutical composition of claim 16 wherein the composition has a pH in a range of 5.5 to 9.5.

18. The pharmaceutical composition of claim 16 wherein the composition has a pH of 7.4.

19. The pharmaceutical composition of claim 16 wherein the composition has a pH of 6.5.

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