BIOLOGICALLY ACTIVE PEPTIDE COMPRISING TYROSYL-SERYL-VALINE (YSV)

Background of the Invention

Field of the Invention

The present invention is related to short peptides and the use thereof. In particular, the present invention is related to short peptides with immune-modifying and anti-cancer properties.

Description of the Related Art

Peptides are known in the art for treatment of diseases and as pharmaceutical compositions. For example, U.S. Patent No. 6,191,113 discloses a peptide that has inhibitory activity for the growth of smooth muscle cells and is therefore useful for preventing and treating pathological conditions associated with growth of smooth muscle cells such as arteriosclerosis, restenosis after angioplasty, luminal stenosis after grafting blood vessels and smooth muscle sarcoma. U.S. 6,184,208 discloses another peptide that is found to modulate physiological processes such as weight gain activity of the epithelial growth zone and hair growth. Furthermore, PCT publication no. WO 03/006492 and U.S. Patent Application No. 10/237,405 suggested that certain peptides and their pharmaceutical compositions are biologically active and capable of modulating immune responses. (References which are cited in the present disclosure are not necessarily prior art and therefore their citation does not constitute an admission that such references are prior art in any jurisdiction.)

It is therefore an object of the present invention to provide short peptides that have biological activity.

Summary of the Invention

One aspect of the present invention relates to the tripeptide Tyrosyl-seryl-valine (YSV), which has been found to contain biological activity. For testing purposes, the peptide L-Tyrosyl-L-seryl-L-valine has been used. Further aspects of the present invention include isolated or purified peptides comprising, consisting essentially of, or consisting of Tyrosyl-seryl-valine. Another aspect relates to substantially pure YSV peptides.

An additional aspect of the present invention comprises an isolated or purified peptide consisting essentially of YSV, where the peptide has an activity selected from the group consisting of modulation of an immune response, stimulation of T lymphocyte

transformation, modulation of a cell proliferative disorder, modulation of the growth of a cancer, modulation of the growth of a liver cancer, modulation of the growth of leukemia cells, modulation of the growth of a cervical cancer, modulation of the growth of a lung cancer and the modulation of the growth of a melanoma.

Additional aspects of the present invention include pharmaceutical compositions comprising a peptide comprising, consisting essentially of, or consisting of the YSV peptide. Other aspects of the present invention relate to pharmaceutical compositions that comprise a peptide that comprises, consists essentially of or consists of a functional derivative of the YSV peptide. Additional aspects include pharmaceutical compositions comprising, consisting essentially of, or consisting of the tripeptide L-Tyrosyl-L-seryl-L-valine.

Another aspect of the present invention relates to a method of making a pharmaceutical composition comprising providing the tripeptide Tyrosyl-seryl-valine and mixing said tripeptide with a pharmaceutical acceptable carrier.

Another aspect of the present invention relates to a method of reducing the effects of a human disease comprising administering a pharmaceutically effective dose of the tripeptide Tyrosyl-seryl-valine to a human. In additional aspects of the present invention, the human disease is selected from the group consisting of a condition whose effects can be reduced by stimulating T lymphocyte transformation and a cell proliferative disorder. In additional aspects of the invention, the cell proliferative disorder is a cancer, including but not limited to liver cancer, leukemia, cervical cancer, lung cancer and melanoma.

Another aspect of the present invention relates to the use of the tripeptide Tyrosyl-seryl-valine as a pharmaceutical composition. Furthermore, the tripeptide may be used to modulate the immune system, and may also be used as a treatment for a cell proliferative disorder. In particular aspects of the invention, the cell proliferative disorder is cancer. In particular aspects of the invention, liver cancer, leukemia, cervical cancer, lung cancer and/or melanoma is treated.

A further aspect of the present invention is directed to a nutritional composition containing Tyrosyl-seryl-valine and the use of the same for the manufacture of a nutritional supplement. Particular aspects of the invention relate to nutritional supplements comprising peptides that comprise, consist essentially of or consist of the tripeptide Tyrosyl-seryl-valine.

In a further aspect of the present invention, enhanced derivatives of YSV or its functional derivatives are provided. The enhanced derivative of the tripeptide Tyrosyl-seryl-valine comprises an enhancement molecule operably linked to the tripeptide Tyrosyl-seryl-valine in such a manner as to improve or augment the therapeutic effectiveness of the tripeptide. The enhancement effect may be that of a prolonged effect, a shortened effect, a delayed onset of effect, a hastened onset of effect, an increased intensity of effect, a decreased intensity of effect, a reduction in side effects, the creation of one or more effects, a delayed subsiding of effect, a hastened subsiding of effect and a targeting of the peptide to a discrete location within an individual. Examples of such enhancement molecules and enhanced derivatives are described below. In some aspects of the invention, the enhanced molecules can modulate, but are not limited to modulating, immune activity and/or the growth of a cancer, where said cancer includes, but is not limited to, cervical carcinoma, liver cancer, and leukemia. Additional aspects of the present invention include methods of enhancing the therapeutic effects of a peptide comprising, consisting essentially of or consisting of YSV or its derivatives, comprising operably linking said peptide to a molecule which enhances the therapeutic effect. In some aspects of the invention, the method is not the inclusion of a peptide which is adjacent to the YSV peptide or its derivative in a naturally occuring peptide. Additional aspects of the present invention include pharmaceutical compositions comprising, consisting essentially of or consisting of enhanced derivatives of YSV or its functional derivatives.

One aspect of the present invention relates to the substantially pure peptide YSV or its functional derivatives disclosed above operably linked to a molecule that enhances their therapeutic effectiveness, also known herein as "enhancement molecules". Such molecules may be prepared and used in any of the ways described in U.S. Provisional Patent Application No. 60/435,796, entitled "Biologically active peptide conjugates", and filed on December 18, 2002, the disclosure of which is incorporated herein by reference in its entirety. Candidate molecules to be operably linked to the peptides and the means for carrying out such linkings are familiar to those with skill in the art. Some molecules that could be operably linked to the YSV peptide and its functional derivatives include, but are not limited to, an organic compound, a carbohydrate, a sugar, a polysaccharide, an amino acid, an amino acid polymer, a peptide, a steroid, a protein, an isolated domain of a protein, a

hapten, an antigen, a lipid molecule, a fatty acid, a bile acid, a polyamine, a protease inhibitor, a silicate and a combination of any of the preceding molecules. The invention also relates to the substantially pure peptide disclosed above and its functional derivatives operably linked to a molecule that enhances its therapeutic effectiveness, wherein said operably linked molecule is not a peptide which is adjacent to the above-disclosed peptide in a naturally occurring peptide. In another aspect of the invention, substantially pure YSV peptide or its functional derivatives can modulate, but are not limited to modulating, immune activity and/or a cell proliferative disorder, such as cancer, where said cancer includes, but is not limited to, cervical carcinoma, liver cancer, leukemia, lung cancer and melanoma. The molecule may be operably linked to the peptide of the invention with a covalent bond or a non-covalent interaction.

In specific embodiments, biologically effective molecules, when operably linked to YSV or its functional derivatives, can alter the pharmacokinetics of the peptide by conferring properties to the peptide as part of a linked molecule. Some of the properties that the operably linked molecules can confer on peptides include, but are not limited to: delivery of a peptide to a discrete location within the body; concentrating the activity of a peptide at a desired location in the body and reducing its effects elsewhere; reducing side effects of treatment with a peptide; changing the permiability of a peptide; changing the bioavailability or the rate of delivery to the body of a peptide; changing the length of the effect of treatment with a peptide; altering the stability of the peptide; altering the rate of the onset and the decay of the effects of a peptide; providing a permissive action by allowing a peptide to have an effect.

Another aspect of the present invention relates to substantially pure peptides comprising, consisting essentially of or consisting of YSV or its functional derivatives operably linked to a molecule which enhances its therapeutic effectiveness, wherein said operably linked molecule is not a peptide which is adjacent to YSV or one of its functional derivatives in a naturally occurring peptide. Some molecules that could be operably linked to the YSV peptide and its functional derivatives include, but are not limited to, an organic compound, a carbohydrate, a sugar, a polysaccharide, an amino acid, an amino acid polymer, a peptide, a steroid, a protein, an isolated domain of a protein, a hapten, an antigen, a lipid molecule, a fatty acid, a bile acid, a polyamine, a protease inhibitor, a silicate and a

combination of any of the preceding molecules. Additional aspects of the invention include substantially pure peptides comprising, consisting essentially of or consisting of YSV peptide or its functional derivatives operably linked to a molecule which enhances its therapeutic effectiveness that can modulate, but are not limited to modulating, immune activity and/or a cell proliferative disorder, such as cancer, where said cancer includes, but is not limited to, cervical carcinoma, liver cancer, leukemia, lung cancer and melanoma. The molecule may be operably linked to the peptide of the invention with a covalent bond or a non-covalent interaction. The effects of the operable linkage between the substantially pure peptides and the molecule which enhances its therapeutic effectiveness can include, but are not limited to: delivery of a peptide to a discrete location within the body; concentrating the activity of a peptide at a desired location in the body and reducing its effects elsewhere; reducing side effects of treatment with a peptide; changing the permiability of a peptide; changing the bioavailability or the rate of delivery to the body of a peptide; changing the length of the effect of treatment with a peptide; altering the stability of the peptide; altering the rate of the onset and the decay of the effects of a peptide; providing a permissive action by allowing a peptide to have an effect.

Another aspect of the present invention relates to hybrid peptides containing the peptide comprising YSV or one of its functional derivatives with an additional peptide sequence attached, where said attached additional sequence is not a sequence found adjacent to the peptide disclosed above in a naturally occurring peptide. In specific embodiments, the hybrid peptides above can modulate, but are not limited to modulating, immune activity and/or a cell proliferative disorder, such as cancer, where said cancer includes, but is not limited to, cervical carcinoma, liver cancer, leukemia, lung cancer and melanoma. In specific embodiments, these attached additional peptide sequences not found adjacent to YSV or its functional derivatives in a naturally occurring peptide, can alter the pharmacokinetics of the peptides of the above described embodiments of the invention by virtue of conferring properties to the peptide as part of a hybrid molecule. Some of the properties that the operably linked molecules can confer on YSV or its functional derivatives include, but are not limited to: delivery of a peptide to a discrete location within the body; concentrating the activity of a peptide at a desired location in the body and reducing its effects elsewhere; reducing side effects of treatment with a peptide; changing the permiability of a peptide;

changing the bioavailability or the rate of delivery to the body of a peptide; changing the length of the effect of treatment with a peptide; altering the stability of the peptide; altering the rate of the onset and the decay of the effects of a peptide; providing a permissive action by allowing a peptide to have an effect.

Another aspect of the present invention relates to a genetic vector comprising, consisting essentially of, or consisting of a first nucleotide sequence encoding the YSV peptide or one of its functional derivatives fused in frame with a second nucleotide sequence encoding a peptide that enhances the therapeutic effectiveness of the aforementioned peptide and that is not adjacent to said YSV peptide or said one of its functional derivatives in a naturally occurring peptide. It also relates to a genetic vector comprising, consisting essentially of, or consisting of a first nucleotide sequence encoding a peptide consisting essentially of the YSV peptide or one of its functional derivatives fused in frame with a second nucleotide sequence encoding a peptide that enhances the therapeutic effectiveness of the aforementioned peptide and that is not adjacent to said YSV peptide or said one of its functional derivatives in a naturally occurring peptide. It further relates to a genetic vector comprising, consisting essentially of, or consisting of a first nucleotide sequence encoding a peptide consisting of the amino acid sequence of the YSV peptide or one of its functional derivatives fused in frame with a second nucleotide sequence encoding a peptide that enhances the therapeutic effectiveness of the aforementioned peptide and that is not adjacent to said YSV peptide or said one of its functional derivatives in a naturally occurring peptide. In specific embodiments, said YSV peptide or said one of its functional derivatives can modulate, but are not limited to modulating, immune activity and/or a cell proliferative disorder, such as cancer, where said cancer includes, but is not limited to, cervical carcinoma, liver cancer, leukemia, lung cancer and melanoma. Some of the properties that the operably linked molecules can confer on said YSV peptide or said one of its functional derivatives include, but are not limited to: delivery of a peptide to a discrete location within the body; concentrating the activity of a peptide at a desired location in the body and reducing its effects elsewhere; reducing side effects of treatment with a peptide; changing the permiability of a peptide; changing the bioavailability or the rate of delivery to the body of a peptide; changing the length of the effect of treatment with a peptide; altering the stability of the peptide; altering the rate of the onset and the decay of the effects of a peptide; providing a

permissive action by allowing a peptide to have an effect. Another aspect of the invention relates to micro-organisms that comprise nucleic acid sequences selected from the list consisting of: the nucleotide sequences of the vectors described above; and a nucleotide sequence comprising a first nucleotide sequence encoding a peptide comprising an amino acid sequence of said YSV peptide or said one of its functional derivatives fused in frame with a second nucleotide sequence encoding a peptide that is not adjacent to said YSV peptide or said one of its functional derivatives in a naturally occurring peptide.

In connection with any of the above-described nucleic acid sequences, the peptides and/or hybrid peptides expressed from these nucleic acid sequences can modulate, but are not limited to modulating, immune activity and/or a cell proliferative disorder, such as cancer, where said cancer includes, but is not limited to, cervical carcinoma, liver cancer, leukemia, lung cancer and melanoma.

A further aspect of the present invention relates to a method of making a pharmaceutical composition comprising providing the YSV peptide or one of its functional derivatives operably linked to a molecule which enhances its therapeutic effect; and formulating said peptide operably linked with said molecule with a pharmaceutically acceptable carrier. The invention also relates to said method wherein said peptide can modulate, but is not limited to modulating, immune activity and/or a cell proliferative disorder, such as cancer, where said cancer includes, but is not limited to, cervical carcinoma, liver cancer, leukemia, lung cancer and melanoma. Some examples of biologically effective molecules that could be attached to said YSV peptide or said one of its functional derivatives include, but are not limited to, an organic compound, a carbohydrate, a sugar, a polysaccharide, an amino acid, an amino acid polymer, a peptide, a steroid, a protein, an isolated domain of a protein, a hapten, an antigen, a lipid molecule, a fatty acid, a bile acid, a polyamine, a protease inhibitor, a silicate and a combination of any of the preceding molecules. The invention also relates to a method of making of pharmaceutical comprising a peptide comprising said YSV peptide or said one of its functional derivatives comprising operably linking said peptide to a molecule which enhances said therapeutic effect, wherein said molecule is not a peptide which is adjacent to said YSV peptide or said one of its functional derivatives in a naturally occurring peptide. The molecule may be operably linked to a peptide of the invention with a covalent bond or a non-covalent interaction. In a specific

embodiment, the properties that said linked molecule can confer on said peptides to enhance their therapeutic effects include, but are not limited to: delivery of a peptide to a discrete location within the body; concentrating the activity of a peptide at a desired location in the body and reducing its effects elsewhere; reducing side effects of treatment with a peptide; changing the permiability of a peptide; changing the bioavailability or the rate of delivery to the body of a peptide; changing the length of the effect of treatment with a peptide; altering the stability of the peptide; altering the rate of the onset and the decay of the effects of a peptide; providing a permissive action by allowing a peptide to have an effect. It also relates to a method of making a pharmaceutical composition comprising providing a substantially pure peptide consisting essentially of the amino acid sequence of YSV peptide or one of its functional derivatives operably linked to a molecule which enhances its therapeutic effect; and formulating said peptide operably linked with said molecule with a pharmaceutically acceptable carrier. It further relates to a method of making a pharmaceutical composition comprising providing a substantially pure peptide consisting of the YSV peptide or one of its functional derivatives operably linked to a molecule which enhances its therapeutic effect; and formulating said peptide operably linked with said molecule with a pharmaceutically acceptable carrier.

Yet a further aspect of the present invention relates to a method of treatment of a human comprising administering a pharmaceutically effective dose of a substantially pure peptide comprising the YSV peptide or one of its functional derivatives to a human, said peptide being operably linked to a molecule which enhances its therapeutic effectiveness. Some examples of biologically effective molecules that could be operably linked to said YSV peptide or said one of its functional derivatives include, but are not limited to, an organic compound, a carbohydrate, a sugar, a polysaccharide, an amino acid, an amino acid polymer, a peptide, a steroid, a protein, an isolated domain of a protein, a hapten, an antigen, a lipid molecule, a fatty acid, a bile acid, a polyamine, a protease inhibitor, a silicate and a combination of any of the preceding molecules. In some embodiments, the properties that said operably linked molecule can confer on said peptides to enhance their therapeutic effects include, but are not limited to: delivery of a peptide to a discrete location within the body; concentrating the activity of a peptide at a desired location in the body and reducing its effects elsewhere; reducing side effects of treatment with a peptide; changing the

permiability of a peptide; changing the bioavailability or the rate of delivery to the body of a peptide; changing the length of the effect of treatment with a peptide; altering the stability of the peptide; altering the rate of the onset and the decay of the effects of a peptide; providing a permissive action by allowing a peptide to have an effect.

In particular embodiments, the peptides used for the treatment of human described above may be used to modulate, but are not limited to modulating, immune activity and/or a cell proliferative disorder, such as cancer, where said cancer includes, but is not limited to, cervical carcinoma, liver cancer, leukemia, lung cancer and melanoma.

Further aspects of the invention include pharmaceutical compositions comprising, consisting essentially of, or consisting of the YSV peptide or its functional derivatives operably linked to a molecule which enhances its therapeutic effect and a pharmaceutically acceptable carrier. The invention also relates to said enhanced YSV peptide or its derivatives where the peptide can modulate, but is not limited to modulating, immune activity and/or a cell proliferative disorder, such as cancer, where said cancer includes, but is not limited to, cervical carcinoma, liver cancer, leukemia, lung cancer and melanoma. Some examples of biologically effective molecules that could be operably linked to said YSV peptide or said one of its functional derivatives include, but are not limited to, an organic compound, a carbohydrate, a sugar, a polysaccharide, an amino acid, an amino acid polymer, a peptide, a steroid, a protein, an isolated domain of a protein, a hapten, an antigen, a lipid molecule, a fatty acid, a bile acid, a polyamine, a protease inhibitor, a silicate and a combination of any of the preceding molecules. In some embodiment, the properties that said operably linked molecule can confer on said peptides to enhance their therapeutic effects include, but are not limited to: delivery of a peptide to a discrete location within the body; concentrating the activity of a peptide at a desired location in the body and reducing its effects elsewhere; reducing side effects of treatment with a peptide; changing the permiability of a peptide; changing the bioavailability or the rate of delivery to the body of a peptide; changing the length of the effect of treatment with a peptide; altering the stability of the peptide; altering the rate of the onset and the decay of the effects of a peptide; providing a permissive action by allowing a peptide to have an effect.

Brief Description of the Drawings

Each of the five figures demonstrates exemplary chemical reactions for linking peptides to steroid molecules.

Figure 1 shows a series of chemical reactions for linking a peptide to an estrone molecule with a covalent bond.

Figure 2 shows a second, alternative set of reactions for creating the same linkage as in Figure 1.

Figure 3 contains a series of chemical reactions designed to link a peptide to a molecule of estradiol with a covalent bond.

Figure 4 contains a second series of chemical reactions for creating the same linkage as in Figure 3.

Figure 5 demonstrates a method of linking a peptide via a covalent bond to a molecule of hydrocortisone.

Detailed Description of the Preferred Embodiment

In the search of short peptide molecules that can be used as immunological modifying, anti-cell proliferative disorder, anti-cancer, and/or anti-sarcoma pharmaceuticals on humans, the inventors of the present invention discovered that the molecule L-Tyrosyl-L-seryl-L-Valine (YSV) has immunological modifying and anti-cancer properties in vitro. This finding suggests that the molecule YSV, larger molecules containing the molecule, including larger peptides and peptides that contain within their sequence the YSV sequence, and functional derivatives of YSV, may be useful as an immunological modifier and/or anti-cell proliferative disorder pharmaceutical or food supplement.

It is understood that it may be possible to add additional amino acids to the amino or carboxyl termini of the YSV peptide as another method of practising the present invention. In such embodiments, the YSV peptide maintains one or more of the therapeutic or functional properties described herein. For example, in some embodiments, one or two amino acids may be added to the disclosed peptide without affecting its biological function. In further embodiments, it may also be possible to add three or four amino acids and still maintain the function of the YSV peptide. These are all referred to as variants of the same peptide. Furthermore, derivatives of the peptide such as conservative replacement of one amino acid for another within the same functional class may be used to practise another

aspect of the present invention. For example, peptides having non-polar or hydrophobic side chains may be possible to substitute one side group for another without reducing biological activity. As a further example, linker/spacer may be inserted into the peptide to form variants, but the variants still retain their active moiety as the original peptide used in this study. These are also considered variants of the peptides. A peptide analogue as used herein, includes peptides that have amino acid molecules which mimic the structure of the natural amino acid, e.g. an analog with a different backbone structure, or D-amino acid substitution. As a further example, although the amino acids used for synthesizing the peptides are in their L optical isomeric form, peptides with one or more of the amino acids in the sequence substituted with the D-form may have similar biological activities. The term "functional derivative" as used in the claims is meant to include fragments, variants, analogues or chemical derivatives of the peptide.

"Substantially pure peptide" refers to peptides that are at least 10%w/w in purity, more preferably 20%, even more preferably 40% and much more preferably 60% and far more preferably larger than 90% pure. In the most preferred embodiment, the purity is larger than 99%. The substantially pure peptide can be used to prepare pharmaceutical and nutritional formulations that may be complex mixtures as described below.

The use of YSV or its functional derivatives in pharmaceutical formulations may be employed as possible treatment for immunological disorders or disease having secondary effect on immunity, e.g. cell proliferative disorders, such as cancer, or infections. The formulations may YSV or its functional derivatives mixed with other active or inactive constituents, including other peptides, e.g. two to several (e.g. 3-5) peptides may be added to the same formulation with or without other ingredients. Alternatively, YSV or its functional derivatives may be used to prepare the formulation together with peptides not listed here. They can be administered in the form of intravenous, intramuscular, intracutaneous, subcutaneous or intradermal. The mode of administration may also be intra-arterial injection that leads directly to the organ of problem. Other modes of administration are transdermal, inhalation as powder or spray, and other forms of delivery known by one in the art. The formulation may also be orally taken, and may contain carriers that can be used to prevent gastric digestion of the peptide after oral intake or any other carriers known in the art (for transdermal such as liposome).

As used herein, the term "hybrid peptide" is used to refer to peptides that contain additional peptides inserted into the original biologically active peptide having the sequence specified above or its functional derivatives, but still retain substantially similar activity. The additional peptides include leader peptides that contain, for example, an amino acid sequence that is recognized by one or more prokaryotic or eukaryotic cell as a signal for secretion of the hybrid protein into the exterior or the cell. The secretion may be a direct secretion, or indirectly through secretory vesicles.

As used in the present specification and claims, the terms "comprise," "comprises," and "comprising" mean "including, but not necessarily limited to". For example, a method, apparatus, molecule or other item which contains A, B, and C may be accurately said to comprise A and B. Likewise, a method, apparatus, molecule or other item which "comprises A and B" may include any number of additional steps, components, atoms or other items as well.

As used herein, the terminology "consisting essentially of" refers to a peptide or polypeptide which includes the amino acid sequence of the YSV peptide or one of its functional derivatives along with additional amino acids at the carboxyl and/or amino terminal ends and which maintains the activity of said peptides provided herein. Thus, as a non-limiting example, where the activity of the YSV peptide or one of its functional derivatives is to modulate immune activity and/or a cell proliferative disorder, such as cancer, a peptide or polypeptide "consisting essentially of" the YSV peptide or one of its functional derivatives will possess the activity of modulating immune activity and/or a cell proliferative disorder, such as cancer, as provided herein with respect to that peptide and will not possess any characteristics in and of itself (i.e. before modification by attachment to one or more biologically active molecules) which materially reduces the ability of the peptide or polypeptide to modulate immune activity and/or a cell proliferative disorder, such as cancer or which constitutes a material change to the basic and novel characteristics of the peptide as a modulator of immune activity. Thus, in the foregoing example, a full length naturally occurring polypeptide which has a primary activity other than modulating immune activity and/or a cell proliferative disorder, such as cancer, and which contains the amino acid sequence of YSV peptide or one of its functional derivatives somewhere therein would not constitute a peptide or polypeptide "consisting essentially of" the YSV peptide or one of its

functional derivatives. Likewise, in the foregoing example, a genetically engineered peptide or polypeptide which has a primary activity other than modulating immune activity and/or a cell proliferative disorder, such as cancer, but includes the amino acid sequence of the YSV peptide or one of its functional derivatives somewhere therein would not constitute a peptide or polypeptide "consisting essentially of" YSV peptide or one of its functional derivatives.

Those skilled in the art can readily determine whether a peptide or polypeptide consists essentially of the YSV peptide or one of its functional derivatives under the foregoing definitions by measuring the activity of the peptide or polypeptide using the assays for modulation of immune activity and/or modulating a cell proliferative disorder, such as cancer, including, but not limited to, cervical carcinoma, liver cancer, and leukemia, which are provided herein with respect to the YSV peptide. Those skilled in the art can also readily determine whether a peptide or polypeptide consists essentially of the YSV peptide or one of its functional derivatives under the foregoing definitions by measuring the activity of the peptide or polypeptide using the assays for modulation of the growth or appearance of a cell-proliferative disorder, including but not limited to cancer, said cancers including but not limited to melanoma, lung cancer, and cancer affecting pulmonary tissues, which are provided herein with respect to the YSV peptide.

In the preferred embodiment, the terminology "consisting essentially of" may also refer to peptides or polypeptides which have less than 20 amino acid residues in addition to the YSV peptide or one of its functional derivatives. In a more preferred embodiment, the same terminology refers to a peptides with less than 15 amino acid residues in addition to the YSV peptide or one of its functional derivatives. In an even more preferred embodiment, the same terminology refers to a peptides with less than 10 amino acid residues in addition the YSV peptide or one of its functional derivatives. In another preferred embodiment, the same terminology refers to peptides or polypeptides with less than 6 amino acids in addition to the YSV peptide or one of its functional derivatives . In another preferred embodiment, the same terminology refers to peptides or polypeptides with less than 4 amino acids in addition to the YSV peptide or one of its functional derivatives. In the most preferred embodiment, the same terminology refers to peptides or polypeptides with less than 2 amino acids in addition to the YSV peptide or one of its functional derivatives.

The pharmaceutical formulation may include any of the known pharmaceutical carriers. Examples of suitable carriers include any of the standard pharmaceutically accepted carrier known to those skilled in the art. These include but are not limited to, physiological saline solution, water, emulsions including oil and water mixtures or triglyceride emulsions, and other types of agents, fillers, coated tablets and capsules. The appropriate carrier may be selected based on the mode of administration of the pharmaceutical composition.

The YSV peptide and its functional derivatives may be administered via intravenous injection, intramuscular injection, intraperitoneal injection, subcutaneous injection, and subcutaneous implantation. The peptide may also be administered in any form of oral administration like tablet, capsule, suspension, solution etc, in the usual form without modification or in slow release form, or with or without gastro-enteric protection. The peptide may further be applied in any form of topic application like ointment, cream, gel, etc., with or without transdermal facilitating device. The peptide may also be interpreted into its genetic sequence and cloned into an expression system, on its own or in combination with other peptide sequences, to generate a resulting peptide molecule to make use of the activity of the peptide as described in this report.

The dose of each peptide may be 1ng – 10g per kg body weight. A preferred dose is 10ng - 10mg per kg, and more preferably 1μg - 1mg per kg for an injection mode of administration. However, the effective dose can be as low as 1ng per kg body weight, since one or more of the peptides may operate through receptors that will induce a cascade of normal physiological response. Alternatively, one or more of the peptides can just be an initiator for a whole cascade of reaction. For an oral intake, the amount may be 1ng - 10g per day per kg body weight, more preferably 0.1μg - 1g per day per kg body weight and even more preferably 1μg - 10mg per day

I. EXPERIMENTS REGARDING THE EFFECTS OF THE YSV PEPTIDE

1.1 Materials for 2.1-2.4

BALB/c Mice, 18-22g weight, provided by Experimental Animal Center, China Medical Science Institute.

YSV was custom manufactured by CS Bio, USA.

Fetal bovine serum (FBS), and RPMI-1640 cell culture medium, Gibco, USA

MTT, and ConA, Sigma, USA

Human hepatocellular carcinoma BEL7402 cells were provided by Cancer Research Department, China Medical Science Institute.

Human leukemia K562 cells were provided by Hematological Disease Research Department, China Medical Science Institute.

Human cervical carcinoma Hela cells were provided by Immunology Department, Tianjin Medical University.

2.1 The effect of YSV on T lymphocyte transformation *in vitro*

2.1.1 Method (as described in Reference 1 and incorporated herein in its entirety)

Healthy mice were sacrificed by cervical dislocation. The spleens were isolated and dispersed aseptically. The spleen lymphocyte suspension was washed and adjusted to a cell density of 5×10^6/ml with RPMI-1640 culture medium containing 10% fetal bovine serum. YSV was diluted with RPMI-1640 culture medium into various concentrations: 2μg/ml, 0.4μg/ml, 0.08μg/ml, 0.016μg/ml. Con A working solution was adjusted to 1mg/ml with RPMI-1640 culture medium.

The reagents were placed onto a 96 wells cell culture plate according to the following: 100μl/well lymphocyte suspension, 20μl/well ConA, and 100μl/well YSV solution of various concentrations, 6 parallel wells for each concentration; 100μl/well lymphocyte suspension and 120μl/well RPMI-1640 culture medium (containing 10% FBS) were added to 12 parallel wells as negative control; 100μl/well lymphocyte suspension, 100μl/well RPMI-1640 culture medium (10% FBS) and 20μl/well ConA were added to 12 parallel wells as positive control.

The cells were incubated for 68 hrs at 37°C, 5% CO_2, and then pelleted by centrifugation at 150g for 10 minutes. After the supernatant was removed, 50μl/well MTT of 1mg/ml in RPMI-1640 was added to the cell pellet and the cells were re-suspended by shaking for 2 minutes. The incubation was continued for 4 hours. The supernatant was removed after centrifugation at 150g for 10 minutes. After blotted dry with filter paper, the cells were mixed with 120μl 40mM HCl-2-propanol and shaken for 3 minutes. OD_{570}nm of each well referenced at 630nm was obtained with an ELISA reader.

2.1.2 Results

Table 1 The effects of YSV on T lymphocytes transformation

Group	Concentration of YSV	N	X±SD (OD)
YSV	2μg/ml	6	0.18±0.00*
YSV	0.4μg/ml	6	0.18±0.01*
YSV	0.08μg/ml	6	0.23±0.00*
YSV	0.016μg/ml	6	0.21±0.01*
Negative control	-	12	0.11±0.01*
Positive control	-	12	0.14±0.00

* comparing to positive control group $P<0.001$

2.1.3 Conclusion

At concentration of 0.016μg/ml to 2μg/ml, YSV was found to be able to stimulate the T lymphocyte transformation activity *in vitro* with statistical significance ($p<0.001$).

2.2 The effect of YSV on the proliferation of cultured human liver cancer BEL7402 cells *in vitro*.

2.2.1 Method (as described in Reference 2 and incorporated herein in its entirety)

Human liver cancer BEL7402 cells at logarithmic growth phase were detached by incubating for 2 to 3 minutes with phosphate buffer saline pH7.4 (PBS) containing 0.05% trypsin and 0.02% EDTA. The cells were examined by inverted phase contrast microscopy. The supernatant was removed after cytoplasm pyknosis and dilatation of cell compartment were observed. A few milliliters of RPMI-1640 culture medium with 10% FBS was added to terminate the digestion. The cells were harvested by gently blowing with a pipette. The suspended cells were collected by centrifugation at 150g for 10 minutes, and washed twice by cold D-Hank's solution with re-suspension and centrifugation. The washed cells were re-suspended in RPMI-1640 medium with 10% FBS and adjusted to density of $5x10^4$/ml. The treated BEL7402 cells were placed onto a 96 wells cell culture plate, 100μl/well. The cells were incubated for 24 hours at 37°C, 5% CO_2 for re-activation and attachment.

The experiment included three testing groups with different concentrations of YSV and a negative control. The final concentrations of YSV in culture medium were 20μg/ml, 10μg/ml, and 5μg/ml. The YSV solution was replaced by RPMI-1640 culture medium with 10% FBS in the negative control group. Each group contained five parallel wells. The cells were incubated for 48 hours at 37°C, 5% CO_2.

The cells were then pelleted by centrifugation at 150g for 10 minutes. After the supernatant was removed, 100μl/well MTT of 0.5mg/ml in RPMI-1640 was added to the cell

pellets and the cells were re-suspended by shaking for 2 minutes. Incubation was continued for 4 hours. The supernatant was removed after centrifugation at 150g for 10 minutes. After blotted dry by filter paper, 100μl/well 40mM HCl-2-propanol was added to the cell pellets and shaken for 3 minutes. OD_{570}nm of each well referenced at 630nm was obtained by using ELISA reader.

2.2.2 Results

Table 2 The inhibition of growth of human liver cancer BEL7402 cells *in vitro* by YSV

Concentration of YSV	N	OD value	% inhibition
20μg/ml	5	0.27±0.01*	23.7
10μg/ml	5	0.22±0.01*	38.0
5μg/ml	5	0.31±0.01*	9.8
Negative control	5	0.36±0.01	

* comparing with negative control, $p<0.05$

2.2.3 Conclusion

At concentration of 5μg/ml to 20μg/ml, YSV was found to be able to inhibit the growth of human hepatocellular carcinoma BEL7402 cells in vitro, with statistical significance ($p<0.05$).

2.3 The effect of YSV on the proliferation of cultured human leukemia K562 cells *in vitro*

2.3.1 Method (as described in Reference 2 and incorporated herein in its entirety)

A human leukemia K562 cell at logarithmic growth phase was adjusted to density of $5x10^4$/ml with RPMI-1640 culture medium containing 10% FBS. 100μl/well of the cell were placed onto a 96 wells cell culture plate. The cells were incubated for 24 hours at 37°C, 5% CO_2. The experiment included five testing groups with different concentrations of YSV in culture medium and a negative control group with culture medium only. The final concentrations of YSV were 40μg/ml, 20μg/ml, 10μg/ml, and 5μg/ml. Each group contained five parallel wells. 100μl/well testing solution was added to the treated cells. The cells were incubated for 48 hours at 37°C, 5% CO_2.

The cells were then pelleted by centrifugation at 150g for 10 minutes. The supernatant was removed and 100μl/well MTT solution of 0.5mg/ml in RPMI-1640 was added to the cell pellets and the cells were re-suspended by shaking for 2 minutes. Incubation was continued for 4 hours. The supernatant was removed after a centrifugation at

150g for 10 minutes. After blotted dry by filter paper, 100μl/well 40mM HCl-2-propanol was added to the cell pellets and shaken for 3 minutes. OD_{570nm} of each well referenced at 630nm was obtained by using ELISA reader.

2.3.2 Results

Table 3 The inhibitory effects of YSV on human leukemia K562 cells *in vitro*

Concentration	N	OD value	% inhibition
40μg/ml	5	0.38 ± 0.00*	10.8
20μg/ml	5	0.38±0.00*	11.3
10μg/ml	5	0.39±0.01*	9.3
5μg/ml	5	0.39±0.01*	9.7
Negative control	5	0.43±0.01	

* comparing with negative control, $p<0.05$.

2.3.3 Conclusion

At concentrations of 5μg/ml to 40μg/ml, YSV was found to be able to inhibit the growth of human leukemia K562 cells *in vitro*, with statistical significance ($p<0.05$).

2.4 The inhibition of growth of human cervical carcinoma Hela cells *in vitro*

2.4.1 Method (as described in Reference 2 and incorporated herein in its entirety)

Human cervical carcinoma Hela cells at logarithmic growth phase were detached by incubating for 2 to 3 minutes with PBS containing 0.05% trypsin and 0.02% EDTA. The cells were examined by inverted phase contrast microscopy. The supernatant was removed after cytoplasm pyknosis and dilatation of cell compartment were observed. A few milliliters of RPMI-1640 medium with 10% FBS were added to terminate the digestion. The cells were harvested by gently blowing with a pipette. The cells were collected by centrifugation at 150g for 10 minutes, and washed twice by cold Hank's solution with re-suspension and centrifugation. The cell pellets were re-suspended in RPMI-1640 medium with 10% FBS and adjusted to density of $5x10^4$/ml. The treated cells were placed onto a 96 wells cell culture plate, 100μl/well. The cells were incubated for 24 hours at 37°C, 5% CO_2 for re-activation and attachment.

The experiment included two testing groups with different concentrations of YSV in culture medium, and a negative control with culture medium only. The final concentrations of YSV were 10μg/ml and 5μg/ml. Each group contained five parallel wells. 100μl/well testing solution was added to the treated cells. The cells were incubated for 48 hours at 37°C, 5% CO_2.

The cells were pelleted by centrifugation at 150g for 10 minutes. The supernatant was discarded and 100μl/well MTT of 0.5mg/ml in RPMI-1640 was added to the cell pellets and the cells were re-suspended by shaking for 2 minutes. Incubation was continued for 4 hours. The supernatant was discarded after centrifugation at 150g for 10 minutes. After blotted dry by filter paper, 100μl/well 40mM HCl-2-propanol was added to the cell pellets and shaken for 3 minutes. OD_{570nm} of each well referenced at 630nm was obtained by using ELISA reader.

2.4.2 Result

Table 4 The inhibitory effect of YSV on the growth of human cervical carcinoma Hela cells *in vitro*

Concentration of YSV	N	OD_{570nm}	% inhibition
10μg/ml	5	0.22 ± 0.01*	19.6
5μg/ml	5	0.22 ± 0.00*	18.4
Negative control	5	0.24 ± 0.01	-

* comparing with negative control, $p<0.05$

2.4.3 Conclusion

At concentration of 5μg/ml to 10μg/ml, YSV was found to be able to inhibit the growth of human cervical carcinoma Hela cells in vitro, with statistical significance ($p<0.05$).

2.5 Effects of YSV on the growth of nude mice-transplanted human leukemia K562

2.5.1 Materials

YSL: custom synthesized by Shenzhen Kangzhe Pharmaceutical Co., Ltd

Saline: China OTSUKA Pharmaceutical Co., Ltd

RPMI-1640 cell culture medium: GIBCO, USA.

Fetal bovine serum (FBS): HYCLONE, USA

Human leukemia K562 cell line: Hematological Disease Research Department, China Medical Science Institute.

Healthy nude mice (BALB/C (nu/nu), SPF, male, 4-5 weeks old, weight 18-22g): Shanghai Tumor Research Department, China Medical Academy of Science.

2.5.2 Methods

2.5.2.1 Cell culture

K-562 cells were maintained in RPMI-1640 medium with 10% FBS at 37°C, 5% CO_2.

2.5.2.2 Preparation of leukemia mice model [3]

K562 cells at log phase were adjusted to 1.6 x 10^8/ml with RPMI-1640. 0.1ml of this was subcutaneously injected to the right flank of the healthy nude mice to form the leukemia mice model.

2.5.2.3 Grouping of animals and administration

The mice bearing human leukemia K562 cells were randomized into groups of: saline control (0.2ml/day), and YSV (160μg/kg/day). The test substance was dissolved in 0.2ml saline and administration of the test substance by intraperitoneal injection was started on the next day after the K562 inoculation, once per day for 30 consecutive days.

2.5.2.4 Monitoring parameters

The general conditions of the mice was examined daily and the tumor size was measured in every 3 – 4 days. Tumor volume (mm^3) V = (1/6)πXYZ where X, Y, and Z were the diameters of the tumor on the three planes.

On the next day after the last test substance administration, the tumors were excised, the weight of tumor was recorded and the tumor volume was measured. Tumor growth inhibition index (%) = (mean tumor weight of control group – mean tumor weight of treatment group) / mean tumor weight of control group x 100)

2.5.2.5 Statistical method

All the data were shown as arithmetic mean ± SD. The data were analyzed using the ANOVA test of the SPSS software. P <0.05 was accepted as having statistical significance.

2.5.3 Results

Table 1 Effect of YSV on the growth of nude mice-transplanted human leukemia K562

Groups	Dosage	N	Tumor weight (g)	Tumor volume (cm3)	Inhibition index (%)
YSV	160μg/kg/day	10	2.95±1.58*	3.15±1.74*	37.9
Saline	0.2ml/day	14	4.75±2.21	5.08±2.15	-

*: Comparing to saline group P<0.05

2.5.4. Conclusion

YSV at suitable dosage was found to be able to inhibit the growth of nude mice-transplanted significantly human leukemia K562, with statistical significance compared with the saline control group (p<0.05).

2.6 Inhibition of YSV on the growth of C57BL/6 mice-transplanted melanoma B16

2.6.1 Materials

2.6.1.1 Peptide

YSV: custom synthesized by Shenzhen Kangzhe Pharmaceutical Co., Ltd, PR China.

2.6.1.2 Control substances and other reagents

Cyclophosphamide (Cy): Shanghai Hualian Pharmaceutical Co., Ltd.

Saline: China Otsuka Pharmaceutical Co., Ltd.

RPMI-1640 cell culture medium: GIBCO, USA.

Fetal bovine serum (FBS): HYCLONE, USA.

Hank's solution: Dingtian Co., Ltd.

2.6.1.3 Cell line

Mouse B16 melanoma cell line: from the Institute of Biochemistry and cell Biology China Medical Academy of Science.

2.6.1.4 Animals

Healthy C57 BL/6 mice (SPF, male, 4-5-week-old, 14-18g): from Academy of Military Medical Sciences.

2.6.2 Method

2.6.2.1 Cell culture

B16 cells were maintained in RPMI-1640 medium with 10% FBS at 37°C, 5% CO_2.

2.6.2.2 Preparation of melanoma mice model [4]

Mouse B16 melanoma culture at log phase was adjusted to 2.5 x 10^6/ml with Hank's solution. 0.2ml of this was subcutaneously injected to the right axilla of healthy C57 BL/6 mice to form the melanoma mice model.

2.6.2.3 Grouping of animals and test substance administration

The mice bearing melanoma B16 were randomized into groups of: saline (0.2ml/day), Cyclophosphamide (Cy) (20mg/kg/day), and YSV (640μg/kg/day and 320μg/kg/day). The test substances were dissolved in 0.2ml saline and applied intraperitoneally once per day for 20 consecutive days to the melanoma mice models started from the next day after the tumor transplantation.

2.6.2.4 Monitoring parameters

From the next day after the tumor implantation, the general conditions of the mice and the growth of the tumor were observed daily.

On the next day after the last test substance administration, the tumors were extirpated and the weights of tumor were measured.

Tumor inhibition rate (%) = (1 - the average weight of tumor in test group/the average weight of tumor in control group) × 100%)

2.6.2.5 Statistical analysis

The results were presented as arithmetic mean ± SD. Statistical analysis was performed using the ANOVA test of the SPSS software. P values <0.05 were taken as statistically significant.

2.6.3 Results

Table 1 The Inhibition of YSV on the growth of melanoma B16 in C57BL/6 mice

Groups	Dosage	N	Tumor weight (g)	The Inhibition rate (%)
YSV	640μg/kg/day	9	1.47±0.92*	44.6
YSV	320μg/kg/day	8	1.60±1.21*	39.6
Cyclophosphamide	20mg/kg/day	9	0.49±0.68*	81.5
Saline	0.2ml/day	10	2.64±0.68	□

*Comparing to saline group p<0.05

2.6.4. Conclusion

At suitable dosage, YSV was found to be able to inhibit the growth of B16 melanoma in C57BL/6 mice, with statistical significance compared with the saline control (p<0.05).

2.7 The Inhibitory Effects of YSV on A549 Human Pulmonary carcinoma Xenograft in Nude Mice

2.7.1 Materials

2.7.1.1 Peptides

YSV: contract synthesized by CS Bio Co., USA

2.7.1.2 Control substances and other reagents

Cyclophosphamide (Cy): Shanghai Hualian Pharmaceutical Co., Ltd.

Saline: China OTSUKA Pharmaceutical Co., Ltd.

Fetal Bovine Serum (FBS): HYCLONE, USA

RPMI-1640 cell culture medium: GIBCO, USA

2.7.1.3 Animals

Healthy BALB/c (nu/nu) athymic nude mice (SPF, 4-5weeks old, weighting 18-22g) were purchased from Shanghai Tumor Academe of China Medical Academy of Science. In the first experiment, female was used. In the second experiment, male was used.

Nude mice bearing xenografts of A549 human pulmonary carcinoma were from Shanghai Tumor Academe of China Medical Academy of Science.

2.7.2 Methods

2.7.2.1 Preparation of pulmonary carcinoma nude mice model [5]

Select A549 human pulmonary carcinoma xenografts of diameter longer than 1cm with good growth on nude mice. Aseptically excise the tumor mass, cut into pieces of 2 – 4 mm^3 and submerged in RPMI1640. Pulmonary carcinoma nude mice model was prepared by transplanting the tumor mass hypodermally to the back of healthy nude mice near to the neck via an incision at the ventral thorax.

2.7.2.2 Grouping and drug administration methods

The nude mice bearing A549 xenografts were randomly divided into groups of: saline control (0.2ml/day), YSV groups of different dosages, and Cy control (0.2mg/kg/day). The test substances were dissolved in 0.2ml saline and administered intraperitoneally to the mice starting from the day after the transplantation, once per day for 40 consecutive days.

2.7.2.3 Monitoring parameters

The general condition of the mice was observed daily. The mice were weighed every 3 – 4 days and the volume of the tumor was measured:

$V = (1/6)\pi XYZ$, where X, Y, and Z were the diameters of the tumor on the 3 planes.

The day after the last injection, the tumors were excised, weighted, and the volume measured. The tumors were examined for signs of necrosis.

Tumor inhibition index = (average tumor weight of saline group - average tumor weight of test group) / average tumor weight of saline group × 100%)

Parts of the tumor with good growth condition and firm texture and without ulceration or necrosis were selected, trimmed and fixed with 10% formaldehyde for pathological examination.

2.7.2.4 Statistical analysis

Statistical analysis was performed with SPSS software using one-way ANOVA analysis.

2.7.3 Results

Table 1 Effects of YSV on the growth of female nude mice-transplanted A549 human pulmonary carcinoma – experiment 1

Groups	Dosage	N	Tumor weight (g)	Tumor volume (cm^3)	Tumor inhibition index (%)
YSV	640μg/kg/day	11	1.56±1.08*	1.17±0.84	37.4
YSV	320μg/kg/day	11	1.69±0.70*	0.84±0.45*	32.2
YSV	160μg/kg/day	12	1.67±0.97*	0.92±0.79*	32.8
Cy	20mg/kg/day	12	1.50±0.75*	1.02±0.47*	39.8
Saline	0.2ml/day	14	2.49±1.02	1.87±1.10	-

* $p<0.05$ vs saline control

Table 2 Effects of YSV on the growth of male nude mice-transplanted human pulmonary carcinoma – experiment 2

Groups	Dosage	N	Tumor weight (g)	Tumor volume (cm^3)	Tumor inhibition index (%)
YSV	160μg/kg/day	8	0.74±0.31*	0.78±0.41	50.8
Cy	20mg/kg/day	10	0.73±0.42*	0.49±0.34*	51.9
Saline	0.2ml/day	10	1.51±0.79	1.34±0.69	-

* $p<0.05$ vs saline control

2.7.4 Conclusion

At suitable dosages, YSV was found to be able to inhibit the growth of nude mice-transplanted A549 human pulmonary carcinoma xenograft, with statistical significance when compared with the saline control ($p < 0.05$).

3. General conclusion

YSV was found to be able to promote the transformation of T lymphocytes, showing that YSV may be useful as an immune modulator for human use.

YSV was found to be able to inhibit the growth of human hepatocellular carcinoma BEL7402 cells, human leukemia K562 cells, and human cervical carcinoma Hela cell *in vitro*, as well as human leukemia K562 cells, murine B16 melanoma cells and A549 human pulmonary carcinoma cells *in vivo*. This shows that YSV may be useful in the treatment of human cell proliferative diseases.

4. References

1. Shuyun Xu, Rulian Bian, Xiu Chen. Methodology of pharmacological experiment (3rd edition). People's Health Publishing House. 2002, p1427

2. Shuyun Xu, Rulian Bian, Xiu Chen. Methodology of pharmacological experiment (3^{rd} edition). People's Health Publishing House. 2002, p1785-1786

3. Potter G K, Shen R N, Chiao J W et al. Nude mice as models for human leukemia studies. Am J Pathol, 1984, 114:360

4. Zhunjiang Xie,Wenqing Liu,Yechun He et al. The inhibition of ginseng glycan and IL-2 on mice melanoma cells in vivo. ACTA ANATOMICA SINICA, 2002, 33(5):538-540.

5. Hanyue, The research and experiment techniques of anticarcinoma drugs.(1st edition).Beijing medical university and China Xiehe medical university united Publishing House.(1997) 299.

II. GENE THERAPY AND METHOD OF TREATMENT

Gene therapy based on the above peptide sequences is performed by designing a nucleic acid sequence that code for one of these peptides. The nucleic acid may be synthesized chemically and operably ligated to a promoter, and cloned into an expression vector. The expression vector is then administered into the human body as the form of gene therapy for expression in the human cell. The term "genetic vectors" as used herein includes these expression vectors. Vectors that can be used for gene therapy includes adeno-associated virus (Mizuno, M. et al. (1998). Jpn J Cancer Res 89, 76-80), LNSX vectors (Miller, A.D. et al. (1993) Methods Enzymol 217, 581-599) and lentivirus (Goldman, M.J. et al. (1997) Hum Gene Ther 8, 2261-2268).

Other vehicles for peptide delivery include expression vectors encoding the desired peptide that can be transferred into an organism which can replicate in the host organism to which it is desired to administer the peptide without significant detrimental effects on the health of the host organism. For example, the expression vectors may be transferred into an organism which is not pathogenic to the host organism to which it is desired to administer the peptide. In some embodiments the expression vector produces the desired peptide in a bacterial or fungal organism which does not have significant detrimental effects on the health of the host organism to which the peptide is to be administered. For example, the expression vector encoding the desired peptide may be an expression vector which produces the desired peptide in an organism such as lactic acid bacteria, E. Coli, or yeast. In one embodiment, the expression vector produces the desired peptide in a microbe normally found in the mammalian gut or a microbe tolerated by the mammalian digestive tract. Some of the microbial species in which the desired peptide can be expressed include, but are not limited to, *Lactobacillus* species, such as *L. acidophilus, L. amylovorus, L. casei, L. crispatus, L. gallinarum, L. gasseri, L. johnsonii, L. paracasei, L. plantarum, L. reuteri, L. rhamnosus* or others; *Bifidobacterium* species, such as *B. adolescentis, B. animalus, B. bifidum, B. breve, B. infantis, B. lactis, B. longum* or others; *Enterococcus faecalis* or *Ent. facium*;

Sporolactobacillus inulinus; *Bacillus subtilis* or *Bacillus cereus*; *Escherichia coli*; *Propionibacterium freudenreichii*; or *Saccharomyces cerevisiae* or *Saccharomyces boulardii.*

Nucleic acid sequences that encode any of the peptides of the present invention, chemically synthesized or produced by other means, including but not limited to the reverse transcription of mRNA to produce cDNA molecules, are incorporated into expression vectors for gene transfer into the desired organisms by methods of genetic engineering familiar to those of skill in the art. The expression vectors may be DNA vectors or RNA vectors. For example, the expression vectors may be based on plasmid or viral genetic elements. The expression vectors may be vectors which replicate extra-chromosomally or vectors which integrate into the chromosome.

The expression vectors comprise a promoter operably linked to a nucleic acid encoding a peptide of the present invention. The promoter may be a regulatable promoter, such as an inducible promoter, or a constitutive promoter. In some embodiments, the promoter may be selected to provide a desired level of peptide expression. In addition, if desired, the expression vectors may comprise other sequences to promote the production, presentation and/or secretion of peptides. In some embodiments a nucleic acid encoding a peptide of the present invention is operably linked to a nucleic acid sequence which directs the secretion of the peptide. For example, the nucleic acid encoding the peptide of the present invention may be operably linked to a nucleic acid encoding a signal peptide.

In some embodiments, the expression vectors which are engineered to encode the peptides of the present invention may be expression vectors which are adapted for expressing the peptide of the present invention in a bacterial species that makes up the normal gut flora of mammals, such as *Lactobacillus* species and *Bacillus subtilis* Examples of such expression vectors can be found in US Patents No. 6,100,388, to Casas, and No. 5,728,571, to Bellini, respectively. These documents are hereby expressly incorporated by reference in their entireties. It will be appreciated that any expression vector which facilitates the expression of a peptide of the present invention in an organism which is not detrimental to the health of the host organism to which the peptide is to be administered may be used.

In some embodiments, the expression vectors which are engineered to encode the peptides of the present invention may be expression vectors which are adapted for expressing the peptide of the present invention in a yeast species that is well tolerated by the mammalian

gut , such as *Saccharomyces cerevisiae;* or, preferably, *Saccharomyces boulardii*, which can colonize the human gut and is used to treat certain forms of diarrhea. Yeast expression vectors can be used that constitutively express heterologous proteins and peptides, are highly stable, thus are well transmitted to progeny cells during mitosis and meiosis and may comprise coding sequence for a signal peptide or peptides that direct high levels of recombinant protein secretion. An example of such a yeast vector is given in US Patent No. 6,391,585, to Jang *et al.*, which is hereby expressly incorporated by reference in its entirety.

The expression vectors encoding the peptides of the present invention may be introduced into the organism in which it is intended to express the peptides through techniques known in the art. These techniques include traditional methods of transforming bacteria, yeast, or other microbes, through the use of chemically competent bacterial cells, electroporation or lithium acetate transformation (for yeast), for example, as well as recent advances in the transformation of bacterial species recalcitrant to these procedures. In some embodiments, the expression vectors are introduced into lactic acid bacteria known to be recalcitrant to transformation using the method disclosed by Leer *et al.* (WO 95/35389), the disclosure of which is incorporated herein by reference in its entirety. The introduced sequences may be incorporated into microbial chromosomal DNA or may remain as extrachromosomal DNA elements.

This genetically engineered microbe containing the expression vector can then be inoculated into the alimentary canal, vagina, trachea etc. to achieve sustained immuno-therapy. In some embodiments, the organisms expressing the peptides of the present invention are ingested in an inactive form or, preferably, in live form. In the gut these microorganisms produce said peptides, release them into the lumen by secretion or by lysis of the microorganism or otherwise present the peptides to the host, whereby the peptides produce their intended effect upon the host organism. In other embodiments, peptides are presented to the host at the mucous membrane of the nasal passages, vagina or the small intestine.

Another method of the treatment is the use of liposomes as a means for delivering the specific nucleic acid to the cells in the human body. The nucleic acid (such as an expression vector containing a nucleic sequence that encodes peptides of sequence ID No.1 to ID No.30) is delivered in an environment that encourages cellular uptake and chromosomal

incorporation as described in Gao, X. and Huang, L. (1995) Gene Ther 2, 710-722 and US 6,207,456. Alternatively, the peptide itself can be encapsulated in the liposome and delivered directly, using a method described in US6,245,427. All the scientific publications and patents indicated above are incorporated herein by reference in their entireties.

The nucleic acid sequences useful for the above-mentioned gene therapy and method of treatment include sequences that code for these peptides and functional derivatives thereof. Any one of the numerous nucleic acid sequences may be used to code for these peptides and their derivatives based on the degenerate codon system.

The following references are incorporated herein by reference in their entireties.

1. Principles of Pre-clinical Research of New Drugs, People's Republic of China. 1993, 7:134-135Shuyun Xu, Rulian Bian, Xiu Chen. Methodology of pharmacological experiment. People's Health Publishing House. 1991, 1221-1234
2. Principle of new drug research in pre-clinic issued by Ministry of Health, People's Republic of China. 1993, 7:140
3. Jinsheng He, Ruizhu Li, Tingyi Zong. The study on MTT reduction method of testing NK cell activity. China Immunology Journal. 1996, 1(6): 356-358
4. Qian Wang. Modern medical experiment method. People's Health Publishing House. 1998, 482-483
5. Principle of new drug research in pre-clinic issued by Ministry of Health, People's Republic of China. 1993, 7: 141
6. Principle of new drug research in pre-clinic issued by Ministry of Health, People's Republic of China. 1993, 7: 132-133
7. Principle of new drug research in pre-clinic issued by Ministry of Health, People's Republic of China. 1993, 7: 128-129
8. Yuanpei Zhang, Huaide Su. Phamalogical experiment (second edition). People's Health Publishing House. 1998, 137-138
9. Jiatai Li, clinical pharmacology(second edition). People's Health Publishing House. 1998, 1338-1339.

III. PEPTIDE CONJUGATIONS TO AND FORMULATIONS WITH YSV AND DERIVATIVES THEREOF

The biologically active peptides of the present invention may be conjugated to other biologically effective or useful molecules to provide an additional effect or use or to enhance their therapeutic effectiveness. Many potential conjugating molecules, their biological

effects and the methods for conjugation of the molecules to peptides are known in the art. For other candidate conjugation partners, chemical reactions for conjugating the instant peptides thereto can be deduced by one skilled in the art without undue experimentation. Effective molecules are described below. Specific examples of how various peptides according to the present invention may be conjugated to their effective molecules and the biological properties of the resulting conjugation product are described. It is understood that other peptides of the instant invention may also be conjugated in similar reactions.

YSV and its derivatives have distinct therapeutic effects on a particular cell or tissue type. One important objective of conjugating molecules to peptide drugs is the targeting of the peptide to a particular location or compartment within the body of an individual being treated. In this way, the peptide drug and its effects can be concentrated at the location of the cell or tissue type on which it has the intended therapeutic effect. This can augment the effect that a similar molar amount of the free, unconjugated peptide would have. Conversely, the dosage of a conjugated peptide drug that is targeted to its therapeutic active site can be significantly lower than the dosage required to get the same therapeutic effect from the free, unconjugated form of the drug.

Another beneficial effect of targeting a peptide drug to the site where its activity is most desired is the reduction of unwanted side effects. A peptide drug that is administered in order to effect a change in a particular cell or tissue type can also act in other locations within an individual, sometimes with detrimental results. By targeting the peptide to the desired location of activity via conjugation to a targeting molecule, the concentration of peptide elsewhere in the individual and the subsequent side effects can be reduced.

Peptides comprising, consisting essentially of, or consisting of one of YSV or functional derivatives thereof can be conjugated to a variety of molecules for targeting to different locations throughout the body of an individual. Any of the conjugation technologies described below for targeting a peptide to a desired location, as well as other conjugation technologies familiar to those skilled in the art, may be employed with any of the peptides of the present invention. For example, the selective delivery of an anti-hepatitis B drug to liver cells has been demonstrated (Fiume *et al.*, Ital J Gastroenterol Hepatol, 29(3):275, 1997, which is incorporated herein by reference in its entirety). In this study, researchers conjugated adenine arabinoside monophosphate (ara-AMP), a phosphorylated

nucleoside analogue active against hepatitis B virus, to lactosaminated human albumin, a galactosyl-terminating macromolecule. Hepatocytes express a receptor protein that interacts with terminal galactosyl residues with high affinity. Through binding to this receptor, the conjugated drug will be selectively taken up by hepatocytes. After absorption, the conjugated drug is delivered to lysosomes, where the bond between the two components of the conjugated drug is cleaved, releasing ara-AMP in its active form. In the study cited above, the conjugated drug was as effective as free ara-AMP in treating patients with chronic hepatitis B infections, but did not cause the clinical side effects, such as neurotoxicity, that the administration of free ara-AMP causes. Such an approach can be used with any of the peptides of the present invention.

In a related study to the one above, by the same research team (Di Stefano *et al.*, Biochem. Pharmacol., 61(4):459, 2001), an anti-cancer chemotherapeutic agent, 5-fluoro 2-deoxyuridine (FUdR), was conjugated to lactosaminated poly-L-lysine in order to target the compound to the liver and treat liver micrometastases. The drug is selectively taken up by liver cells, which cleave the bond between FUdR and the targeting molecule. A portion of the free FUdR will then exit the liver cells and a localized therapeutic concentration of the anti-cancer agent is created. This concentration is sufficient for pharmacological activity on the metastatic cells that have infiltrated the liver. Because the drug is selectively concentrated in the liver, the dosage of the conjugated drug can be significantly less than the smallest pharmacologically active dosage of the free, unconjugated compound. This strategy can be utilized with any of the peptides of the present invention. For instance, conjugation of lactosaminated poly-L-lysine to ysv could significantly reduce the dosage necessary to treat a hepatitis B infection and liver cancer in an individual.

The targeting of compounds to particular tissues or cell types within the body has been achieved for a number of different tissues or cell types. For example, tumor cells often express abnormally high levels of peptide hormone receptors on their surfaces, such as bombesin, lutenizing hormone-releasing hormone, and somatostatin. In one study, the anti-cancer compound paclitaxel (taxol) has been selectively targeted to hormone-secreting tumor cells that express somatostatin receptors at a high density by conjugating the drug with octreotide, an analog of somatostatin. The ostreotide-conjugated taxol was just as effective as free taxol but with reduced toxicity to normal cells (Huang *et al.*, Chem. Biol., 7(7):453,

2000). Several peptides of the present invention, such as CMS024 and CMS034, have shown potent anti-tumor activity in animal studies. Using the techniques of Huang *et al.* to conjugate these peptides to octreotide would create a potential anti-cancer treatment specifically targeting tumor cells expressing high levels of somatostatin. This approach can be adapted to target tumor cells overexpressing any number of peptide hormone receptors. In another example of targeting a drug to a specific tissue type, poly (L-aspartic acid) was used as a carrier molecule to target drug delivery to colon cells specifically (Leopold *et al.*, J. Pharmacokinet. Biopharm., 23(4):397, 1995).

Beyond the specific targeting of a peptide drug to a particular cell or tissue type, conjugation of peptides comprising, consisting essentially of, or consisting of YSV or a functional derivative thereof to carrier molecules can provide other ways to enhance the delivery of peptide drugs, thereby augmenting or otherwise improving their therapeutic effects. Any of the conjugation technologies described below may be used with any of the peptides of the present invention, as with other technologies familiar to those skilled in the art. The effectiveness of any drug will be hampered if the compound cannot be delivered to its target efficiently. A drug must be transported, actively or otherwise, to the site of its activity without substantial loss of activity due to metabolic processing or degradation. Peptide drugs are subject to the activity of peptidases and, as highly charged molecules, can be refractory to transport across lipid cell membranes and endothelial cell membranes, such as the blood-brain barrier. Conjugation to other molecules provides a way to protect peptides from degradation and to enhance the absorption of peptide drugs into cells or anatomical compartments that would normally exclude the compounds.

By allowing peptides access to locations within the body from which they would normally be excluded, conjugation techniques can open up new routes for administration of the drug. In Patel *et al.*, Bioconjugate Chem., 8(3):434, 1997, the chemistry of which is detailed in Example 5 below and which is incorporated herein by reference in its entirety, researchers conjugated a peptide drug known to be a potent analgesic, the heptapeptide deltorphin, to an organic molecule that was specifically designed to allow the peptide to cross the blood-brain barrier. This allows the drug to be administered intravenously instead of by intracerebro ventricular injection.

The carrier molecule in Patel *et al.* was designed to specifically target those endothelial cells that comprise the blood-brain barrier in addition to allowing the peptide to get across the barrier. Endothelial cell membranes throughout the body, including the blood brain barrier, are heterogeneous with regards to the sequence specificity and concentration of membrane-bound endopeptidases that are displayed on their surfaces. The design of the molecule exploits this characteristic to enable targeting of the carrier molecule and its cargo. The molecule contains three fatty acid chains whose free ends are capped with the dipeptide Arg-Pro, which will interact preferentially with the endopeptidases of the blood brain barrier. The transport of the charged peptide drug molecule is then enabled by the lipophilic fatty acids chains. Thus the dipeptide-capped triglyceride molecule permits both the targeting and the transport across the blood brain barrier.

Conjugation methods can also enhance the kinetics of a peptide drug's activity. Any of the conjugation technologies described below for enhancing the kinetics of a peptide's activity as well as other conjugation technologies familiar to those skilled in the art may be employed with a peptide comprising, consisting essentially of or consisting of the YSV peptide or a functional derivative. Patel *et al.* found that the conjugated form of the analgesic peptide was not only able to enter the brain from the bloodstream, but had sustained action in comparison to the free peptide as well. The intravenously administered drug took longer to have a therapeutic effect, but the effect lasted longer and decreased more slowly than the effect of the free peptide injected intracranially. The researchers found that the conjugated peptide molecule is remarkably stable in serum, yet had no effect when injected intracerebro ventricularly, indicating that the carrier molecule is likely degraded and removed during its transport from the bloodstream to the brain. They suspect that the time required to transport the conjugate and degrade the carrier molecule is the cause of the altered kinetics. Regardless of the mechanics of the delay, in a clinical setting, the intravenous stability of the conjugated peptide molecule and the prolonged onset and activity of the drug's effects would mean that it could be administered less frequently. A less frequent and thus more convenient dosing schedule enhances the practical value of the drug as a treatment option.

As would be apparent to a person of skill in the art, the techniques and procedures of Patel *et al.* are readily adaptable to the delivery of any peptides that fall within a limited size range, including any of the peptides of the present invention. For example, a peptide of the

present invention that exhibits an anti-cancer effect, such as YSV, could be conjugated to the same molecule used by Patel *et al.*. In the treatment of an individual with brain cancer, the conjugated molecule would allow YSV access to the brain from the bloodstream and allow YSV to exert its effects on the tumor tissue in the brain. Modifications to alter the targeting of the carrier molecule would also be apparent to such a person. The targeting feature of the carrier molecule is a function of the identity of the two amino acids that comprise the dipeptide mask at the end of the fatty acid chains. The Arg-Pro dipeptide interacts preferentially with the set of membrane-bound endopeptidases found on the surface of the blood brain barrier's endothelial membrane. Other endothelial cells and membranes could potentially be targeted by other dipeptide combinations.

Conjugation has also been used by researchers to create peptide drugs that can be effectively absorbed through the digestive tract or transdermally. Any of the conjugation technologies for enhancing absorption described below, as well as other conjugation technologies familiar to those skilled in the art, may be used to enhance the absorption of a peptide comprising, consisting essentially of or consisting of one of YSV or a functional derivative thereof. Kramer *et al.* describe a procedure for the coupling of peptide drugs to bile acids. The absorption rate for the conjugated molecule following oral delivery of the compound is significantly enhanced as compared to the peptide alone (J. Biol. Chem., 269(14): 10621, 1994). Toth *et al.* (J. Med.Chem., 42(19):4010, 1999) describe the conjugation of a peptide drug with anti-tumor properties to lipoamino acids (LAA) or liposaccharides (LS), in order to increase the absorption rate and enhance the delivery of the anti-cancer peptide to its active site. In their study, a derivative of somatostatin that shows strong anti-proliferative properties, but has impaired pharmokinetics, is conjugated to either LAA or LS. The resulting conjugate drug has improved absorption profiles across skin and gut epithelium and increased resistance to degradation while still active against tumor cells. These techniques would be very useful in conjunction with any of the peptides of the present invention. By increasing the rate of absorption of the molecule across the intestinal epithelium, more of the peptide can be delivered to the bloodstream and exert its effect on the individual being treated.

Conjugation may also be used to provide sustained release of a peptide drug. Any of the conjugation technologies for providing sustained release, as well as other conjugation

technologies familiar to those skilled in the art, may be used to provide sustained release of a peptide comprising, consisting essentially of or consisting of YSV or a functional derivative thereof. As seen above in the work of Patel *et al.*, the sustained delivery of a peptide drug can be achieved with conjugation methods. Another example is the work of Kim *et al.* (Biomaterials, 23:2311, 2002), where recombinant human epidermal growth factor (rhEGF) was conjugated to polyethylene glycol (PEG) before microencapsulation in biodegradable poly(lactic-co-glycolic acid) (PLGA) microspheres. Microencapsulation in PLGA has been used by several groups to deliver various growth factors and morphogenic proteins (Meinel *et al.*, J. Controlled Rel., 70:193, 2001). Through conjugation to PEG, rhEGF became resistant to forming water-insoluble aggregates and to adsorption to the water-organic phase interface during micelle formation with PLGA as compared to unconjugated, free rhEGF. The pharmokinetics of the formulation with the conjugated hormone were improved, showing longer lasting, steadier and overall greater drug activity than with the free hormone, which the researchers speculate is due to the enhanced physical stability of the hormone conjugated to PEG. A similar strategy could be employed to create sustained release formulations of any of the peptides of the present invention. For example, YSV has potent stimulatory effects on cells of the immune system. By conjugating PEG to this peptide and incorporating the conjugated drug into PLGA microspheres, the stimulatory effects of YSV on an individual can be longer lasting and more stable, as the dosing of the drug, as it is being released from its PEG conjugate, is more even and ensures a more constant delivery of the peptide drug to the immune system.

Prolonged release of a peptide drug can significantly enhance its activity. Any of the conjugation technologies for providing prolonged release of a peptide described below, as well as other conjugation technologies familiar to those skilled in the art, may be used to provide prolonged release of a peptide comprising, consisting essentially of or consisting of YSV or a functional derivative thereof. Oldham *et al.* (Int. J. Oncology, 16:125, 2000) compares the anticancer agent paclitaxel against a new form of the drug, paxlitaxel conjugated to poly(L-glutamic acid) (PG-TXL). PG-TXL appeared to have superior anti-tumor activity compared to free paclitaxel, suggesting that the drug has superior pharmokinetic properties or maybe even a superior method of action. However, investigators found that PG-TXL exerted its effects by the same mechanism of action as the free drug,

inducing cell cycle arrest by disturbing the polymerization of microtubules subunits. Evidence suggests that the superior anti-tumor activity of the conjugated drug arises from a continuous and steady release of the free drug from the conjugate, maintaining its therapeutic concentration for a longer period as compared to administration of the free peptide. The addition of poly(L-glutamic acid) tail to a peptide of the invention with anti-cancer properties, such as CMS008, could enhance its tumor-killing ability as well.

The enzymatic degradation of peptides may, in some cases, reduce the effectiveness of the peptides as drugs. Any of the conjugation technologies for reducing enzymatic degradation of a peptide described below, as well as other conjugation technologies familiar to those skilled in the art, may be used to reduce the enzymatic degradation of a peptide comprising, consisting essentially of or consisting of YSV or a functional derivative thereof. Researchers have developed numerous approaches to protect peptides from luminally secreted proteases in the gut as well as membrane-bound peptidases. The latter are found on the surface of all mucosal tissues, the crossing of which is often the route of entry for peptide drugs. Bernkop-Schurch *et al.* (J. Drug Target., 7:55, 1999) report the creation of peptide drug formulations containing inhibitors of pepsin. An analogue of pepstatin was covalently attached to mucoadhesive polymers; this novel pepsin inhibitor was included in tablets containing insulin. After incubation under laboratory conditions simulating digestion, all of the insulin from control tablets was metabolised, whereas nearly 50% of the insulin from tablets containing the inhibitor was protected from degradation. In another study, the same group utilized protease inhibitors at dosages that would normally cause toxic side effects to inhibit degradation of biologically active peptides (Bernkop-Schnurch *et al.*, Adv. Drug Del. Rev., 52:127, 2001). This approach utilizes chitosan, an aminopolysaccharide related to cellulose that is extracted from chitin, a major structural polysaccaride found in crustaceans and other organisms. By conjugating the protease inhibitors to chitosan and including this conjugated molecule in the formulation of the peptide drug, significant inhibition of digestive tract proteases was seen, increasing the bioavailability of the peptide, without the side effects that would be expected with administration of free protease inhibitors. In the study, a variety of protease inhibitors alone and in combination were utilized for conjugation to the chitosan carrier. A chitosan-EDTA conjugate inhibited endogenous proteases as well, by binding mineral co-factors required by certain proteases for activity. As would be readily apparent to

one with skill in the art, a large number of possible combinations between carrier molecules and effector moieties could be created to provide beneficial properties to peptide formulations, any of which could easily be adapted for use with a peptide of the present invention. By creating a formulation for oral delivery of the peptide using protease inhibitors bound to chitosan, oral delivery of YSV could be used in place of intramuscular injections. This approach does not rule out using the more absorbable, conjugated version of YSV (discussed in a paragraph above) in this formulation, to create an even greater level of bioavailability for this peptide and its derivatives.

In addition to being targeted to a location by another molecule, peptides themselves can serve as the molecule that targets. Any of the conjugation technologies for using a peptide to target a molecule to a desired location described below, as well as other conjugation technologies familiar to those skilled in the art, may be used with a peptide comprising, consisting essentially of or consisting of one of YSV or a functional derivative thereof. For example, researchers have taken the anticancer drug difluoromethylornithine (DFMO) and conjugated it to a peptide for targeting purposes. DFMO is a highly cytotoxic agent that is effective in killing a variety of tumor cell types. However, since it is rapidly cleared from the body, its therapeutic value is limited. In this study, DFMO has been conjugated to a particular fragment of α melanotropin and an analogue of the fragment containing two amino acid substitutions that was shown to bind preferentially to the melanotropin receptors on a human melanoma cell line (Suli-Vargha *et al.*, J. Pharm. Sci., 86:997, 1997). To facilitate the liberation of DFMO from the peptide fragments by aminopeptidases, the drug was conjugated to the N-terminal ends of the peptides. The researchers found that the conjugated drugs are more effective at killing melanoma cells that the unconjugated drug alone.

The effects of the peptides of the present invention may be due in part to a targeting ability inherent in the peptides themselves. For instance, like the α melanotropin fragment, a particular peptide of the invention may bind to a certain receptor found on the surface of a distinct type of cell. By using that peptide as a conjugant, a drug could be targeted to the location of those cells within the body of an individual being treated with the drug.

Peptides as conjugates can serve functions other than targeting. Any of the conjugation technologies for enhancing the therapeutic effectiveness of a peptide described

below, as well as other conjugation technologies familiar to those skilled in the art, may be used to enhance the therapeutic effectiveness of a peptide comprising, consisting essentially of or consisting of YSV or a functional derivative thereof. Fitzpatrick *et al.* have improved upon a conjugated anticancer agent by using a peptide spacer between the two molecules (Anticancer Drug Design, 10:1, 1995). Methotrexate had already been conjugated to human serum albumen (HSA) to increase its uptake by and activity against tumor cells. Once taken up by a cell, some of the methotrexate is liberated from the conjugate by enzymes in the lysosome and can then exert its cytotoxic effects. By inserting a four amino acid linker peptide between the methotrexate and the HSA that is easily digested by lysosomal enzymes, the amount of active methotrexate generated within cells from the conjugate molecule was increased. The peptides of the present invention may be exerting their effects through specific interaction with particular enzymes. By incorporating a peptide of the invention into a conjugated molecule as a linker segment between a drug and its carrier molecule, or in addition to another linker segment, the pharmacokinetics can be altered. This can create a pro-drug that is more resistant or more susceptible to the activity of proteases and subsequently increase or decreasing the rate of drug molecule release from the conjugate. As seen in the examples of conjugated chemotherapy agents above, altering that rate of drug molecule delivery can greatly enhance the effectiveness of a drug.

The effects of a drug on a particular cell may be altered depending upon other factors such as the activation state of a cell or the presence of other molecular signals near or within the cell. In some cases, in order for a drug to have an effect, another molecule or signal needs to be present. Damjancic *et al.* (Exp. Clin. Endocrin., 95:315, 1990) studied the effects of human atrial natriuretic peptide (hANP) on patients with deficient endogenous glucocorticoid synthesis. The peptide was given to patients during a withdrawal of glucocorticoid therapy or during subsequent resumption of therapy using dexamethasone. Patients responded to hANP with an increase in diuresis and sodium excretion only when the peptide hormone was given during concomitant dexamethasone treatment. Treatment with hANP during withdrawal of glucocorticoid therapy had no effect. The effect of concurrent steroid hormone administration can also be to enhance the activity of a peptide. In a report from Zhu *et al.* (Acta Pharm. Sinica, 28:166, 1993), the activity of the analgesic peptide kyotorphin (KTP) was significantly enhanced by conjugation to hydrocortisone via a short

linker segment, as compared to the action of the peptide alone. No effect was seen with the administration of hydrocortisone alone.

The results of these studies illustrate the ability of steroid hormones as conjugated molecules or as ingredients in formulations can allow or enhance the activity of biologically active peptides. Any of the peptides of the present invention may also be modulated or activated by conjugation to or co-application of steroid hormones. The techniques of Zhu *et al.* can be readily adapted for conjugation of steroid molecules to peptide of the present invention. Figures 1 through 5 also provide exemplary step-wise synthesis reactions for linking steroid hormones to any of the peptides of the present invention.

The examples presented above provide exemplary ways to augment the usefulness and the activities of any of the peptides of the invention. Further developments in this field will help overcome the barriers to creating effective peptide-based clinical treatments. As would be apparent to one with skill in the art, the techniques, reagents and protocols developed for use in peptide biochemistry, pharmaceutical research and clinical testing are all readily appliable to any of the peptides of the present invention.

EXAMPLE 1

Delivery of peptides through genetically engineered *Lactobacillus* bacterial species

The following is provided as one exemplary method to deliver peptides of this invention to a host as described above. A DNA sequence that encodes YSV is synthesized by chemical means and this DNA sequence is inserted into an expression vector using standard techniques of genetic engineering familiar to those skilled in the art. The expression vector selected contains a constitutive promoter functional in *Lactobacilli*, a multiple cloning site for the introduction of DNA sequences in a specific 5' to 3' orientation as well as a selectable marker gene that confers resistance to an antibiotic (to aid in cloning procedures) and may comprise other sequences to assist in the production and/or secretion of the peptides, such as signal peptide sequences. An example of such a vector is provided by U.S. Patent No. 5,592,908, to Pavla, which is incorporated herein by reference in its entirety. Briefly, this patent discusses several known promoters that function in *Lactobacillus* species,

as well as a method for discovering novel promoters in said bacteria, any of which may be operably linked to a nucleic acid encoding a peptide of the present invention to express the peptide in *Lactobacilli.* A nucleic acid encoding a signal peptide, such as peptides comprising of 16 to 35 mostly hydrophobic amino acids that are active in *Lactobacillus lactis* described in U.S. Patent No. 5,529,908, cited above, is interposed between the promoter and the nucleic acid encoding the peptide of the present invention such that the nucleic acid encoding the signal peptide is in frame with the nucleic acid encoding the peptide of the present invention.

In addition to the coding sequence of the peptide, the DNA sequence synthesized may comprise sequences to aid in the ligation and cloning of said DNA into the expression vector. For example, restriction enzyme recognition sites that correspond to ones found in the multiple cloning site of the vector can be incorporated into the synthesized DNA at the 5' and 3' ends of the sequence, so that the sequence can be cloned in proper orientation within the vector. Both the vector and the synthesized DNA are digested with the particular restriction enzymes, then purified. Ligation reactions with the vector and the synthesized DNA are followed by transformation into a suitable strain of *E. Coli.* The transformed bacteria are plated on media containing the antibiotic to which the vector confers resistance. A colony of transformed bacteria is selected for growth cultures and plasmid preparation procedures; the presence of the synthesized DNA in the correct orientation is confirmed.

This expression vector is then transformed into a bacterial host cell of a *Lactobacillus* species, such as *L. acidophilus*. Transformed cells are selected for by virtue of the selectable marker found within the vector sequence and the secretion of the peptide may be verified by performing a western blot, performing gel electrophoresis of peptides present in the growth medium or other standard techniques. A transformed colony of bacteria is chosen and used to prepare large-scale cultures of the genetically engineered bacteria. A culture of the genetically engineered bacteria expressing the desired peptide is grown up and at least a portion thereof is administered to the alimentary canal, vagina, trachea or other area of the host organism in which the bacteria are able to replicate. If desired, the bacterial cultures can be treated in a variety of ways to produce a supplement for enteric consumption by the host. These treatments include lyophilization or other methods of preserving the bacteria, in addition to combining the bacteria with carrier agents, such as solutions, solvents, dispersion

media, delay agents, emulsions and the like. The use of these agents to prepare supplements is well known in the art. For example, the bacteria can be used to make cultured milk products or other foodstuffs for human consumption, such that the organism expressing the peptide colonizes the gut of the host organism. A number of different methods for incorporating specific strains of lactic acid bacteria into foodstuffs such as yogurt, kimchee, cheese and butter are disclosed in U.S. Patent No. 6,036,952, to Oh, which is incorporated herein by reference in its entirety. Upon consuming the bacteria through one of any number of routes, the engineered organisms can colonize the gut and allow the presentation and/or absorption of the peptides of this invention via the mucosal layer of the gut.

EXAMPLE 2

Delivery of peptides through a genetically engineered form of *Bacillus subtilis*

The following is provided as another exemplary method to deliver peptides of this invention to a host as described above. A DNA sequence that encodes one of the peptides listed in table A above is synthesized by chemical means and this DNA sequence is inserted into an expression vector via techniques of genetic engineering, all techniques being known in the art. The expression vector selected comprises a shuttle vector, such as pTZ18R (Pharmacia, Piscataway, NJ), capable of being propagated in both *E. Coli* and *B. Subtilis* and containing an antibiotic resistance gene for selecting colonies of transformed bacteria. This vector can contain a constitutive promoter active in *B. subtilis*, such as a promoter derived from the Sac B gene of *B. subtilis* as well as a nucleotide sequence encoding a signal peptide active in *B. subtilis* that directs efficient export of expressed heterologous proteins from the bacterial cell. An example of such a vector is disclosed in U.S. Patent No. 6,268,169, to Fahnestock, the disclosure of which is incorporated herein by reference in its entirety. Briefly, as detailed above, the DNA encoding a peptide of this invention will be synthesized with restriction enzymes sites and/or other sequences to facilitate cloning of the DNA through techniques familiar to those with skill in the art. After transformation into *E. Coli.*, plating, selection and propagation of the plasmid to create a plasmid stock, the plasmid is then be transformed into *B. subtilis* and transformants are selected by virtue of resistance to an antibiotic in the plating media.

Peptide production in and secretion from the genetically engineered *B. subtilis* is verified using techniques well known to those with skill in the art, such as radiolabeling of peptides for autoradiographic detection after SDS-PAGE analysis or Western blotting.

A culture of genetically engineered bacteria is grown up and at least a portion thereof is administered to the alimentary canal, vagina, trachea or other area of the host organism in which the bacteria are able to replicate.

EXAMPLE 3

Delivery of peptides through genetically engineered *Saccharomyces* yeast species

The following is provided as another exemplary method to deliver peptides of this invention to a host as described above. A DNA sequence that encodes one of the peptides listed in table A above is synthesized by chemical means and this DNA sequence is inserted into an expression vector via techniques of genetic engineering, all techniques being known in the art. The expression vector selected comprises a stably maintained yeast protein expression vector, comprising a constitutive yeast promoter such as pADH1, sites for replication of the vector in both yeast and *E. Coli*, a gene or genes that confer prototrophy to an auxotrophic yeast mutant for selection purposes, a multiple cloning site (MCS) and, if desired, sequences that code for a signal peptide. Vectors such as this are commercially available and well known in the art or can be readily constructed using standard techniques After insertion of the synthesized DNA into the yeast vector, transformation into *E. Coli*, plating of transformed *E. Coli* onto selective media, selection of a transformed bacterial colony and preparation of plasmid DNA from a growth culture of bacteria from said colony, the vector is transformed into *Saccharomyces cerevisiae* via well-known techniques such as lithium acetate transformation or electroporation. The strain of *Saccharomyces cerevisiae* selected for transformation is a mutant auxotrophic strain that will require a gene on the plasmid in order to grow on minimal media plates. Transformed yeast colonies are isolated by plating the yeast on growth media lacking the gene provided on the vector. Only those yeast that have received the vector and its selective gene and are expressing that gene product will be able to grow into colonies on the minimal media. Verification of peptide secretion can be obtained by performing a Western blot, performing gel electrophoresis of peptides present in the growth medium or other standard techniques.

A transformed colony of yeast is chosen and used to prepare large scale cultures. A culture of the genetically engineered yeast expressing the desired peptide is grown up and at least a portion thereof is administered to the alimentary canal, vagina, trachea or other area of the host organism in which the bacteria are able to replicate. If desired, the yeast cultures can be treated in a variety of ways to produce a supplement for enteric consumption by the host. These treatments include lyophilization or other methods of preserving yeast, in addition to combining the bacteria with carrier agents, such as solutions, solvents, dispersion media, delay agents, emulsions and the like. The use of these agents to prepare supplements is well known in the art. In another embodiment, the transformed yeast are used in the creation of food products, such as fermented milk products like yogurt and kefir, by techniques known to those skilled in the art. As with live lactic acid bacterial cultures in these foodstuffs, the transformed yeast colonize the gut at least transiently and serve to present peptides to the host via the gut lumen.

EXAMPLE 4

Targeting of a peptide to a particular location

The following is provided as an exemplary method to selectively deliver a peptide of this invention to a particular compartment, organ, cell type or location within the body. In this case, nephritis is treated by targeting YSV to tissues in the kidney of an individual. YSV is linked by covalent bonds via chemical reactions known in the art to low molecular weight (LMW) lysozyme, a commercially available protein moiety that concentrates specifically in renal tissue. Techniques for achieving conjugation of molecules to LMW lysozyme are documented (Folgert *et al.*, Br. J. Pharmcology, 136:1107, 2002). General techniques for conjugating proteins or peptides to one another are also taught in the literature of the field (Fischer *et al.*, Bioconj. Chem., 12:825, 2001). The newly created conjugated peptide sample is then purified away from chemical reagents used in the linking process by chromotography methods such as cation exchange FPLC and/or gradient centrifugation. Once purified, the conjugated peptide is administered to an individual in need of therapy for nephritis. For anti-nephritic activity, YSV is preferentially targeted to renal tissue by virtue of the link between it and the LMW lysozyme, which is selectively absorbed and catabolized by cells of the proximal tubules of the kidney. This preferential delivery allows a greater anti-nephritic effect compared to that of a molar equivalent amount of YSV by itself. Inversely, it can

reduce the amount of peptide drug required to achieve a certain level of anti-nephritic activity.

EXAMPLE 5

Enhancing the delivery of a peptide to its active site

The following is presented as an exemplary method to increase the delivery of a neuroactive peptide to the brain. A peptide of the present invention that exerts its effects on receptors expressed by neurons of the brain is synthesized by chemical methods known to those with skill in the art. Alternatively, it can be expressed by an engineered microorganism and recovered from a culture of such organisms, as detailed in examples above. Once obtained in a purified form, the peptide is utilized in a series of organic chemical reactions to create a triglyceride ester conjugated moiety, attached to the peptide. The conjugated moiety consists of a quaternary substituted carbon center joined to the peptide of the invention through an amide bond with the terminal carboxyl carbon of the peptide. The other three groups attached to the quarternary carbon center consist of carbon ester linkages to 16 carbon fatty acid chains. The fatty acid chains themselves end in terminal dipeptide group, known as a peptide mask, which makes the chains more hydrophilic and targets them to the blood-brain barrier's endothelial cell membrane specifically. The procedure for this synthesis is explained at length in Patel *et al.*, Bioconjugate Chem., 8(3):434, 1997, and utilizes common reagents and equipment familiar to those with skill in the art.

Once introduced into an individual at a peripheral location, the compound travels throughout the body via the circulatory system, interacting with the endothelial membrane of the blood brain barrier. Step-wise degradation of the dipeptide mask and the lipid chains during the transport of the molecule across the epithelial layer of the blood-brain barrier results in the release of the peptide of the invention into the brain compartment. There the peptide can interact with receptors on the surface of neurons to exert its effect on brain function. The time required for the drug to reach the blood brain barrier and be transported to the brain, with the concomitant degradation of the carrier moiety, alters the kinetics of the drug's activity, creating a more stable and longer lasting effect as compared to the intracerebro ventricular injection of the free peptide.

EXAMPLE 6

Creating peptide formulations that are resistant to enzymatic degradation

The following is provided as an exemplary method for creating a formulation of a biologically active peptide for oral administration that is resistant to the activity of proteases and peptidases found in and along the surface of the digestive tract. In this example, YSV is utilized in the making of a pharmaceutical formulation for oral administration to a patient. As described in Larionova *et al.* (Int. J. Pharma., 189:171, 1999), the peptide is used in the creation of microparticles with soluble starch and a protease inhibitor, aprotinin, that is a strong inhibitor of a variety of luminally secreted and brush border membrane-bound proteases. Briefly, soluble starch, the protease inhibitor aprotinin and the peptide of the invention are dissolved in an aqueous buffer. The ratios of soluble starch, aprotinin peptide are determined by experimental methods familiar to one with skill in the art; for example, Larionova *et al.* utilized *in vitro* simulated digestion assays to determine the ratios and preparation conditions most effective for the protein used in their study. The aqueous solution is emulsified under mechanical agitation in cyclohexane (1:3 ratio, v/v) containing 5% Span-80, a non-ionic surfactant. A terephthaloyl chloride solution in chloroform is added to the emulsion and stirring is continued 30 minutes, during which the starch molecules are cross-linked with the aprotinin and the peptide. The microparticles created in that process are washed with sequentially with cyclo-hexane, a 95% ethanol solution with 2% v/v Tween 85 detergent, 95% ethanol and water. The microparticles are resuspended in water and lyophilized. The lyophilized compound can be placed into gelatin capsules for oral delivery to the individual in need of treatment.

Once ingested, the compound is released as the gelatin capsule dissolved. The microparticles are broken down in the small intestine by the action of α amylase on the starch molecules, leading to the gradual release of aprotinin and the peptide of the invention. The concurrent release of the potent protease inhibitor aprotinin at the same time and location of the peptide decreases the enzymatic degradation of the peptide and increases the proportion of intact peptide available for absorption through the gut membrane.

While the present invention has been described using the aforementioned methods and data and the specific example of YSV, it is understood that this is an example only and should not be taken as limitation to the present invention. It should also be understood that

YSV represents one embodiment of the present invention and the same principle of the present invention can also apply to other functionally equivalent peptides that have been modified without affecting the biological function of YSV. For example, equivalents of YSV include those that have conservative amino acid substitutions (i.e. one of the Y, S or V replaced by another amino acid having a residue within the same biochemical type such as hydrophobic, hydrophilic, positive or negatively charged groups) Another example of an equivalent peptide to YSV is a slightly longer peptide, such as one or two amino acids longer, that retain the same biological activities.

What is claimed is:

1. A pharmaceutical composition comprising a peptide comprising the sequence of L-Tyrosyl-L-Seryl-L-Valine admixed with a pharmaceutical carrier.
2. A pharmaceutical composition comprising a peptide consisting essentially of the sequence of L-Tyrosyl-L-Seryl-L-Valine admixed with a pharmaceutical carrier.
3. The pharmaceutical composition according to claim 2, wherein the dose of said peptide for injection mode of administration is 10 ng – 10 mg per kg body weight.
4. The pharmaceutical composition according to claim 2, wherein the dose of said peptide for injection mode of administration is 1ug – 1 mg per kg body weight.
5. The pharmaceutical composition according to claim 2, wherein the dose of said peptide for injection mode of administration is 1 ng per kg body weight.
6. The pharmaceutical composition according to claim 2, wherein the amount of said peptide for oral administration is 0.1ug – 1 g per day per kg body weight.
7. The pharmaceutical composition according to claim 2, wherein the amount of said peptide for oral administration is 1ug – 10 mg per day per kg body weight.
8. A pharmaceutical composition comprising a peptide consisting of the sequence of L-Tyrosyl-L-Seryl-L-Valine admixed with a pharmaceutical carrier.
9. The pharmaceutical composition according to claim 8, wherein the dose of said peptide for injection mode of administration is 10 ng – 10 mg per kg body weight.
10. The pharmaceutical composition according to claim 8, wherein the dose of said peptide for injection mode of administration is 1ug – 1 mg per kg body weight.
11. The pharmaceutical composition according to claim 8, wherein the dose of said peptide for injection mode of administration is 1 ng per kg body weight.
12. The pharmaceutical composition according to claim 8, wherein the amount of said peptide for oral administration is 0.1ug – 1 g per day per kg body weight.
13. The pharmaceutical composition according to claim 8, wherein the amount of said peptide for oral

administration is 1ug – 10 mg per day per kg body weight.

14. A pharmaceutical composition comprising a peptide comprising a functional derivative of the sequence of L-Tyrosyl-L-Seryl-L-Valine admixed with a pharmaceutical carrier.

15. A pharmaceutical composition comprising a peptide consisting essentially of a functional derivative of the sequence of L-Tyrosyl-L-Seryl-L-Valine admixed with a pharmaceutical carrier.

16. A pharmaceutical composition comprising a peptide consisting of a functional derivative of the sequence of L-Tyrosyl-L-Seryl-L-Valine admixed with a pharmaceutical carrier.

17. A method of making a pharmaceutical composition comprising providing a substantially pure peptide comprising the sequence of L-Tyrosyl-L-Seryl-L-Valine and mixing said sequence with a pharmaceutically acceptable carrier.

18. A method of making a pharmaceutical composition comprising providing a substantially pure peptide consisting essentially of the sequence of L-Tyrosyl-L-Seryl-L-Valine and mixing said sequence with a pharmaceutical acceptable carrier.

19. A method of making a pharmaceutical composition comprising providing a substantially pure peptide consisting of the sequence of L-Tyrosyl-L-Seryl-L-Valine and mixing said sequence with a pharmaceutical acceptable carrier.

20. A method of making a pharmaceutical composition comprising providing a substantially pure peptide comprising a functional derivative of the sequence of L-Tyrosyl-L-Seryl-L-Valine and mixing said sequence with a pharmaceutically acceptable carrier.

21. A method of making a pharmaceutical composition comprising providing a substantially pure peptide consisting essentially of a functional derivative of the sequence of L-Tyrosyl-L-Seryl-L-Valine and mixing said sequence with a pharmaceutical acceptable carrier.

22. A method of making a pharmaceutical composition comprising providing a substantially pure peptide consisting of a functional derivative of the sequence of L-Tyrosyl-L-Seryl-L-Valine and mixing said sequence with a pharmaceutical acceptable carrier.

23. Use of the sequence of L-Tyrosyl-L-Seryl-L-Valine in the manufacture of a medicament for

reducing the condition of a human disease, wherein said human disease is selected from the group consisting of a condition that can be reduced by stimulating T lymphocyte transformation and a cell proliferative disorder.

24. Use of the sequence according to claim 23, wherein said cell proliferative disorder is cancer.

25. Use of the sequence according to claim 24, wherein said cancer is selected from the group consisting of liver cancer, leukemia, lung cancer, melanoma and cervical cancer.

26. Use of the sequence according to claim 23, wherein said disease may be treated by modulation of immune system.

27. A nutritional supplement, comprising a tripeptide of amino acid sequence L-Tyrosyl-L-Seryl-L-Valine.

28. The nutritional supplement of claim 27 further comprises a suitable carrier.

29. The nutritional supplement of claim 27, wherein said tripeptide comprises an isolated, purified tripeptide.

30. A method of making a nutritional supplement, comprising obtaining a tripeptide of amino acid sequence L-Tyrosyl-L-Seryl-L-Valine.

31. The method according to 30 wherein said nutritional supplement further comprises a suitable carrier.

32. Use of the sequence of L-Tyrosyl-L-Seryl-L-Valine in the manufacture of a nutritional supplement for administering a nutritionally effective amount to an individual in need thereof.

33. The use according to claim 32, wherein said tripeptide is administered as a nutritional formulation further comprising a suitable carrier.

34. The use according to claim 32, wherein said tripeptide is administered orally.