MODIFIED ARGININE DEIMINASE

Related Applications

This application is a continuation in part application of U.S. Patent Application Serial No. 09/023,809, allowed, which claims priority to U.S. Provisional Patent Application Serial No. 60/046,200, filed on May 12, 1997.

Field of the Invention

The present invention is directed to arginine deiminase modified with polyethylene glycol, to methods for treating cancer, and to methods for treating and/or inhibiting metastasis.

Background of the Invention

Malignant melanoma (stage 3) and hepatoma are fatal diseases which kill most patients within one year of diagnosis. In the United States, approximately 16,000 people die from these diseases annually. The incidence of melanoma is rapidly increasing in the United States and is even higher in other countries, such as Australia. The incidence of hepatoma, in parts of the world where hepatitis is endemic, is even greater. For example, hepatoma is one of the leading forms of cancer in Japan and Taiwan. Effective treatments for these diseases are urgently needed.

Selective deprivation of essential amino acids has been used to treat some forms of cancer. The best known example is the use of L-asparaginase to lower levels of asparagine as a treatment for acute lymphoblastic leukemia. The L-asparaginase most frequently used is isolated from *E. coli*. However, clinical use of this enzyme is compromised by its inherent antigenicity and short circulating half-life, as described by Y.K. Park, et al, *Anticancer Res.*, 1:373-376 (1981). Covalent modification of *E. coli* L-

asparaginase with polyethylene glycol reduces its antigenicity and prolongs its circulating half-life, as described, for example, by Park, *Anticancer Res., supra;* Y. Kamisaki et al, *J. Pharmacol. Exp. Ther.*, 216:410-414 (1981); and Y. Kamisaki et al, *Gann.*, 73:47-474 (1982). Although there has been a great deal of effort to identify other essential amino acid degrading enzymes for the treatment of cancer, none have been approved, primarily because deprivation of essential amino acids, by definition, results in numerous, and severe, side effects.

It has been reported that enzymes which degrade non-essential amino acids, such as arginine, may be an effective means of controlling some forms of cancer. For example, arginine deiminase (ADI) isolated from *Pseudomonas pudita* was described by J.B. Jones, "The Effect of Arginine Deiminase on Murine Leukemic Lymphoblasts," Ph.D. Dissertation, The University of Oklahoma, pages 1-165 (1981). Although effective in killing tumor cells *in vitro*, 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. Arginine deiminase derived from *Mycoplasma arginini* is described, for example, by Takaku et al, *Int. J. Cancer, 51*:244-249 (1992), and U.S. Patent No. 5,474,928, the disclosures of which are hereby incorporated by reference herein in their entirety. However, a problem associated with the therapeutic use of such a heterologous protein is its antigenicity. The chemical modification of arginine deiminase from *Mycoplasma arginini*, via a cyanuric chloride linking group, with polyethylene glycol 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.

There is a need for compositions which degrade non-essential amino acids and which do not have the problems associated with the prior art. The present invention is directed to these, as well as other, important ends.

Summary of the Invention

The present invention is directed to arginine deiminase modified with polyethylene glycol. In a preferred embodiment, the arginine deiminase is modified with polyethylene glycol, having a total weight average molecular weight of about 1,000 to about 50,000, directly or through a biocompatible linking group.

Another embodiment of the invention is directed to methods of treating cancer, including, for example, sarcomas, hepatomas and melanomas. The invention is also directed to methods of treating and/or inhibiting the metastasis of tumor cells.

These and other aspects of the present invention will be elucidated in the following detailed description of the invention.

Brief Description of the Drawings

Figure 1 depicts the amino acid sequences of arginine deiminase cloned from *Mycoplasma arginini* (the top amino acid sequence SEQ ID NO: 1, identified as ADIPROT), *Mycoplasma arthritides* (the middle amino acid sequence SEQ ID NO: 2, identified as ARTADIPRO), and *Mycoplasma hominus* (the bottom amino acid sequence SEQ ID NO: 3, identified as HOMADIPRO).

Figures 2A and 2B are graphs showing the effect of a single dose of native arginine deiminase and arginine deiminase modified with polyethylene glycol (*e.g.*, molecular weight 5,000) on serum arginine levels and serum citrulline levels in mice.

Figure 3 is a graph showing the effects on serum arginine levels when PEG10,000 is covalently bonded to ADI via various linking groups.

Figure 4 is a graph showing the effect that the linking group and the molecular weight of the polyethylene glycol have on citrulline production in mice injected with a single dose of PEG-ADI.

Figures 5A and 5B are graphs showing the dose response that ADI-SS-PEG5,000 had on serum arginine and citrulline levels. **Figures 5C and 5D** are graphs showing the dose response that ADI-SS-PEG20,000 had on serum arginine and citrulline levels.

Figure 6 is a graph showing the antigenicity of native ADI, ADI-SS-PEG5,000, and ADI-SS-PEG20,000.

Figure 7 is a graph showing the effect that treatments with ADI-SS-PEG5,000, ADI-SS-PEG12,000 or ADI-SS-PEG20,000 had on tumor size in mice which were injected with SK-mel 2 human melanoma cells.

Figure 8 is a graph showing the effect that treatments with ADI-PEG20,000 had on tumor size in mice which were injected with SK-mel 28, SK-mel 2 or M24-met human melanoma cells.

Figure 9 is a graph showing the effect that treatments with ADI-PEG5,000, ADI-PEG12,000 or ADI-PEG20,000 had on the survival of mice which were injected with human hepatoma SK-Hep1 cells.

Figure 10 depicts the amino acid sequences of arginine deiminase cloned from *Steptococcus pyogenes* (the top amino acid sequence SEQ ID NO: 6, identified as STRADIPYR) and *Steptococcus pneumoniae*(the bottom amino acid sequence SEQ ID NO: 7, identified as STRADIPNE).

Figure 11 depicts the amino acid sequences of arginine deiminase cloned from *Borrelia burgdorferi* (the top amino acid sequence SEQ ID NO: 8, identified as BORADIBUR) and *Borrelia afzelii* (the bottom amino acid sequence SEQ ID NO: 9, identified as BORADIAFZ).

Figure 12 depicts the amino acid sequence of *Qiardia intestinalis* (the top amino acid sequence SEQ ID NO: 10, identified as QIAADIINT), *Clostridium perfringens* (the middle amino acid sequence SEQ ID NO: 11, identified as CLOADIPER) and *Bacillus licheniformis* (the bottom amino acid sequence SEQ ID NO: 12, identified as BACADILIC).

Figure 13 depicts the amino acid sequence of *Enterococcus faecalis* (the top amino acid sequence SEQ ID NO: 13, identified as ENTADIFAE) and *Lactobacillus sake* (the bottom amino acid sequence SEQ ID NO: 14, identified as LACADISAK).

Detailed Description of the Invention

Normal cells do not require arginine for growth, since they can synthesize arginine from citrulline in a two step process catalyzed by argininosuccinate synthase and argininosuccinate lyase. In contrast, melanomas, hepatomas and some sarcomas do not express arginosuccinate synthase; therefore, they are auxotrophic for arginine. This metabolic difference may be capitalized upon to develop a safe and effective therapy to treat these forms of cancer. Arginine deiminase catalyzes the conversion of arginine to citrulline, and may be used to eliminate arginine. Thus, arginine deiminase may be utilized as a treatment for melanomas, hepatomas and some sarcomas.

Native arginine deiminase may be found in microorganisms and is antigenic and rapidly cleared from circulation in a patient. These problems may be overcome by

covalently modifying arginine deiminase with polyethylene glycol (PEG). Arginine deiminase covalently modified with polyethylene glycol (with or without a linking group) may be hereinafter referred to as "ADI-PEG." When compared to native arginine deiminase, ADI-PEG retains most of its enzymatic activity, is far less antigenic, has a greatly extended circulating half-life, and is much more efficacious in the treatment of tumors.

"Polyethylene glycol" or "PEG" refers to mixtures of condensation polymers of ethylene oxide and water, in a branched or straight chain, represented by the general formula $H(OCH_2CH_2)_nOH$, wherein n is at least 4. "Polyethylene glycol" or "PEG" is used in combination with a numeric suffix to indicate the approximate weight average molecular weight thereof. For example, PEG5,000 refers to polyethylene glycol having a total weight average molecular weight of about 5,000; PEG12,000 refers to polyethylene glycol having a total weight average molecular weight of about 12,000; and PEG20,000 refers to polyethylene glycol having a total weight average molecular weight of about 20,000.

"Melanoma" may be a malignant or benign tumor arising from the melanocytic system of the skin and other organs, including the oral cavity, esophagus, anal canal, vagina, leptomeninges, and/or the conjunctivae or eye. The term "melanoma" includes, for example, acral-lentiginous melanoma, amelanotic melanoma, benign juvenile melanoma, lentigo maligna melanoma, malignant melanoma, nodular melanoma, subungual melanoma and superficial spreading melanoma.

"Hepatoma" may be a malignant or benign tumor of the liver, including, for example, hepatocellular carcinoma.

"Patient" refers to an animal, preferably a mammal, more preferably a human.

"Biocompatible" refers to materials or compounds which are generally not injurious to biological functions and which will not result in any degree of unacceptable toxicity, including allergenic and disease states.

Throughout the present disclosure, the following abbreviations may be used: PEG, polyethylene glycol; ADI, arginine deiminase; SS, succinimidyl succinate;

SSA, succinimidyl succinamide; SPA, succinimidyl propionate; and NHS, N-hydroxy-succinimide.

The present invention is based on the unexpected discovery that ADI modified with polyethylene glycol provides excellent results in treating certain types of cancer and inhibiting the metastasis of cancer. ADI may be covalently bonded to polyethylene glycol with or without a linking group, although a preferred embodiment utilizes a linking group.

In the present invention, the arginine deiminase gene may be derived, cloned or produced from any source, including, for example, microorganisms, recombinant biotechnology or any combination thereof. For example, arginine deiminase may be cloned from microorganisms of the genera *Mycoplasma*, *Clostridium, Bacillus*, *Borrelia*, *Enterococcus, Streptococcus, Lactobacillus*, *Qiardia*. It is preferred that arginine deiminase is cloned from *Mycoplasma pneumoniae*, *Mycoplasma hominus*, *Mycoplasma arginini*, *Steptococcus pyogenes*, *Steptococcus pneumoniae*, *Borrelia burgdorferi*, *Borrelia afzelii*, *Qiardia intestinalis*, *Clostridium perfringens, Bacillus licheniformis*, *Enterococcus faecalis*, *Lactobacillus sake*, or any combination thereof. In particular, the arginine deiminase used in the present invention may have one or more of the amino acid sequences depicted in Figures 1 and 10-13.

In certain embodiments of the present invention, it is preferred that arginine deiminase is cloned from microorganisms of the genus *Mycoplasma*. More preferably, the arginine deiminase is cloned from *Mycoplasma arginini, Mycoplasma hominus, Mycoplasma arthritides*, or any combination thereof. In particular, the arginine deiminase used in the present invention may have one or more of the amino acid sequences depicted in Figure 1.

In one embodiment of the present invention, the polyethylene glycol (PEG) has a total weight average molecular weight of about 1,000 to about 50,000; more preferably from about 3,000 to about 40,000, more preferably from about 5,000 to about 30,000; more preferably from about 8,000 to about 30,000; more preferably from about 11,000 to about 30,000; even more preferably from about 12,000 to about 28,000; still more preferably from about 16,000 to about 24,000; even more preferably from about

18,000 to about 22,000; even more preferably from about 19,000 to about 21,000, and most preferably about 20,000. Generally, polyethylene glycol with a molecular weight of 30,000 or more is difficult to dissolve, and yields of the formulated product are greatly reduced. The polyethylene glycol may be a branched or straight chain, preferably a straight chain. Generally, increasing the molecular weight of the polyethylene glycol decreases the immunogenicity of the ADI. The polyethylene glycol having a molecular weight described in this embodiment may be used in conjunction with ADI, and, optionally, a biocompatible linking group, to treat cancer, including, for example, melanomas, hepatomas and sarcomas, preferably melanomas.

In another embodiment of the present invention, the polyethylene glycol has a total weight average molecular weight of about 1,000 to about 50,000; preferably about 3,000 to about 30,000; more preferably from about 3,000 to about 20,000; more preferably from about 4,000 to about 12,000; still more preferably from about 4,000 to about 10,000; even more preferably from about 4,000 to about 8,000; still more preferably from about 4,000 to about 6,000; with about 5,000 being most preferred. The polyethylene glycol may be a branched or straight chain, preferably a straight chain. The polyethylene glycol having a molecular weight described in this embodiment may be used in conjunction with ADI, and optionally, a biocompatible linking group, to treat cancer, including, for example, melanomas, hepatomas and sarcomas, preferably hepatomas.

The linking group used to covalently attach ADI to PEG may be any biocompatible linking group. As discussed above, "biocompatible" indicates that the compound or group is non-toxic and may be utilized *in vitro* or *in vivo* without causing injury, sickness, disease or death. PEG can be bonded to the linking group, for example, via an ether bond, an ester bond, a thiol bond or an amide bond. Suitable biocompatible linking groups include, for example, an ester group, an amide group, an imide group, a carbamate group, a carboxyl group, a hydroxyl group, a carbohydrate, a succinimide group (including, for example, succinimidyl succinate (SS), succinimidyl propionate (SPA), succinimidyl carboxymethylate (SCM), succinimidyl succinamide (SSA) or N-hydroxy succinimide (NHS)), an epoxide group, an oxycarbonylimidazole group (including, for example, carbonyldimidazole (CDI)), a nitro phenyl group (including, for example,

nitrophenyl carbonate (NPC) or trichlorophenyl carbonate (TPC)), a trysylate group, an aldehyde group, an isocyanate group, a vinylsulfone group, a tyrosine group, a cysteine group, a histidine group or a primary amine. Preferably, the biocompatible linking group is an ester group and/or a succinimide group. More preferably, the linking group is SS, SPA, SCM, SSA or NHS; with SS, SPA or NHS being more preferred, and with SS or SPA being most preferred.

Alternatively, ADI may be coupled directly to PEG (i.e., without a linking group) through an amino group, a sulfhydral group, a hydroxyl group or a carboxyl group.

ADI may be covalently bonded to PEG, via a biocompatible linking group, using methods known in the art, as described, for example, by Park et al, *Anticancer Res., 1:373-376 (1981)*; and Zaplipsky and Lee, *Polyethylene Glycol Chemistry: Biotechnical and Biomedical Applications*, J.M. Harris, ed., Plenum Press, NY, Chapter 21 (1992), the disclosures of which are hereby incorporated by reference herein in their entirety.

The attachment of PEG to ADI increases the circulating half-life of ADI. Generally, PEG is attached to a primary amine of ADI. Selection of the attachment site of polyethylene glycol on the arginine deiminase is determined by the role of each of the sites within the active domain of the protein, as would be known to the skilled artisan. PEG may be attached to the primary amines of arginine deiminase without substantial loss of enzymatic activity. For example, ADI cloned from *Mycoplasma arginini, Mycoplasma arthritides* and *Mycoplasma hominus* has about 17 lysines that may be modified by this procedure. In other words, the 17 lysines are all possible points at which ADI can be attached to PEG via a biocompatible linking group, such as SS, SPA, SCM, SSA and/or NHS. PEG may also be attached to other sites on ADI, as would be apparent to one skilled in the art in view of the present disclosure.

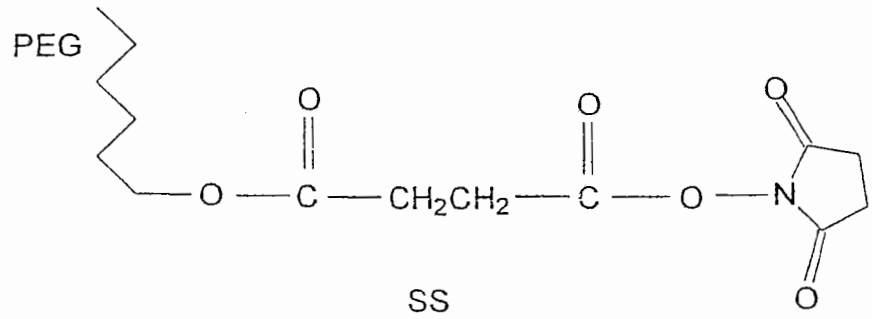
From 1 to about 30 PEG molecules may be covalently bonded to ADI. Preferably, ADI is modified with about 7 to about 15 PEG molecules, more preferably from about 9 to about 12 PEG molecules. In other words, about 30% to about 70% of the primary amino groups in arginine deiminase are modified with PEG, preferably about 40% to about 60%, more preferably about 45% to about 55%, and most preferably about 50% of the primary amino groups in arginine deiminase are modified with PEG. When PEG is covalently bonded to the end terminus of ADI, preferably only 1 PEG molecule is utilized.

Increasing the number of PEG units on ADI increases the circulating half life of the enzyme. However, increasing the number of PEG units on ADI decreases the specific activity of the enzyme. Thus, a balance needs to be achieved between the two, as would be apparent to one skilled in the art in view of the present disclosure.

In the present invention, a common feature of the most preferred biocompatible linking groups is that they attach to a primary amine of arginine deiminase via a maleimide group. Once coupled with arginine deiminase, SS-PEG has an ester linkage next to the PEG, which may render this site sensitive to serum esterase, which may release PEG from ADI in the body. SPA-PEG and PEG2-NHS do not have an ester linkage, so they are not sensitive to serum esterase.

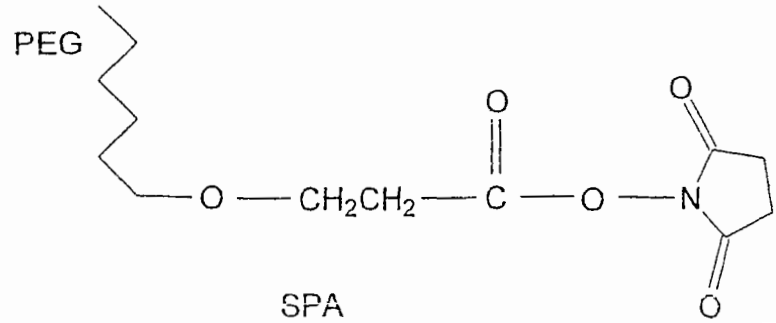
In the present invention, the particular linking groups do not appear to influence the circulating half-life of PEG-ADI or its specific enzyme activity. However, it is critical to use a biocompatible linking group in the present invention. PEG which is attached to the protein may be either a single chain, as with SS-PEG, SPA-PEG and SC-PEG, or a branched chain of PEG may be used, as with PEG2-NHS. The structural formulas of the preferred linking groups in the present invention are set forth below.

SS-PEG:



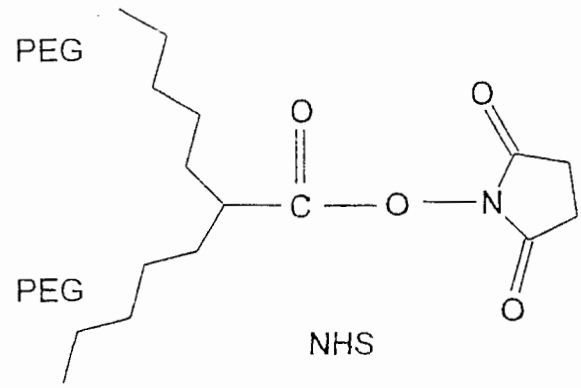
SS

SPA-PEG:



SPA

PEG2-NHS:



A therapeutically effective amount of one of the compounds of the present invention is an amount that is effective to inhibit tumor growth. Generally, treatment is initiated with small dosages which can be increased by small increments until the optimum effect under the circumstances is achieved. Generally, a therapeutic dosage of compounds of the present invention may be from about 1 to about 200 mg/kg twice a week to about once every two weeks. For example, the dosage may be about 1 mg/kg once a week as a 2 ml intravenous injection to about 20 mg/kg once every 3 days. The optimum dosage with ADI-SS-PEG5,000 may be about twice a week, while the optimum dosage with ADI-SS-PEG20,000 may be from about once a week to about once every two weeks. PEG-ADI may be mixed with a phosphate buffered saline solution, or any other appropriate solution known to those skilled in the art, prior to injection. The PEG-ADI formulation may be administered as a solid (lyophilate) or as a liquid formulation, as desired.

The methods of the present invention can involve either *in vitro* or *in vivo* applications. In the case of *in vitro* applications, including cell culture applications, the compounds described herein can be added to the cells in cultures and then incubated. The compounds of the present invention may also be used to facilitate the production of monoclonal and/or polyclonal antibodies, using antibody production techniques well known in the art. The monoclonal and/or polyclonal antibodies can then be used in a wide variety of diagnostic applications, as would be apparent to one skilled in the art.

The *in vivo* means of administration of the compounds of the present invention will vary depending upon the intended application. As one skilled in the art will

recognize, administration of the PEG-ADI composition of the present invention can be carried out, for example, orally, intranasally, intraperitoneally, parenterally, intravenously, intralymphatically, intratumorly, intramuscularly, interstitially, intra-arterially, subcutaneously, intraocularly, intrasynovial, transepithelial, and transdermally.

Examples

The invention is further demonstrated in the following examples, which are for purposes of illustration, and are not intended to limit the scope of the present invention.

Example 1: ***Production of Recombinant ADI***

Cultures of *Mycoplasma arginini* (ATCC 23243), *Mycoplasma hominus* (ATCC 23114) and *Mycoplasma arthritides* (ATCC 23192) were obtained from the American Type Culture Collection, Rockville, Maryland.

Arginine deiminase was cloned from *Mycoplasma arginini*, *Mycoplasma hominus* and *Mycoplasma arthritides* and expressed in *E. coli* as previously described by S. Misawa et al, *J. Biotechnology,* 36:145-155 (1994), the disclosure of which is hereby incorporated herein by reference in its entirety. The amino acid sequences of arginine deiminase from each of the above species is set forth in Figure 1. The top amino acid sequence, identified as ADIPROT, is from *Mycoplasma arginini;* the middle amino acid sequence, identified as ARTADIPRO, is from *Mycoplasma arthritides;* and the bottom amino acid sequence, identified as HOMADIPRO, is from *Mycoplasma hominus.* Each of the amino acid sequences are more than 96% conserved. Characterization, by methods known to those skilled in the art, of each of the proteins with respect to specific enzyme activity, K_m, V_{max} and pH optima revealed that they were biochemically indistinguishable from each other. The pH optima was determined using a citrate buffer (pH 5-6.5), a phosphate buffer (pH 6.5-7.5) and a borate buffer (pH 7.5-8.5). The K_m and V_{max} were determined by incubating the enzyme with various concentrations of arginine and quantifying citrulline production. The K_m for the various enzymes was about 0.02 to 0.06 μM and the V_{max} was about 15-20 μmol/min/mg, the values of which are within standard error of each other.

The arginine deiminase genes were amplified by polymerase chain reaction

using the following primer pair derived from the published sequence of *M. arginini*, as described, for example, by T. Ohno et al, *Infect. Immun.*, 58:3788-3795 (1990), the disclosure of which is hereby incorporated by reference herein in its entirety:

SEQ ID NO: 4, 5'-GGGATCCATGTCTGTATTTGACAGT-3'

SEQ ID NO: 5, 5'-TGAAAGCTTTTACTACCACTTAACATCTTTACG-3'

The polymerase chain reaction products were cloned as a Bam H1-Hind III fragment into expression plasmid pQE16. DNA sequence analysis indicated that the fragment derived from *M. arginini* by PCR had the same sequence for the arginine deiminase gene as described by Ohno et al, *Infect. Immun., supra*. The five TGA codons in the ADI gene which encode tryptophan in *Mycoplasma* were changed to TGG codons by oligonucleotide-directed mutagenesis prior to gene expression in *E. coli*, as taught, for example, by J.R. Sayers et al, *Biotechniques*, 13:592-596 (1992). Recombinant ADI was expressed in inclusion bodies at levels of 10% of total cell protein.

The proteins from each of the above three species of *Mycoplasma* have approximately 95% homology and are readily purified by column chromatography. Approximately 200 mg of pure protein may be isolated from 1 liter of fermentation broth. Recombinant ADI is stable for about 2 weeks at 37°C and for at least 8 months when stored at 4°C. As determined by methods known to those skilled in the art, the proteins had a high affinity for arginine (0.04 μM), and a physiological pH optima of about 7.2 to about 7.4.

Example 2: ***Renaturation and Purification of Recombinant ADI***

ADI protein was renatured, with minor modifications, as described by Misawa et al, *J. Biotechnology*, 36:145-155 (1994), the disclosure of which is hereby incorporated herein by reference in its entirety. 100 g of cell paste was resuspended in 800 ml of 10 mM K_2PO_4 pH 7.0, 1 mM EDTA (buffer 1) and the cells were disrupted by two passes in a Microfluidizer (Microfluidics Corporation, Newton, MA). Triton X-100 was added to achieve a final concentration of 4% (v/v). The homogenate was stirred for 30 min at 4°C, then centrifuged for 30 min at 13,000 g. The pellet was collected and resuspended in one liter of buffer 1 containing 0.5% Triton X-100. The solution was diafiltered against 5 volumes of denaturation buffer (50 mM Tris HCl, pH 8.5, 10 mM

DTT) using hollow-fiber cartridges with 100 kD retention rating (Microgon Inc., Laguna Hills, CA). Guanidine HCl was added to achieve a final concentration of 6 M and the solution was stirred for 15 min at 4°C. The solution was diluted 100-fold into refolding buffer 1, 10 mm K_2PO_4, pH 7.0 and stirred for 48 hours at 15°C, particulates were removed by centrifugation at 15,000 x g.

The resulting supernatant was concentrated on a Q Sepharose Fast Flow (Pharmacia Inc., Piscataway, NJ) column preequilabrated in refolding buffer. ADI was eluted using refolding buffer containing 0.2 M NaCl. The purification procedure yielded ADI protein, which was >95% pure as estimated by SDS-PAGE analysis. 8 g of pure renatured ADI protein was produced from 1 kg of cell paste which corresponds to 200 mg purified ADI per liter of fermentation.

ADI activity was determined by micro-modification of the method described by Oginsky et al, *Meth. Enzymol.*, (1957) 3:639-642. 10 µl samples in 0.1 m Na_2PO_4, pH 7.0 (BUN assay buffer) were placed in a 96 well microliter plate, 40 µl of 0.5 mM arginine in BUN assay buffer was added, and the plate was covered and incubated at 37°C for 15 minutes. 20 µl of complete BUN reagent (Sigma Diagnostics) was added and the plate was incubated for 10 minutes at 100°C. The plate was then cooled to 22°C and analyzed at 490 nm by a microliter plate reader (Molecular Devices, Inc). 1.0 IU is the amount of enzyme which converts 1 µmole of L-arginine to L-citrulline per minute. Protein concentrations were determined using Pierce Coomassie Blue Protein Assay Reagent (Pierce Co., Rockford, IL) with bovine serum albumin as a standard.

The enzyme activity of the purified ADI preparations was 17-25 IU/mg.

Example 3: ***Attachment of PEG to ADI***

PEG was covalently bonded to ADI in a 100 mM phosphate buffer, pH 7.4. Briefly, ADI in phosphate buffer was mixed with a 100 molar excess of PEG. The reaction was stirred at room temperature for 1 hour, then the mixture was extensively dialized to remove unincorporated PEG.

A first experiment was performed where the effect of the linking group used in the PEG-ADI compositions was evaluated. PEG and ADI were covalently bonded via four different linking groups: an ester group or maleimide group, including SS, SSA,

SPA and SSPA, where the PEG had a total weight average molecular weight of 5,000, 10,000, 12,000, 20,000, 30,000 and 40,000; an epoxy group, PEG-epoxy, where the PEG had a total weight average molecular weight of 5,000; and a branched PEG group, PEG2-NHS, where the PEG had a total weight average molecular weight of 10,000, 20,000 and 40,000.

5.0 IU of the resulting compositions were injected into mice (5 mice in each group). To determine the serum levels of arginine, the mice were bled from the retro orbital plexus (100 ul). Immediately following collection an equal volume of 50% (w/v) of trichloroacetic acid was added. The precipitate was removed by centrifugation (13,000 x g for 30 minutes) and the supernatant removed and stored frozen at -70°C. The samples were then analyzed using an automated amino acid analyzer and reagents from Beckman Instruments using protocols supplied by the manufacturer. The limits of sensitivity for arginine by this method was approximately 2-6 μM and the reproducibility of measurements within about 8%. The amount of serum arginine was determined by amino acid analysis. As can be seen from the results in Figure 3, the linking group covalently bonding the PEG and ADI did not have an appreciable effect on the ability of ADI to reduce serum arginine *in vivo*. In other words, the linking group may not be critical to the results of the experiment, except that a non-toxic linking group must be used for *in vivo* applications.

A second experiment was performed wherein the effect of the linking group and molecular weight of PEG on serum citrulline levels *in vivo* was evaluated. Mice (5 in each group) were given various compositions of ADI and PEG-ADI in an amount of 5.0 IU. To determine the serum levels of citrulline, the mice were bled from the retro orbital plexus (100 ul). Immediately following collection an equal volume of 50% (w/v) of trichloroacetic acid was added. The precipitate was removed by centrifugation (13,000 x g for 30 minutes) and the supernatant removed and stored frozen at -70°C. The samples were then analyzed using an automated amino acid analyzer and reagents from Beckman Instruments using protocols supplied by the manufacturer. The limits of sensitivity for citrulline by this method was approximately 2-6 μM and the reproducibility of measurements within about 8%. The amount of citrulline was determined, and the area

under the curve approximated and expressed as μmol days.

In Figure 4, the open circles indicate the amount of citrulline produced by native ADI, the filled circles are ADI-SC-PEG, the open squares are ADI-SS-PEG, the open triangles are ADI-SPA-PEG, and the filled triangles are branched chain PEG-NHS-PEG_2. The results in Figure 4 demonstrate that the molecular weight of the PEG determines the effectiveness of the PEG-ADI composition. The effectiveness of the PEG-ADI compositions is not necessarily based on the method or means of attachment of the PEG to ADI, except that a biocompatible linking group must be used for *in vivo* applications.

The results in Figure 4 also demonstrate that the optimal molecular weight of PEG is 20,000. Although PEG30,000 appears to be superior to PEG20,000 in terms of its pharmacodynamics, PEG30,000 is less soluble, which makes it more difficult to work with. The yields, which were based on the recovery of enzyme activity, were about 90% for PEG5,000 and PEG12,000; about 85% for PEG20,000 and about 40% for PEG30,000. Therefore, PEG20,000 is the best compromise between yield and circulating half life, as determined by citrulline production.

In a third experiment, the dose response of serum arginine depletion and the production of citrulline with ADI-SS-PEG5,000 and ADI-SS-PEG20,000 was determined. Mice (5 in each group) were given a single injection of 0.05 IU, 0.5 IU or 5.0 IU of either ADI-SS-PEG5,000 or ADI-SS-PEG20,000. At indicated times, serum was collected, as described above, and an amino acid analysis was performed to quantify serum arginine (Figures 5A and 5C) and serum citrulline (Figures 5B and 5D). Both formulations induced a dose dependent decrease in serum arginine and an increase in serum citrulline. However, the effects induced by ADI-SS-PEG20,000 were more pronounced and of longer duration than the effects induced by ADI-SS-PEG5,000.

Example 4: ***Selectivity of ADI Mediated Cytotoxicity***

The selectivity of arginine deiminase mediated cytotoxicity was demonstrated using a number of human tumors. Specifically, human tumors were tested *in vitro* for sensitivity to ADI-SS-PEG5,000 (50 ng/ml). Viability of cultures was determined after 7 days. For a culture to be defined as "inhibited," greater than 95% of the

cells must take up Trypan blue dye. A host of normal cells were also tested, including endothelial cells, smooth muscle cells, epithelial cells and fibroblasts, and none were inhibited by ADI-SS-PEG5,000. Although arginine deiminase has no appreciable toxicity towards normal, and most tumor cells, ADI-SS-PEG5,000 greatly inhibited all human melanomas and hepatomas that were commercially available from the ATCC, MSKCC and Europe.

Table 1: Specificity of Arginine Deiminase Cytotoxicity

Tumor Type	Number of Tumors Tested	Tumors inhibited (%)
Brain	16	0
Colon	34	0
Bladder	3	0
Breast	12	0
Kidney	5	0
Sarcoma	11	64
Hepatoma	17	100
Melanoma	37	100

In a parallel set of experiments, mRNA was isolated from the tumors. Northern blot analyses, using the human argininosuccinate synthase cDNA probe, indicated complete concordance between the sensitivity to arginine deiminase treatment and an inability to express argininosuccinate synthase. This data suggests that ADI toxicity results from an inability to induce argininosuccinate synthase. Therefore, these cells cannot synthesize arginine from citrulline, and are unable to synthesize the proteins necessary for growth.

Example 5: ***Circulating Half-Life***

Balb C mice (5 in each group) were injected intravenously with a single 5.0 IU dose of either native arginine deiminase or various formulations of arginine deiminase modified with polyethylene glycol, as indicated in Figures 2A and 2B. To determine the

serum levels of arginine and citrulline, the mice were bled from the retro orbital plexus (100 ul). Immediately following collection an equal volume of 50% (w/v) of trichloro-acetic acid was added. The precipitate was removed by centrifugation (13,000 x g for 30 minutes) and the supernatant removed and stored frozen at -70°C. The samples were then analyzed using an automated amino acid analyzer and reagents from Beckman Instruments using protocols supplied by the manufacturer. The limits of sensitivity for arginine by this method was approximately 6 pM and the reproducibility of measurements within about 8%.

A dose dependent decrease in serum arginine levels, as shown by the solid circles in Figure 2A, and a rise in serum citrulline, as shown by the open triangles in Figure 2B, were detected from the single dose administration of native ADI (filled circles) or ADI-SS-PEG (open triangles). However, the decrease in serum arginine and rise in serum citrulline was short lived, and soon returned to normal. The half life of arginine depletion is summarized in the Table below.

Table 2: Half-Life of Serum Arginine Depletion

Compound	Half-Life in Days
Native ADI	1
ADI-SS-PEG5,000	5
ADI-SS-PEG12,000	15
ADI-SS-PEG20,000	20
ADI-SS-PEG30,000	22

This experiment demonstrates that normal cells and tissues are able to convert the citrulline back into arginine intracellularly while melanomas and hepatomas cannot because they lack argininosuccinate synthetase.

Example 6: ***Antigenicity of PEG modified ADI***

To determine the antigenicity of native ADI, ADI-SS-PEG5,000, and ADI-

SS-PEG20,000, the procedures described in, for example, Park, *Anticancer Res., supra,* and Kamisaki, *J. Pharmacol. Exp. Ther., supra,* were followed.. Briefly, Balb C mice (5 in each group) were intravenously injected weekly for 12 weeks with approximately 0.5 IU (100 μg of protein) of native ADI, ADI-SS-PEG5,000 or ADI-SS-PEG20,000. The animals were bled (0.05 ml) from the retro orbital plexus at the beginning of the experiment and at weeks 4, 8 and 12. The serum was isolated and stored at -70°C. The titers of anti-ADI IgG were determined by ELISA. 50 μg of ADI was added to each well of a 96 well micro-titer plate and was incubated at room temperature for 4 hours. The plates were rinsed with PBS and then coated with bovine serum albumin (1 mg/ml) to block nonspecific protein binding sites, and stored over night at 4°C. The next day serum from the mice was diluted and added to the wells. After 1 hour the plates were rinsed with PBS and rabbit anti-mouse IgG coupled to peroxidase was added to the wells. The plates were incubated for 30 min and then the resulting UV absorbance was measured using a micro-titer plate reader. The titer was defined as the highest dilution of the serum which resulted in a two-fold increase from background absorbance (approximately 0.50 OD).

The results are shown in Figure 6. The open circles represent the data obtained from animals injected with native ADI, which was very antigenic. The filled circles represent the data obtained from the animals injected with ADI-SS-PEG5,000, while the open triangles represent the data obtained from the animals injected with ADI-SS-PEG20,000. As can be seen from Figure 6, ADI-SS-PEG5,000 and ADI-SS-PEG20,000 are significantly less antigenic than native ADI. For example, as few as 4 injections of native ADI resulted in a titer of about 10^6, while 4 injections of any of the PEG-ADI formulations failed to produce any measurable antibody. However, after 8 injections, the ADI-PEG5,000 had a titer of about 10^2, while ADI-PEG20,000 did not induce this much of an immune response until after 12 injections. The results demonstrate that attaching PEG to ADI blunts the immune response to the protein.

Example 7: ***Tumor Inhibition of Human Melanomas***

The effect of PEG-ADI on the growth of human melanoma (SK-Mel 28) in nude mice was determined. Nude mice (5 in each group) were injected with 10^6 SK-mel 2

human melanoma cells which were allowed to grow until the tumors reached a diameter of about 3-5 mm. The mice were left untreated (open circles) or were treated once a week for 8 weeks with 5.0 IU of ADI-SS-PEG5,000 (filled triangles), ADI-SS-PEG12,000 (open triangles) or ADI-SS-PEG20,000 (filled circles). The tumor size was measured weekly, and the mean diameter of the tumors is presented in Figure 7.

Figure 8 shows the effectiveness of ADI-SS-PEG20,000 on three human melanomas (SK-mel 2, SK-mel 28, M24-met) grown *in vivo* in nude mice. Nude mice (5 in each group) were injected with 10^6 SK-mel 2, SK-mel 28 or M24-met human melanoma cells. The tumors were allowed to grow until they were approximately 3-5 mm in diameter. Thereafter, the animals were injected once a week with 5.0 IU of ADI-SS-PEG20,000. The results are shown in Figure 8, and show that PEG-ADI inhibited tumor growth and that eventually the tumors began to regress and disappear. Because the tumors did not have argininosuccinate synthatase, they were unable to synthesize proteins (because ADI eliminated arginine and the tumors could not make it) so that the cells "starved to death."

Since M24-met human melanoma is highly metastatic, the animals injected with M24-met human melanoma cells were sacrificed after 4 weeks of treatment and the number of metastases in the lungs of the animals was determined. The control animals had an average of 32 metastases, while the animals treated with ADI-SS-PEG20,000 did not have any metastases. The results appear to indicate that ADI-SS-PEG20,000 not only inhibited the growth of the primary melanoma tumor, but also inhibited the formation of metastases.

It is of interest to note that in over 200 animals tested, the average number of metastases in the control group was 49 ± 18, while only a single metastasis was observed in 1 treated animal.

Example 8: ***Tumor Inhibition of Human Hepatomas***

The ability of PEG-ADI to inhibit the growth of a human hepatoma *in vivo* was tested. Nude mice (5 in each group) were injected with 10^6 human hepatoma SK-Hep1 cells. The tumors were allowed to grow for two weeks and then the animals were treated once a week with 5.0 IU of SS-PEG5,000-ADI (solid circles), SS-PEG12,000-ADI

(solid triangles) or SS-PEG20,000-ADI (open triangles). The results are set forth in Figure 9. The untreated animals (open circles) all died within 3 weeks. In contrast, animals treated with ADI had a far longer life expectancy, as can be seen from Figure 9. All the surviving mice were euthanized after 6 months, and necropsy indicated that they were free of tumors.

Surprisingly, PEG5,000-ADI is most effective in inhibiting hepatoma growth *in vivo*. The exact mechanism by which this occurs is unknown. Without being bound to any theory of the invention works, it appears that proteins formulated with SS-PEG5,000-ADI become sequestered in the liver. Larger molecular weights of PEG do not, which may be due to the uniqueness of the hepatic endothelium and the spaces (fenestrae) being of such a size that larger molecular weights of PEG-ADI conjugates are excluded.

Example 9: ***Application to Humans***

PEG5,000-ADI and PEG20,000-ADI were incubated *ex vivo* with normal human serum and the effects on arginine concentration was determined by amino acid analysis, where the enzyme was found to be fully active and capable of degrading all the detectable arginine with the same kinetics as in the experiments involving mice. The reaction was conducted at a volume of 0.1 ml in a time of 1 hour at 37°C. Additionally, the levels of arginine and citrulline in human serum are identical with that found in mice. PEG-proteins circulate longer in humans than they do in mice. For example, the circulating half life of PEG conjugated adenosine deiminase, asparaginase, glucocerbrocidase, uricase, hemoglobulin and superoxide dismutase all have a circulating half life that is 5 to 10 times longer than the same formulations in mice. What this has meant in the past is that the human dose is most often 1/5 to 1/10 of that used in mice. Accordingly, PEG-ADI should circulate even longer in humans than it does in mice.

Each of the patents, patent applications and publications described herein are hereby incorporated by reference herein in their entirety.

Various modifications of the invention, in addition to those described herein, will be apparent to one skilled in the art in view of the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

What is claimed is:

1. A compound comprising arginine deiminase covalently bonded via a linking group to polyethylene glycol, wherein the polyethylene glycol has a total weight average molecular weight of from about 1,000 to about 40,000, and wherein the linking group is selected from the group consisting of a succinimide group, an amide group, an imide group, a carbamate group, an ester group, an epoxy group, a carboxyl group, a hydroxyl group, a carbohydrate, a tyrosine group, a cysteine group, a histidine group and combinations thereof.

2. The compound of claim 1, wherein said linking group is a succinimide group.

3. The compound of claim 2, wherein said succinimide group is succinimidyl succinate, succinimidyl propionate, succinimidyl carboxymethylate, succinimidyl succinamide, N-hydroxy succinimide or combinations thereof.

4. The compound of claim 3, wherein said succinimide group is succinimidyl succinate, succinimidyl propionate or combinations thereof.

5. The compound of claim 1, wherein said arginine deiminase is derived from a microorganism of the genus *Mycoplasma*.

6. The compound of claim 5, wherein said microorganism is selected from the group consisting of *Mycoplasma arginini, Mycoplasma hominus*, *Mycoplasma arthritides* and combinations thereof.

7. The compound of claim 1, wherein said arginine deiminase is derived from a microorganism of the genus *Steptococcus*.

8. The compound of claim 7, wherein said microorganism is selected from the group consisting of *Steptococcus pyogenes*, *Steptococcus pneumoniae* and combinations thereof.

9. The compound of claim 1, wherein said arginine deiminase is derived from a microorganism of the genus *Borrelia*.

10. The compound of claim 9, wherein said microorganism is selected from the group consisting of *Borrelia burgdorferi*, *Borrelia afzelii*, and combinations thereof.

11. The compound of claim 1, wherein said arginine deiminase is derived from a microorganism of the genus *Qiardia*.

12. The compound of claim 11, wherein said microorganism is *Qiardia intestinalis*.

13. The compound of claim 1, wherein said arginine deiminase is derived from a microorganism of the genus *Clostridium*.

14. The compound of claim 13, wherein said microorganism is *Clostridium perfringens*.

15. The compound of claim 1, wherein said arginine deiminase is derived from a microorganism of the genus *Enterococcus*.

16. The compound of claim 15, wherein said microorganism is *Enterococcus faecalis*.

17. The compound of claim 1, wherein said arginine deiminase is derived from a microorganism of the genus *Lactobacillus*.

18. The compound of claim 17, wherein said microorganism is *Lactobacillus sake*.

19. The compound of claim 1, wherein said arginine deiminase is derived from a microorganism of the genus *Bacillus*.

20. The compound of claim 19, wherein said microorganism is *Bacillus licheniformis*.

21. The compound of claim 1, wherein said microorganism is selected from the group consisting of *Mycoplasma pneumoniae*, *Mycoplasma hominus*, *Mycoplasma arginini*, *Steptococcus pyogenes*, *Steptococcus pneumoniae*, *Borrelia burgdorferi*, *Borrelia afzelii*, *Qiardia intestinalis*, *Clostridium perfringens*, *Bacillus licheniformis*, *Enterococcus faecalis*, *Lactobacillus sake*, and combinations thereof.

22. The compound of claim 1, wherein said arginine deiminase is covalently bonded to about 7 to about 15 polyethylene glycol molecules.

23. The compound of claim 22, wherein said arginine deiminase is covalently bonded to about 9 to about 12 polyethylene glycol molecules.

24. The compound of claim 1, wherein said polyethylene glycol has a total weight average molecular weight of from about 10,000 to about 30,000.

25. A method of enhancing the circulating half life of arginine deiminase comprising modifying said arginine deiminase by covalently bonding said arginine deiminase via a linking group to polyethylene glycol, wherein the polyethylene glycol has a total weight average molecular weight of from about 1,000 to about 40,000, and wherein the linking group is selected from the group consisting of a succinimide group, an amide group, an imide group, a carbamate group, an ester group, an epoxy group, a carboxyl group, a hydroxyl group, a carbohydrate, a tyrosine group, a cysteine group, a histidine group and combinations thereof.

26. A method of enhancing the tumoricidal activity of arginine deiminase comprising modifying said arginine deiminase by covalently bonding said arginine deiminase via a linking group to polyethylene glycol, wherein the polyethylene glycol has a total weight average molecular weight of from about 1,000 to about 40,000, and wherein the linking group is selected from the group consisting of a succinimide group, an amide group, an imide group, a carbamate group, an ester group, an epoxy group, a carboxyl group, a hydroxyl group, a carbohydrate, a tyrosine group, a cysteine group, a histidine group and combinations thereof.

27. A compound according to claim 1 for use in therapy.

28. Use of a compound according to claim 1 in the manufacture of a medicament for the treatment of tumors.

29. The use of claim 28, wherein said tumor is a melanoma.

30. The use of claim 29, wherein said polyethylene glycol has a total weight average molecular weight of about 20,000.

31. The use of claim 28, wherein said tumor is a hepatoma.

32. The use of claim 31, wherein said polyethylene glycol has a total weight average molecular weight of about 5,000.

33. The use of claim 29 or claim 31, wherein said linking group is a succinimide group.

34. The use of claim 33, wherein said succinimide group is succinimidyl succinate, succinimidyl propionate, succinimidyl carboxymethylate, succinimidyl succinamide, N-hydroxy succinimide or combinations thereof.

35. The use of claim 28, wherein said tumor is a sarcoma.

36. Use of a compound according to claim 1 in the manufacture of a medicament for the prevention and treatment of metastases.

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Asn Glu Lys Leu Lys Ile Val Glu Glu Lys Gly Asp Leu Ala Glu Leu
305                 310                 315                 320

Leu Ala Gln Asn Leu Gly Val Glu Lys Val His Leu Ile Arg Cys Gly
                325                 330                 335

Gly Gly Asn Ile Val Ala Ala Ala Arg Glu Gln Trp Asn Asp Gly Ser
            340                 345                 350

Asn Thr Leu Thr Ile Ala Pro Gly Val Val Val Val Tyr Asp Arg Asn
        355                 360                 365

Thr Val Thr Asn Lys Ile Leu Glu Glu Tyr Gly Leu Arg Leu Ile Lys
    370                 375                 380

Ile Arg Gly Ser Glu Leu Val Arg Gly Arg Gly Gly Pro Arg Cys Met
385                 390                 395                 400

Ser Met Pro Phe Glu Arg Glu Glu Val
                405
```

<210> 8
<211> 410
<212> PRT
<213> Borrelia burgdorferi

<400> 8

```
Met Glu Glu Glu Tyr Leu Asn Pro Ile Asn Ile Phe Ser Glu Ile Gly
  1               5                  10                  15

Arg Leu Lys Lys Val Leu Leu His Arg Pro Gly Glu Glu Leu Glu Asn
             20                  25                  30

Leu Thr Pro Leu Ile Met Lys Asn Phe Leu Phe Asp Asp Ile Pro Tyr
         35                  40                  45

Leu Lys Val Ala Arg Gln Glu His Glu Val Phe Val Asn Ile Leu Lys
```

50 55 60

Asp Asn Ser Val Glu Ile Glu Tyr Val Glu Asp Leu Val Ser Glu Val
65 70 75 80

Leu Ala Ser Ser Val Ala Leu Lys Asn Lys Phe Ile Ser Gln Phe Ile
85 90 95

Leu Glu Ala Glu Ile Lys Thr Asp Gly Val Ile Asn Ile Leu Lys Asp
100 105 110

Tyr Phe Ser Asn Leu Thr Val Asp Asn Met Val Ser Lys Met Ile Ser
115 120 125

Gly Val Ala Arg Glu Glu Leu Lys Asp Cys Glu Phe Ser Leu Asp Asp
130 135 140

Trp Val Asn Gly Ser Ser Leu Phe Val Ile Asp Pro Met Pro Asn Val
145 150 155 160

Leu Phe Thr Arg Asp Pro Phe Ala Ser Ile Gly Asn Gly Ile Thr Ile
165 170 175

Asn Lys Met Tyr Thr Lys Val Arg Arg Arg Glu Thr Ile Phe Ala Glu
180 185 190

Tyr Ile Phe Lys Tyr His Ser Ala Tyr Lys Glu Asn Val Pro Ile Trp
195 200 205

Phe Asn Arg Trp Glu Glu Thr Ser Leu Glu Gly Gly Asp Glu Phe Val
210 215 220

Leu Asn Lys Asp Leu Leu Val Ile Gly Ile Ser Glu Arg Thr Glu Ala
225 230 235 240

Gly Ser Val Glu Lys Leu Ala Ala Ser Leu Phe Lys Asn Lys Ala Pro
245 250 255

Phe Ser Thr Ile Leu Ala Phe Lys Ile Pro Lys Asn Arg Ala Tyr Met
260 265 270

His Leu Asp Thr Val Phe Thr Gln Ile Asp Tyr Ser Val Phe Thr Ser
275 280 285

Phe Thr Ser Asp Asp Met Tyr Phe Ser Ile Tyr Val Leu Thr Tyr Asn
290 295 300

Ser Asn Ser Asn Lys Ile Asn Ile Lys Lys Glu Lys Ala Lys Leu Lys

```
305                 310                 315                 320

Asp Val Leu Ser Phe Tyr Leu Gly Arg Lys Ile Asp Ile Ile Lys Cys
                325                 330                 335

Ala Gly Gly Asp Leu Ile His Gly Ala Arg Glu Gln Trp Asn Asp Gly
            340                 345                 350

Ala Asn Val Leu Ala Ile Ala Pro Gly Glu Val Ile Ala Tyr Ser Arg
        355                 360                 365

Asn His Val Thr Asn Lys Leu Phe Glu Glu Asn Gly Ile Lys Val His
    370                 375                 380

Arg Ile Pro Ser Ser Glu Leu Ser Arg Gly Arg Gly Gly Pro Arg Cys
385                 390                 395                 400

Met Ser Met Ser Leu Val Arg Glu Asp Ile
                405                 410


<210> 9
<211> 409
<212> PRT
<213> Borrelia afzelii

<400> 9
Met Glu Glu Tyr Leu Asn Pro Ile Asn Ile Phe Ser Glu Ile Gly Arg
  1               5                  10                  15

Leu Lys Lys Val Leu Leu His Arg Pro Gly Glu Glu Leu Glu Asn Leu
             20                  25                  30

Thr Pro Phe Ile Met Lys Asn Phe Leu Phe Asp Asp Ile Pro Tyr Leu
         35                  40                  45

Glu Val Ala Arg Gln Glu His Glu Val Phe Ala Ser Ile Leu Lys Asn
     50                  55                  60

Asn Leu Val Glu Ile Glu Tyr Ile Glu Asp Leu Ile Ser Glu Val Leu
 65                  70                  75                  80

Val Ser Ser Val Ala Leu Glu Asn Lys Phe Ile Ser Gln Phe Ile Leu
                 85                  90                  95

Glu Ala Glu Ile Lys Thr Asp Phe Thr Ile Asn Leu Leu Lys Asp Tyr
            100                 105                 110
```

Phe Ser Ser Leu Thr Ile Asp Asn Met Ile Ser Lys Met Ile Ser Gly
115 120 125

Val Val Thr Glu Glu Leu Lys Asn Tyr Thr Ser Ser Leu Asp Asp Leu
130 135 140

Val Asn Gly Ala Asn Leu Phe Ile Ile Asp Pro Met Pro Asn Val Leu
145 150 155 160

Phe Thr Arg Asp Pro Phe Ala Ser Ile Gly Asn Gly Val Thr Ile Asn
165 170 175

Lys Met Phe Thr Lys Val Arg Gln Arg Glu Thr Ile Phe Ala Glu Tyr
180 185 190

Ile Phe Lys Tyr His Pro Val Tyr Lys Glu Asn Val Pro Ile Trp Leu
195 200 205

Asn Arg Trp Glu Glu Ala Ser Leu Glu Gly Gly Asp Glu Leu Val Leu
210 215 220

Asn Lys Gly Leu Leu Val Ile Gly Ile Ser Glu Arg Thr Glu Ala Lys
225 230 235 240

Ser Val Glu Lys Leu Ala Ile Ser Leu Phe Lys Asn Lys Thr Ser Phe
245 250 255

Asp Thr Ile Leu Ala Phe Gln Ile Pro Lys Asn Arg Ser Tyr Met His
260 265 270

Leu Asp Thr Val Phe Thr Gln Ile Asp Tyr Ser Val Phe Thr Ser Phe
275 280 285

Thr Ser Asp Asp Met Tyr Phe Ser Ile Tyr Val Leu Thr Tyr Asn Pro
290 295 300

Ser Ser Ser Lys Ile His Ile Lys Lys Glu Lys Ala Arg Ile Lys Asp
305 310 315 320

Val Leu Ser Phe Tyr Leu Gly Arg Lys Ile Asp Ile Ile Lys Cys Ala
325 330 335

Gly Gly Asp Leu Ile His Gly Ala Arg Glu Gln Trp Asn Asp Gly Ala
340 345 350

Asn Val Leu Ala Ile Ala Pro Gly Glu Ile Ile Ala Tyr Ser Arg Asn
355 360 365

```
His Val Thr Asn Lys Leu Phe Glu Glu Asn Gly Ile Lys Val His Arg
    370                 375                 380

Ile Pro Ser Ser Glu Leu Ser Arg Gly Arg Gly Gly Pro Arg Cys Met
385                 390                 395                 400

Ser Met Pro Leu Ile Arg Glu Asp Ile
                405
```

<210> 10
<211> 580
<212> PRT
<213> Qiardia intestinalis

<400> 10

```
Met Thr Asp Phe Ser Lys Asp Lys Glu Lys Leu Ala Gln Ala Thr Gln
  1               5                  10                  15

Gly Gly Glu Asn Glu Arg Ala Glu Ile Val Val Val His Leu Pro Gln
             20                  25                  30

Gly Thr Ser Phe Leu Thr Ser Leu Asn Pro Glu Gly Asn Leu Leu Glu
         35                  40                  45

Glu Pro Ile Cys Pro Asp Glu Leu Arg Arg Asp His Glu Gly Phe Gln
     50                  55                  60

Ala Val Leu Lys Glu Lys Gly Cys Arg Val Tyr Met Pro Tyr Asp Val
 65                  70                  75                  80

Leu Ser Glu Ala Ser Pro Ala Glu Arg Glu Val Leu Met Asp Gln Ala
                85                  90                  95

Met Ala Ser Leu Lys Tyr Glu Leu His Ala Thr Gly Ala Arg Ile Thr
            100                 105                 110

Pro Lys Met Lys Tyr Cys Val Ser Asp Glu Tyr Lys Arg Lys Val Leu
        115                 120                 125

Ser Ala Leu Ser Thr Arg Asn Leu Val Asp Val Ile Leu Ser Glu Pro
    130                 135                 140

Val Ile His Leu Ala Pro Gly Val Arg Asn Thr Ala Leu Val Thr Asn
145                 150                 155                 160

Ser Val Glu Ile His Asp Ser Asn Asn Met Val Phe Met Arg Asp Gln
                165                 170                 175
```

```
Gln Ile Thr Thr Arg Arg Gly Ile Val Met Gly Gln Phe Gln Ala Pro
            180                 185                 190

Gln Arg Arg Arg Glu Gln Val Leu Ala Leu Ile Phe Trp Lys Arg Leu
        195                 200                 205

Gly Ala Arg Val Val Gly Asp Cys Arg Glu Gly Gly Pro His Cys Met
    210                 215                 220

Leu Glu Gly Gly Asp Phe Val Pro Val Ser Pro Gly Leu Ala Met Met
225                 230                 235                 240

Gly Val Gly Leu Arg Ser Thr Tyr Val Gly Ala Gln Tyr Leu Met Ser
                245                 250                 255

Lys Asp Leu Leu Gly Thr Arg Arg Phe Ala Val Val Lys Asp Cys Phe
            260                 265                 270

Asp Gln His Gln Asp Arg Met His Leu Asp Cys Thr Phe Ser Val Leu
        275                 280                 285

His Asp Lys Leu Val Val Leu Asp Asp Tyr Ile Cys Ser Gly Met Gly
    290                 295                 300

Leu Arg Tyr Val Asp Glu Trp Ile Asp Val Gly Ala Asp Ala Val Lys
305                 310                 315                 320

Lys Ala Lys Ser Ser Ala Val Thr Cys Gly Asn Tyr Val Leu Ala Lys
                325                 330                 335

Ala Asn Val Glu Phe Gln Gln Trp Leu Ser Glu Asn Gly Tyr Thr Ile
            340                 345                 350

Val Arg Ile Pro His Glu Tyr Gln Leu Ala Tyr Gly Cys Asn Asn Leu
        355                 360                 365

Asn Leu Gly Asn Asn Cys Val Leu Ser Val His Gln Pro Thr Val Asp
    370                 375                 380

Phe Ile Lys Ala Asp Pro Ala Tyr Ile Ser Tyr Cys Lys Ser Asn Asn
385                 390                 395                 400

Leu Pro Asn Gly Leu Asp Leu Val Tyr Val Pro Phe Arg Gly Ile Thr
                405                 410                 415

Arg Met Tyr Gly Ser Leu His Cys Ala Ser Gln Val Val Tyr Arg Thr
            420                 425                 430
```

```
Pro Leu Ala Pro Ala Ala Val Lys Ala Cys Glu Gln Glu Gly Asp Gly
        435                 440                 445

Ile Ala Ala Ile Tyr Glu Lys Asn Gly Glu Pro Val Asp Ala Ala Gly
    450                 455                 460

Lys Lys Phe Asp Cys Val Ile Tyr Ile Pro Ser Ser Val Asp Asp Leu
465                 470                 475                 480

Ile Asp Gly Leu Lys Ile Asn Leu Arg Asp Asp Ala Ala Pro Ser Arg
                485                 490                 495

Glu Ile Ile Ala Asp Ala Tyr Gly Leu Tyr Gln Lys Leu Val Ser Glu
            500                 505                 510

Gly Arg Val Pro Tyr Ile Thr Trp Arg Met Pro Ser Met Pro Val Val
        515                 520                 525

Ser Leu Lys Gly Ala Ala Lys Ala Gly Ser Leu Lys Ala Val Leu Asp
    530                 535                 540

Lys Ile Pro Gln Leu Thr Pro Phe Thr Pro Lys Ala Val Glu Gly Ala
545                 550                 555                 560

Pro Ala Ala Tyr Thr Arg Tyr Leu Gly Leu Glu Gln Ala Asp Ile Cys
                565                 570                 575

Val Asp Ile Lys
            580
```

<210> 11
<211> 413
<212> PRT
<213> Clostridium perfringens

<400> 11

```
Met Arg Asp Asp Arg Ala Leu Asn Val Thr Ser Glu Ile Gly Arg Leu
  1               5                  10                  15

Lys Thr Val Leu Leu His Arg Pro Gly Glu Glu Ile Glu Asn Leu Thr
             20                  25                  30

Pro Asp Leu Leu Asp Arg Leu Leu Phe Asp Asp Ile Pro Tyr Leu Lys
         35                  40                  45

Val Ala Arg Glu Glu His Asp Ala Phe Ala Gln Thr Leu Arg Glu Ala
```

```
     50                  55                  60

Gly Val Glu Val Leu Tyr Leu Glu Val Leu Ala Ala Glu Ala Ile Glu
 65                  70                  75                  80

Thr Ser Asp Glu Val Lys Gln Gln Phe Ile Ser Glu Phe Ile Asp Glu
                 85                  90                  95

Ala Gly Val Glu Ser Glu Arg Leu Lys Glu Ala Leu Ile Glu Tyr Phe
            100                 105                 110

Asn Ser Phe Ser Asp Asn Lys Ala Met Val Asp Lys Met Met Ala Gly
        115                 120                 125

Val Arg Lys Glu Glu Leu Lys Asp Tyr His Arg Glu Ser Leu Tyr Asp
    130                 135                 140

Gln Val Asn Asn Val Tyr Pro Phe Val Cys Asp Pro Met Pro Asn Leu
145                 150                 155                 160

Tyr Phe Thr Arg Glu Pro Phe Ala Thr Ile Gly His Gly Ile Thr Leu
                165                 170                 175

Asn His Met Arg Thr Asp Thr Arg Asn Arg Glu Thr Ile Phe Ala Lys
            180                 185                 190

Tyr Ile Phe Arg His His Pro Arg Phe Glu Gly Lys Asp Ile Pro Phe
        195                 200                 205

Trp Phe Asn Arg Asn Asp Lys Thr Ser Leu Glu Gly Gly Asp Glu Leu
    210                 215                 220

Ile Leu Ser Lys Glu Ile Leu Ala Val Gly Ile Ser Gln Arg Thr Asp
225                 230                 235                 240

Ser Ala Ser Val Glu Lys Leu Ala Lys Lys Leu Leu Tyr Tyr Pro Asp
                245                 250                 255

Thr Ser Phe Lys Thr Val Leu Ala Phe Lys Ile Pro Val Ser Arg Ala
            260                 265                 270

Phe Met His Leu Asp Thr Val Phe Thr Gln Val Asp Tyr Asp Lys Phe
        275                 280                 285

Thr Val His Pro Gly Ile Val Gly Pro Leu Glu Val Tyr Ala Leu Thr
    290                 295                 300

Lys Asp Pro Glu Asn Asp Gly Gln Leu Leu Val Thr Glu Glu Val Asp
```

305 310 315 320

Thr Leu Glu Asn Ile Leu Lys Lys Tyr Leu Asp Arg Asp Ile Lys Leu
325 330 335

Ile Lys Cys Gly Gly Gly Asp Glu Ile Ile Ala Ala Arg Glu Gln Trp
340 345 350

Asn Asp Gly Ser Asn Thr Leu Ala Ile Ala Pro Gly Glu Val Val Val
355 360 365

Tyr Ser Arg Asn Tyr Val Thr Asn Glu Ile Leu Glu Lys Glu Gly Ile
370 375 380

Lys Leu His Val Ile Pro Ser Ser Glu Leu Ser Arg Gly Arg Gly Gly
385 390 395 400

Pro Arg Cys Met Ser Met Pro Leu Ile Arg Glu Asp Leu
405 410

<210> 12
<211> 413
<212> PRT
<213> Bacillus licheniformis

<400> 12
Met Ile Met Thr Thr Pro Ile His Val Tyr Ser Glu Ile Gly Pro Leu
1 5 10 15

Lys Thr Val Met Leu Lys Arg Pro Gly Arg Glu Leu Glu Asn Leu Thr
20 25 30

Pro Glu Tyr Leu Glu Arg Leu Leu Phe Asp Asp Ile Pro Phe Leu Pro
35 40 45

Ala Val Gln Lys Glu His Asp Gln Phe Ala Glu Thr Leu Lys Gln Gln
50 55 60

Gly Ala Glu Val Leu Tyr Leu Glu Lys Leu Thr Ala Glu Ala Leu Asp
65 70 75 80

Asp Ala Leu Val Arg Glu Gln Phe Ile Asp Glu Leu Leu Thr Glu Ser
85 90 95

Lys Ala Asp Ile Asn Gly Ala Tyr Asp Arg Leu Lys Glu Phe Leu Leu
100 105 110

```
Thr Phe Asp Ala Asp Ser Met Val Glu Gln Val Met Ser Gly Ile Arg
        115                 120                 125

Lys Asn Glu Leu Glu Arg Glu Lys Lys Ser His Leu His Glu Leu Met
    130                 135                 140

Glu Asp His Tyr Pro Phe Tyr Leu Asp Pro Met Pro Asn Leu Tyr Phe
145                 150                 155                 160

Thr Arg Asp Pro Ala Ala Ala Ile Gly Ser Gly Leu Thr Ile Asn Lys
                165                 170                 175

Met Lys Glu Pro Ala Arg Arg Arg Glu Ser Leu Phe Met Arg Tyr Ile
            180                 185                 190

Ile Asn His His Pro Arg Phe Lys Gly His Glu Ile Pro Val Trp Leu
        195                 200                 205

Asp Arg Asp Phe Lys Phe Asn Ile Glu Gly Gly Asp Glu Leu Val Leu
    210                 215                 220

Asn Glu Glu Thr Val Ala Ile Gly Val Ser Glu Arg Thr Thr Ala Gln
225                 230                 235                 240

Ala Ile Glu Arg Leu Val Arg Asn Leu Phe Gln Arg Gln Ser Arg Ile
                245                 250                 255

Arg Arg Val Leu Ala Val Glu Ile Pro Lys Ser Arg Ala Phe Met His
            260                 265                 270

Leu Asp Thr Val Phe Thr Met Val Asp Arg Asp Gln Phe Thr Ile His
        275                 280                 285

Pro Ala Ile Gln Gly Pro Glu Gly Asp Met Arg Ile Phe Val Leu Glu
    290                 295                 300

Arg Gly Lys Thr Ala Asp Glu Ile His Thr Thr Glu Glu His Asn Leu
305                 310                 315                 320

Pro Glu Val Leu Lys Arg Thr Leu Gly Leu Ser Asp Val Asn Leu Ile
                325                 330                 335

Phe Cys Gly Gly Gly Asp Glu Ile Ala Ser Ala Arg Glu Gln Trp Asn
            340                 345                 350

Asp Gly Ser Asn Thr Leu Ala Ile Ala Pro Gly Val Val Val Thr Tyr
        355                 360                 365
```

```
Asp Arg Asn Tyr Ile Ser Asn Glu Cys Leu Arg Glu Gln Gly Ile Lys
    370                 375                 380

Val Ile Glu Ile Pro Ser Gly Glu Leu Ser Arg Gly Arg Gly Gly Pro
385                 390                 395                 400

Arg Cys Met Ser Met Pro Leu Tyr Arg Glu Asp Val Lys
                405                 410
```

<210> 13
<211> 408
<212> PRT
<213> Enterococcus faecalis

<400> 13

```
Met Ser His Pro Ile Asn Val Phe Ser Glu Ile Gly Lys Leu Lys Thr
  1               5                  10                  15

Val Met Leu His Arg Pro Gly Lys Glu Leu Glu Asn Leu Met Pro Asp
             20                  25                  30

Tyr Leu Glu Arg Leu Leu Phe Asp Asp Ile Pro Phe Leu Glu Lys Ala
         35                  40                  45

Gln Ala Glu His Asp Ala Phe Ala Glu Leu Leu Arg Ser Lys Asp Ile
     50                  55                  60

Glu Val Val Tyr Leu Glu Asp Leu Ala Ala Glu Ala Leu Ile Asn Glu
 65                  70                  75                  80

Glu Val Arg Arg Gln Phe Ile Asp Gln Phe Leu Glu Glu Ala Asn Ile
                85                  90                  95

Arg Ser Glu Ser Ala Lys Glu Lys Val Arg Glu Leu Met Leu Glu Ile
            100                 105                 110

Asp Asp Asn Glu Glu Leu Ile Gln Lys Ala Ile Ala Gly Ile Gln Lys
        115                 120                 125

Gln Glu Leu Pro Lys Tyr Glu Gln Glu Phe Leu Thr Asp Met Val Glu
    130                 135                 140

Ala Asp Tyr Pro Phe Ile Ile Asp Pro Met Pro Asn Leu Tyr Phe Thr
145                 150                 155                 160

Arg Asp Asn Phe Ala Thr Met Gly His Gly Ile Ser Leu Asn His Met
                165                 170                 175
```

Tyr Ser Val Thr Arg Gln Arg Glu Thr Ile Phe Gly Gln Tyr Ile Phe
180 185 190

Asp Tyr His Pro Arg Phe Ala Gly Lys Glu Val Pro Arg Val Tyr Asp
195 200 205

Arg Ser Glu Ser Thr Arg Ile Glu Gly Gly Asp Glu Leu Ile Leu Ser
210 215 220

Lys Glu Val Val Ala Ile Gly Ile Ser Gln Arg Thr Asp Ala Ala Ser
225 230 235 240

Ile Glu Lys Ile Ala Arg Asn Ile Phe Glu Gln Lys Leu Gly Phe Lys
245 250 255

Asn Ile Leu Ala Phe Asp Ile Gly Glu His Arg Lys Phe Met His Leu
260 265 270

Asp Thr Val Phe Thr Met Ile Asp Tyr Asp Lys Phe Thr Ile His Pro
275 280 285

Glu Ile Glu Gly Gly Leu Val Val Tyr Ser Ile Thr Glu Lys Ala Asp
290 295 300

Gly Asp Ile Gln Ile Thr Lys Glu Lys Asp Thr Leu Asp Asn Ile Leu
305 310 315 320

Cys Lys Tyr Leu His Leu Asp Asn Val Gln Leu Ile Arg Cys Gly Ala
325 330 335

Gly Asn Leu Thr Ala Ala Ala Arg Glu Gln Trp Asn Asp Gly Ser Asn
340 345 350

Thr Leu Ala Ile Ala Pro Gly Glu Val Val Val Tyr Asp Arg Asn Thr
355 360 365

Ile Thr Asn Lys Ala Leu Glu Glu Ala Gly Val Lys Leu Asn Tyr Ile
370 375 380

Pro Gly Ser Glu Leu Val Arg Gly Arg Gly Gly Pro Arg Cys Met Ser
385 390 395 400

Met Pro Leu Tyr Arg Glu Asp Leu
405

<210> 14

<211> 409
<212> PRT
<213> Lactobacillus sake

<400> 14

```
Met Thr Ser Pro Ile His Val Asn Ser Glu Ile Gly Lys Leu Lys Thr
  1               5                  10                  15

Val Leu Leu Lys Arg Pro Gly Lys Glu Val Glu Asn Ile Thr Pro Asp
             20                  25                  30

Ile Met Tyr Arg Leu Leu Phe Asp Asp Ile Pro Tyr Leu Pro Thr Ile
         35                  40                  45

Gln Lys Glu His Asp Gln Phe Ala Gln Thr Leu Arg Asp Asn Gly Val
     50                  55                  60

Glu Val Leu Tyr Leu Glu Asn Leu Ala Ala Glu Ala Ile Asp Ala Gly
 65                  70                  75                  80

Asp Val Lys Glu Ala Phe Leu Asp Lys Met Leu Asn Glu Ser His Ile
                 85                  90                  95

Lys Ser Pro Gln Val Gln Ala Ala Leu Lys Asp Tyr Leu Ile Ser Met
            100                 105                 110

Ala Thr Leu Asp Met Val Glu Lys Ile Met Ala Gly Val Arg Thr Asn
        115                 120                 125

Glu Ile Asp Ile Lys Ser Lys Ala Leu Ile Asp Val Ser Ala Asp Asp
    130                 135                 140

Asp Tyr Pro Phe Tyr Met Asp Pro Met Pro Asn Leu Tyr Phe Thr Arg
145                 150                 155                 160

Asp Pro Ala Ala Ser Met Gly Asp Gly Leu Thr Ile Asn Lys Met Thr
                165                 170                 175

Phe Glu Ala Arg Gln Arg Glu Ser Met Phe Met Glu Val Ile Met Gln
            180                 185                 190

His His Pro Arg Phe Ala Asn Gln Gly Ala Gln Val Trp Arg Asp Arg
        195                 200                 205

Asp His Ile Asp Arg Met Glu Gly Gly Asp Glu Leu Ile Leu Ser Asp
    210                 215                 220

Lys Val Leu Ala Ile Gly Ile Ser Gln Arg Thr Ser Ala Gln Ser Ile
```

225 230 235 240

Glu Glu Leu Ala Lys Val Leu Phe Ala Asn His Ser Gly Phe Glu Lys
245 250 255

Ile Leu Ala Ile Lys Ile Pro His Lys His Ala Met Met His Leu Asp
260 265 270

Thr Val Phe Thr Met Ile Asp Tyr Asp Lys Phe Thr Ile His Pro Gly
275 280 285

Ile Gln Gly Ala Gly Gly Met Val Asp Thr Tyr Ile Leu Glu Pro Gly
290 295 300

Asn Asn Asp Glu Ile Lys Ile Thr His Gln Thr Asp Leu Glu Lys Val
305 310 315 320

Leu Arg Asp Ala Leu Glu Val Pro Glu Leu Thr Leu Ile Pro Cys Gly
325 330 335

Gly Gly Asp Ala Val Val Ala Pro Arg Glu Gln Trp Asn Asp Gly Ser
340 345 350

Asn Thr Leu Ala Ile Ala Pro Gly Val Val Val Thr Tyr Asp Arg Asn
355 360 365

Tyr Val Ser Asn Glu Asn Leu Arg Gln Tyr Gly Ile Lys Val Ile Glu
370 375 380

Val Pro Ser Ser Glu Leu Ser Arg Gly Arg Gly Gly Pro Arg Cys Met
385 390 395 400

Ser Met Pro Leu Val Arg Arg Lys Thr
405