Abstract:

A novel recombinant bifunctional fusion protein and its preparation and use thereof. Said fusion protein includes an extracellular domain of type I transmembrane protein and an extracellular domain of type II transmembrane protein, which are connected by the element of IgG. The bifunctional protein can block the binding of two ligands to their endogenous receptors simultaneously and inhibit the biological activity of ligands, and can be used in treating tumor.

Fig. 1

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(54) Title: A novel recombinant bifunctional fusion protein and its preparation and use

(57) Abstract: Provided is a recombinant bifunctional fusion protein and its preparation and use thereof. Said fusion protein includes an extracellular domain of type I transmembrane protein and an extracellular domain of type II transmembrane protein, which are connected by the element of IgG. The bifunctional protein can block the binding of two ligands to their endogenous receptors simultaneously and inhibit the biological activity of ligands, and can be used in treating tumor.
A NOVEL RECOMBINANT BIFUNCTIONAL FUSION PROTEIN AND ITS PREPARATION AND USE

FIELD OF INVENTION

This invention relates to the biomedical area, in particular, this invention relates to a novel recombinant bifunctional fusion protein and the preparation and use thereof. This invention also relates to a genetic engineering technical platform used to produce a pharmaceutical composition comprising the novel recombinant bifunctional fusion protein and the use thereof.

BACKGROUND OF INVENTION

Tens of millions of people have lost their lives because of tumor, hepatic fibrosis, rheumatism, and AIDS every year, causing economic losses of hundreds of millions of dollars. The binding of a receptor on the surface of cell membrane and its corresponding ligand is an important reason for the occurrence and development of diseases. Therefore, there is a bright future for the development of corresponding protein-ligand pharmaceuticals.

The known protein drugs so far include growth factor, hormonal protein, zymoprotein, cytokine, interferon, erythropoietin, fusion protein and the like. Except for fusion proteins, other protein pharmaceuticals are all homogeneous proteins comprising only one kind of protein component. Although the existing fusion protein pharmaceuticals (such as Etanercept and Rilonacept), produced by the confusion of the extracellular domain of a receptor protein with the Fc fragment of human IgG, are composed of two protein components, they still play only one function of blocking the binding of the endogenous receptor and its corresponding ligand. For example, Etanercept blocks the binding of tumor necrosis factor (TNF-a) and type II tumor necrosis factor receptor (TNFRII), and plays a useful part in rheumatism treatment. Rilonacept which blocks the biological activity of IL-1 is used to treat Familial cold inflammatory syndrome and Muckle-Wells syndrome.

However, no recombinant bifunctional fusion protein pharmaceuticals used to treat the above-described diseases have been reported so far. Therefore, the development of
bifunctional fusion protein pharmaceuticals is of great significance to extend survival time of patients, improve their living quality and reduce mortality rate.

SUMMARY OF INVENTION

In the light of the foregoing background, it is an object of this invention to provide a genetic engineering platform used to produce recombinant bifunctional fusion proteins, such as TpRII-Fc-CLEC2, and its use.

Accordingly, the present invention, in the first aspect, provides a monomer protein having the structure shown in formulae \( \text{la} \) or \( \text{lb} \):

\[
\begin{align*}
\text{A-B-C} & \quad (\text{la}), \\
\text{C-B-A} & \quad (\text{lb})
\end{align*}
\]

wherein,

- A is an element of an extracellular domain of type I transmembrane protein;
- B is an element of IgG;
- C is an element of an extracellular domain of type II transmembrane protein; and
- "-" is a peptide bond or a peptide linker that links the elements above.

In an exemplary embodiment of the present invention, the monomer protein has one or more features selected from the group consisting of:

- the extracellular domain of the type I transmembrane protein is selected from the group consisting of: TGF-\(\beta\)RI, TGF-pRII, SIRPla, RANK, VEGFR1, VEGFR2, CTLA4 or the combination thereof.

  In another exemplary embodiment, the extracellular domain of the type I transmembrane protein has the functions selected from the group consisting of: inhibiting immunity, promoting migration and invasion of tumor cells, promoting angiogenesis of neoplastic tissues, promoting tumor metastasis and the like.

  The element of the extracellular domain of the type II transmembrane protein is selected from the group consisting of: CLEC-2, DECTIN-1, NKG2D, DC-SIGN, Mincle, or the combination thereof.

  In another exemplary embodiment, the element of the extracellular domain of the type II transmembrane protein has the functions selected from the group consisting of:
activating platelets, promoting aggregation of platelets, and promoting dendritic cells (DC) infection of HIV and the like.

In another exemplary embodiment, A is an element of an extracellular domain of TGF-β receptor II (TβRII).

In another exemplary embodiment, B is an Fc fragment of human IgGl.

In another exemplary embodiment, C is an element of an extracellular domain of CLEC2.

In another exemplary embodiment, the linker has 1-30 amino acid residues in length, further preferably, the linker has 2-15 amino acid residues in length.

In another exemplary embodiment, the monomer protein has one or more functions selected from the group consisting of:

a) binding two kinds of ligands simultaneously;

b) inhibiting TGF-β mediated Smad2 phosphorylation;

c) inhibiting the binding of monoclonal antibody 18H5 and podoplanin-positive tumor cells; and

d) inhibiting migration and invasion of tumor cells.

The present invention, in the second aspect, provides a bifunctional dimer protein composed of the monomer protein according to the first aspect.

In another exemplary embodiment, the dimer protein has a structure shown in either formula Ila or lib:

A-B-C

or

C-B-A

\[\text{A-B-C (Ila), or C-B-A (lib)}\]

wherein,

A is an element of an extracellular domain of type I transmembrane protein;

B is an element of IgGl;

C is an element of an extracellular domain of type II transmembrane protein;

"-" is a peptide bond or a peptide linker that links the above elements;

"-" denotes a disulfide bond.

The present invention, in the third aspect, provides an isolated polynucleotide
encoding the monomer protein according to the first aspect of the present invention.

The present invention, in the fourth aspect, provides a vector containing the polynucleotide according to the third aspect of the present invention.

The present invention, in the fifth aspect, provides a host cell containing the vector according to the fourth aspect or whose genome is integrated with the polynucleotide according to the third aspect of the present invention.

In another exemplary embodiment, the host cell is CHO.

The present invention, in the sixth aspect, provides a method for producing the fusion protein, which comprises the steps of:

culturing the host cells according to the fifth aspect of the present invention under suitable conditions to express the fusion protein; and
isolating the monomer protein or dimer protein.

The present invention, in the seventh aspect, provides a pharmaceutical composition containing the bifunctional monomer protein according to the first aspect or the dimer protein according to the second aspect and a pharmaceutically acceptable carrier.

The present invention, in the eighth aspect, provides a use of the bifunctional monomer protein according to the first aspect and/or the dimer protein according to the second aspect in the preparation of a pharmaceutical composition for treating diseases.

In another exemplary embodiment, the said diseases are selected from the group consisting of: tumor, hepatic fibrosis, and AIDS.

**BRIEF DESCRIPTION OF FIGURES**

Fig.1 is an illustration of the structure of the recombinant bifunctional fusion protein TpRII-Fc-CLEC2.

Fig.2 shows the working principle of the bifunctional protein. On one hand, TβMI
binds to TGF-β, preventing TGF-β from binding to the receptor on the surface of cell membrane, and lowering TGF-β's biological activity; on the other hand, CLEC-2 can bind to podoplanin, which is located on the membrane of tumor cells, blocking the binding of podoplanin to CLEC-2, which is on the surface of platelet membrane, thus preventing tumor cell-induced platelet aggregation.

Fig. 3 shows the structure of the vector that can express the recombinant bifunctional protein, from N-terminal to C-terminal are genes encoding TβMI, Fc and CLEC-2, respectively.

Fig. 4A shows the results of an ELISA binding assay and recombinant bifunctional protein TpRII-Fc-CLEC2 shows three components: when the anti-Fc antibody was used, a hybrid band was detected in both the recombinant bifunctional protein TpRII-Fc-CLEC2 and recombinant monofunctional protein TpRII-Fc. When the anti-CLEC2 antibody was used, a hybrid band was detected only in bifunctional protein TpRII-Fc-CLEC2. When the anti-TpRII antibody was used, a hybrid band was detected in both recombinant bifunctional protein TpRII-Fc-CLEC2 and monofunctional protein TpRII-Fc. Fig. 4B shows an SDS-PAGE gel of monomer protein TpRII-Fc-CLEC2 and dimer protein TpRII-Fc-CLEC2.

Fig. 5A shows that the recombinant bifunctional protein TpRII-Fc-CLEC2 could bind to TGF-βI and the concentration of Effective Binding (ED₅₀) is 3nM. Fig. 5B shows the results of a flow cytometric analysis, thereby confirming that TpRII-Fc-CLEC2 could bind to a podoplanin-expressing cell line (OVCAR-8) and to a similar extent as 18H5 (a monoclonal antibody against podoplanin).

Fig. 6 shows that TGF-βI-induced Smad2 phosphorylation was inhibited by TpRII-Fc-CLEC2, in a dose-dependent manner.

Fig. 7 shows TpRII-Fc-CLEC2 could inhibit tumor cell migration in a dose-dependent manner.

Fig. 8 shows TpRII-Fc-CLEC2 could inhibit tumor cell invasion in a dose-dependent manner. Fig. 8A shows that PC-3 cells could not invade without existence of TGF-βI and that there were few cells on the lower surface of the membrane, and the addition of TGF-βI could significantly induce the PC-3 cells' invasion from the upper surface to the lower surface. Addition of TpRII-Fc-CLEC2 in the upper surface of the membrane could completely inhibit TGF-βI-induced PC-3 cell
invasion, while there were no similar observations when only control protein (human hlgG) was added. Fig. 8B shows the number of invaded cells in each group.

Fig. 9 shows the results of a flow cytometric analysis in each group, indicating that TpPJI-Fc-CLEC2 could inhibit binding of 18H5 to OVCAR-8 cells.

Fig. 10 shows the results of a flow cytometric analysis, indicating that TpRII-Fc-CLEC2 could bind podoplanin in a dose-dependent manner.

**DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS**

In order to overcome defects of existing single-function fusion protein pharmaceuticals, a genetic engineering technical platform which could produce the novel recombinant bifunctional fusion protein drugs (such as TpRII-Fc-CLEC2) was constructed through intensive and extensive researches. The invention is completed on the basis above.

In one exemplary embodiment, recombinant bifunctional fusion protein TpRII-Fc-CLEC2 has one or more functions selected from the group consisting of:

a) binding TGF-β and podoplanin two (ligand of CLEC2) simultaneously;
b) inhibiting TGF-β mediated Smad2 phosphorylation;
c) inhibiting binding of monoclonal antibody 18H5 to podoplanin-positive tumor cells;
d) inhibiting migration and invasion of tumor cells in a dose-dependent manner.

In one exemplary embodiment, a starting vector plg-Fc was modified, extracellular domains of TGF-β receptor II and CLEC-2 were amplified by PCR, and the amplified products were cloned to the modified vector plg-Fc, generating a new vector plg-TpRII-Fc-CLEC2. The expression products, recombinant bifunctional fusion protein TpRII-Fc-CLEC2, can bind to two ligands (TGF-β and podoplanin) simultaneously. Because TGF-β is able to promote migration of tumors, stimulate angiogenesis of neoplastic tissues, promote tumor bone metastasis, stimulate hepatic stellate cells, and induce the production of an extracellular matrix, cytoskeleton proteins, cytokine and acceptors located on the surface of cell membrane, the binding of protein TpRII-Fc-CLEC2 and TGF-β could be used to treat tumor and hepatic fibrosis. Since CLEC2, a HIV-attachment factor, facilitate platelet-mediated HIV capture and dissemination, the protein TpRII-Fc-CLEC2 is expected to be used in treating AIDS.

—6—
Besides inductions of tumor metastasis, TGF-β could also inhibit immunity, so the binding of protein TpPJI-Fc-CLEC2 and TGF-β can be used to increase tumor immunity.

**Type I transmembrane proteins and their extracellular domain**

Type I transmembrane proteins are characterized by an N-terminus outside of cell membrane and an C-terminus inside of cell membrane. Type I transmembrane proteins usually include members of immunoglobulin super family (CD4, CD8, CD28, CTLA4, CD80, CD86, SIRPla, etc.), receptor kinases (TGF-β receptor, EGFR, VEGFR, PDGFR, HGF receptor, etc.), and cytokine receptors (TNF receptor, RANK, IL-6 receptor, CSF1 receptor, c-kit, etc.). Type I transmembrane proteins could induce various biological reactions after their binding to corresponding ligands.

There is no special restriction on the type I transmembrane protein used in this invention. Thus, all the type I transmembrane proteins with biological activity can be used in this invention.

In a preferred embodiment, the type I transmembrane proteins include, but not limited to: TGF-pRII, TNF-pR, SIRPla, RANK, VEGFRI, VEGFR2, CTLA4 or a combination thereof.

**Type II transmembrane proteins and their extracellular domain**

Type II transmembrane proteins are characterized by a C-terminus outside of cell membrane and a N-terminus inside of cell membrane. Usually type II transmembrane proteins are C-Type lectins or C-Type lectin-like receptors. The extracellular domain of the receptor includes a carbohydrate-binding domain (or referred to as C-Type Lectin Domain, CTLD). Proteins with carbohydrate-binding domain are involved with various functions such as cell adhesion, platelet activation, immunity of pathogens, inducement of apoptosis, etc.

There is no special restriction on the type II transmembrane proteins used in this invention. Thus, all the type II transmembrane proteins with biological activity can be used in this invention.

Preferred type II transmembrane proteins include, but not limited to: CLEC-2, DECTIN-1, NKG2D, DC-SIGN, Mincle or a combination thereof.
The Element of IgG

A suitable element of IgG includes, but not limited to, an element of IgG derived from human beings or other animals, or the mutants and derivatives thereof. A preferred element which may be used is the element of human IgG.

Human IgG includes four subgroups: IgG1, IgG2, IgG3, and IgG4. These four subgroups have great similarities in structure, all having one variable-region (VH) and three constant regions (CH1, CH2, and CH3). The element of Fc is composed of two constant regions (CH2-CH3), and a disulfide bond of region CH2 makes two Fc elements into a homologous dimer through a covalent bond. Under normal physiological conditions of the human body, the concentration of IgG1, IgG2, and IgG3 and IgG4 ranks first, second, and third, respectively, in the human plasma.

A preferred element of IgG is human IgG1, including variants or derivatives thereof.

Bifunctional Protein and its Preparation

In this invention, the term "recombinant bifunctional protein", "bifunctional protein in this invention", "protein in this invention" and "bifunctional protein" have the same meanings and can be interchangeably used. The fusion protein having the structure as shown in formula 1a or 1b is composed of three elements: the element of an extracellular domain of a type I transmembrane protein, the element of IgG and the element of an extracellular domain of a type II transmembrane protein. A typical example of the fusion protein is TpRII-Fc-CLEC2. The protein in this invention may be a monomer protein or polymer protein (such as dimer protein), in addition, the term "recombinant bifunctional protein" also includes its active fragment and derivatives thereof.

As used herein, the term "isolated" refers to a substance which has been isolated from the original environment. For a naturally occurring substance, the original environment is the natural environment. Such as the polynucleotide and polypeptide in a naturally occurring state in the viable cells are not isolated or purified. However, if the same polynucleotide and polypeptide have been isolated from other components naturally accompanying them, they are isolated or purified.
As used herein, the term "isolated recombinant bifunctional protein" means that the recombinant bifunctional protein essentially does not contain other proteins, lipids, carbohydrate or other substances associated therewith in nature. Those skilled in the art may purify the recombinant bifunctional protein by standard protein purification techniques. Essentially purified protein forms a single main band on a non-reducing PAGE gel.

The polynucleotide according to the invention may be in the forms of DNA and RNA. DNA includes cDNA, genomic DNA, synthetic DNA, etc., in single strand or double strand form. A single strand DNA may be an encoding strand or non-encoding strand.

The invention further relates to the variants of the foregoing polynucleotide which encode a polypeptide having the same amino acid sequence as that of invention, or the fragments, analogues, or derivatives thereof. The variant of the polynucleotide may be a naturally occurring allelic variant of the polynucleotide or a non-naturally occurring variant of the polynucleotide. Such nucleotide variants include substitution, deletion, and insertion variants. As known in the art, the allelic variant is a substitution form of polynucleotide, which may be formed by a substitution, deletion, and insertion of one or more nucleotides without substantially changing the functions of the encoded polypeptide.

As used herein, the term "primer" means the oligo nucleotides which can match with a template and start the synthesis of DNA chains under the action of DNA polymerases. The primer may be naturally occurring RNA or DNA, any natural nucleotides in any form, or even a non-naturally occurring variant of the nucleotides, such as LNA or ZNA. The primer is complementary, generally (or mainly) with a special sequence on one chain of the template. It is necessary that the primer is complementary at least largely with one chain of the template before elongation. For example, adding a sequence which is uncomplimentary with the template to the 5'-end of a primer of which, however, 3'-end is complimentary with the template still makes the primer complementary with the template. As long as the primer is long enough, the partially, but not completely, complimentary primer and the template can form a complex for amplification.

The full-length recombinant protein nucleotide sequence or its fragments can be
prepared by PCR amplification, recombinant method and synthetic method. For PCR amplification, one can obtain said sequences by designing primers based on the nucleotide sequence disclosed herein, especially the ORF, and using a cDNA library commercially available or prepared by routine techniques in the art as a template. When the sequence is long, it is usually necessary to perform two or more PCR amplifications and link the amplified fragments together correctly.

Once the sequence is obtained, one can produce lots of the sequences by recombinant methods. Usually, said sequence is cloned into a vector which is then transformed into a host cell. The sequence is isolated from the proliferated host cells using conventional techniques.

Further, the sequence can be synthesized, especially when the fragment is short. Typically, a number of small fragments are synthesized and linked together to obtain a long sequence.

The method of amplification of DNA/RNA by PCR is preferably used to obtain the gene of the invention. The primers used in PCR can be properly selected according to the polynucleotide sequence information of the invention disclosed herein and synthesized by conventional methods. The amplified DNA/RNA fragments can be isolated and purified by conventional methods such as gel electrophoresis.

The invention further relates to a vector comprising the polynucleotide of the invention, a genetically engineered host cell transformed with the vector of the invention or directly with the sequence encoding the recombinant bifunctional protein, and a method for producing the protein of the invention by recombinant techniques.

The recombinant bifunctional protein can be expressed or produced by a conventional recombinant DNA technology, using the polynucleotide sequence of the invention. Generally, it comprises the following steps of:

1. Transfecting or transforming appropriate host cells with the polynucleotide or its variants encoding the recombinant bifunctional protein of the invention or the vector containing the polynucleotide;
2. Culturing the host cells in an appropriate medium; and
3. Isolating or purifying the protein from the medium or cells.

The methods known to the artisans in the art can be used to construct an expression vector containing the DNA sequence of the recombinant bifunctional protein
and appropriate regulatory components for transcription/translation. These methods include in vitro recombinant DNA techniques, DNA synthesis techniques, in vivo recombinant techniques, etc. The DNA sequence is efficiently linked to a proper promoter in the expression vector to direct the synthesis of mRNA. The expression vector may further comprise a ribosome-binding site for initiating the translation, transcription terminator and the like.

The expression vector preferably comprises one or more selective marker genes to provide a phenotype for selecting the transformed host cells, such as the dehydrofolate reductase, neomycin resistance gene and GFP (green fluorescent protein) for eukaryotic cells, as well as tetracycline or ampicillin resistance gene for _E. coli_.

The vector containing said DNA sequence and proper promoter or other regulatory elements can be transformed into appropriate host cells to express the protein.

The 'host cell' includes prokaryote, such as bacteria; primary eukaryote, such as yeast; and advanced eukaryotic, such as mammalian cells. The representative examples are _E. coli_, _Streptomyces_; fungal cells, yeast; plant cells; insect cells such as Drosophila S2 or Sf9; animal cells such as CHO, COS, or 293 cells.

The recombinant transformation of the host cells with the DNA might be carried out by conventional techniques known to the artisans. Where the host is prokaryotic, such as _E. coli_, the competent cells capable of DNA uptake, can be prepared from the cells harvested after exponential growth phase and subsequently treated by the CaCl₂ method using known procedures. Alternatively, MgCl₂ can be used. The transformation can also be carried out by electroporation. When the host is a eukaryote, transfection of DNA such as calcium phosphate co-precipitates, conventional mechanical procedures such as micro-injection, electroporation, or liposome-mediated transfection may be used.

The transformants are cultured conventionally to express the recombinant bifunctional protein of the invention. According to the used host cells, the medium for cultivation can be selected from various conventional mediums. The host cells are cultured under conditions suitable for its growth until the host cells grow to an appropriate cell density. Then, the selected promoter is induced by appropriate means (such as temperature shift or chemical induction) and the cells are cultured for an additional period.
In the above methods, the recombinant protein may be included in the cells, or expressed on the cell membrane, or secreted out. If desired, the physical, chemical and other properties can be utilized in various isolation methods to isolate and purify the recombinant protein. These methods are well-known to the artisans and include, but are not limited to, conventional renaturation treatment, treatment by protein precipitant (such as salt precipitation), centrifugation, cell lysis by osmosis, sonication, supercentrifugation, molecular sieve chromatography or gel chromatography, adsorption chromatography, ion exchange chromatography, HPLC, any other liquid chromatography, and the combination thereof.

**Antibody**

This invention also includes polyclonal and monoclonal antibodies (mAbs), preferably mAbs which are specific for the polypeptides encoded by the recombinant protein DNA or the fragments thereof. The terms "antibody" and "ligand" have same meanings and can be interchangeably used. The term "specific" means that an antibody is capable of specifically binding to the recombinant bifunctional protein gene products or fragments thereof. Preferably, the antibody binds to the protein gene products or fragments thereof and does not substantially recognize nor bind to other antigenically unrelated molecules. Antibodies which bind to the recombinant bifunctional protein and block the protein and those which do not affect the function of the recombinant bifunctional protein are included in the invention. Antibodies which bind to the modified or unmodified recombinant bifunctional protein are also included in the invention.

The present invention includes not only intact monoclonal or polyclonal antibodies, but also immunologically-active antibody fragments, such as a Fab' or (Fab)_2 fragments, an antibody light chain, an antibody heavy chain, a genetically engineered single chain Fv molecule, or a chimerical antibody.

**Peptide Linker**

This invention also includes a recombinant bifunctional protein which selectively contains a peptide linker. The size and complexity of the linker may affect the biological activity of the recombinant protein. The peptide linker usually has a length
and flexibility enough to ensure that the two protein elements connected with the linker in between have enough freedom in space to exert their functions and avoid influences of the formation of α-helix and β-fold on the stability of the recombinant bifunctional protein.

A too short peptide linker could cause internal spatial resistance inside the recombinant protein and influence the correct folding of the protein. A too long peptide linker may increase immunogenicity of the recombinant protein and affect the biological activity and function of the recombinant bifunctional protein.

There is no special restriction on the length of the linker. The linker is usually 4-44 amino acid residues in length. Preferably, the linker has 6-27 amino acid residues, and, further preferably, 12-15 amino acid residues.

**Pharmaceutical Composition and Method of Administration Thereof**

This invention also provides a pharmaceutical composition comprising a safe and effective amount of the recombinant bifunctional fusion protein in combination with a therapeutically acceptable carrier. The protein could be prepared in a non-toxic, inert, therapeutically acceptable aqueous medium at a certain pH. The pH typically is about 5-8, preferably 6-8, although the pH may vary according to the properties of the formulated composition and the diseases to be treated by the composition.

As used herein, the term 'safe and effective amount' refers to an amount of a compound sufficient to substantially improve the condition of the humans and/or animals in need. Preferably, the effective amount is 0.000001-90wt%, further preferably, the effective amount is 0.1-50wt%, and, particularly preferably, the effective amount is 5-40wt%.

As used herein, the 'pharmaceutically acceptable' composition is an agent applicable to humans and/or animals without causing side-effects (such as toxicity, stimulation and allergy), and is an agent with a reasonable value of benefit/risk. The term "pharmaceutically acceptable carrier" means an agent suitable for administration, including all sorts of excipients and thinners.

The pharmaceutical composition of this invention contains a safe and effective amount of the recombinant bifunctional fusion protein in combination with a therapeutically acceptable carrier. Such a carrier includes but not limited to saline,
buffer solution, glucose, water, glycerin, ethanol, or the combination thereof. The pharmaceutical formulation should be suitable for a delivery method. The pharmaceutical composition may be in the form of injections that are made by conventional methods, using physiological saline or other aqueous solutions containing glucose or other adjuvants. The pharmaceutical composition in the form of tablet or capsule may be prepared by routine methods. The pharmaceutical composition in the form of such as injections, solutions, tablets, and capsules, should be manufactured under sterile conditions. The active ingredient is administered in a therapeutically effective amount. The pharmaceutical composition of this invention can be made into slow-release formulation.

The precise amount of the pharmaceutical composition will depend upon various factors, such as delivery methods, the subject health, and the like, and is within the judgment of the skilled clinician. In a preferred embodiment, the amount of pharmaceutical compositions will be decided by the artisans according to several factors (such as clinical trial). The said factors include but are not limited to pharmacokinetic parameters (such as biological utilization, metabolism and half-life of the said protein), severity of the disease, body weight, immunological conditions and method to administration thereof. Typically, the therapeutically effective amount is about 0.00001mg-50mg/kg body weight in most cases, and preferably about 0.0001mg-10mg/kg body weight. Separated dose or proportional reduced dose of several times every day will be administered according to the urgent requirement. The formulated pharmaceutical composition is administered in conventional routes including but not limited to oral, intramuscular, intraperitoneal, intravenous, subcutaneous, intradermal or topical administration.

There are many advantages of the monomer and dimer or polymer fusion protein of the present invention which includes but not limited to:

1) binding simultaneously with TGF-β and podoplanin two (ligand of CLEC2);
2) inhibiting TGF-β mediated Smad2 phosphorylation;
3) inhibiting the binding of monoclonal antibody 18H5 and podoplanin-positive tumor cells;
4) inhibiting migration and invasion of tumor cells in a dose-dependent manner;
5) inhibiting the binding of HIV and dendritic cells (DC);
6) blocking immunosuppression of TGF-β, enhancing immunity; and
7) blocking TGF-P-induced hepatic fibrosis.

The following exemplary embodiments further describe the present invention. Although the description referred to particular embodiments, it will be clear to one skilled in the art that the present invention may be practiced with variation of these specific details. Hence this invention should not be construed as limited to the embodiments set forth herein. Further, for the embodiments in which details of the experimental methods are not described, such methods are carried out according to conventional conditions such as those described in Sambrook et al. Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Pres, 1989), or suggested by the manufacturers.

Example 1

Construction of Vector plg-TpRII-Fc-CLEC2

1. Modification of plg-Fc vector by deleting the stop codon at the C terminal of Fc.

To generate the bifunctional protein linked with the Fc fragment of human IgGl, the Fc encoding gene was amplified by using primer 1 (SEQ ID NO: 1) and primer 2 (SEQ ID NO: 2) (Table 1). The amplified product was cloned into the EcoRI/ Xhol cloning site of the starting vector plg-Fc (commercially available from CLONETECH).

2. PCR amplification of the extracellular domain of type II TGF-beta receptor

The cDNA sequence encoding the extracellular domain (ECD) of type II receptor was amplified by PCR using primer 3 (SEQ ID NO: 3) and 4 (SEQ ID NO: 4) (Table 1) from RNA isolated from breast cancer cell lines MDA-MB-231 (commercially available from the Chinese Academy of Sciences). The PCR condition was as follows: 92°C for 30sec, 58°C for 30sec, and 72°C for 30sec for 25 cycles with a final extension at 72°C for 10min. The amplified product was cloned into the HindIII/EcoRI cloning site of the modified vector plg-Fc, generating a new vector plg-TpRII-Fc.

3. PCR amplification of the extracellular domain of CLEC-2

The cDNA sequence encoding the extracellular domains (ECD) of CLEC-2 was amplified by PCR using primers 5 (SEQ ID NO: 5) and 6(SEQ ID NO: 6) (Table 1)
from RNA isolated from human peripheral blood mononuclear cells (PBMC). The PCR condition was as follows: 92°C for 30sec, 58°C for 30sec, and 72°C for 30sec for 25 cycles with a final extension at 72°C for 10min. The amplified PCR product was cloned into the Xhol/Xbal cloning site of the modified vector plg-TpRII-Fc, generating a vector plg-TpRII-Fc-CLEC2.

Table 1

<table>
<thead>
<tr>
<th>Name of Primers</th>
<th>SEQ ID NO.</th>
<th>Primer Sequence (From 5' to 3')</th>
<th>Target Gene</th>
<th>Restriction Enzyme</th>
<th>Restriction Cutting Site</th>
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<tr>
<td>Primer 1</td>
<td>1</td>
<td>CGGAATTCCAGGCCCCAAATCGTTGTG</td>
<td>Human IgG Fc</td>
<td>GAATTC</td>
<td>EcoRI</td>
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<td>Primer 2</td>
<td>2</td>
<td>CATGCTCGAGITTTACCCCGAGACAGGGGAG</td>
<td>Human IgG Fc</td>
<td>CTGCAG</td>
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<td>3</td>
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<td>ΤβRI</td>
<td>AAGCTT</td>
<td>HindIII</td>
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<td>ΤβRI</td>
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<td>CLEC-2</td>
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<td>CLEC-2</td>
<td>TCTAGA</td>
<td>Xbal</td>
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The length of the polynucleotide sequence (SEQ ID NO: 7) encoding the bifunctional recombinant protein TpRII-Fc-CLEC2 is 1779bp. The 1-552 region bp of the whole sequence is the gene encoding TpRII, the region bp 553-558 (GAATTC) is the restriction enzyme cutting site of EcoRI, the region bp 559-1254 is the gene encoding Fc, the region bp 1255-1260 (CTGCAG) is the restriction enzyme cutting site of Xhol, the region bp 1261-1776 is the gene encoding CLEC-2, and TAA is the stop codon.

Figure 1 shows the structure of the dimeric recombinant bifunctional protein TpRII-Fc-CLEC2.
Figure 2 shows the working principle of the bifunctional protein. On one hand, the TβM1 binds to TGF-β, preventing TGF-P's binding to the receptor on the surface of cell membrane, and lowering TGF-P's biological activity; on the other hand, CLEC-2 can bind to podoplanin which is located on the membrane of tumor cells, blocking podoplanin's binding to CLEC-2 which is on the surface of platelet membrane, and preventing tumor cell-induced platelet aggregation.

Figure 3 shows the structure of the recombinant bifunctional protein, SEQ ID NO: 8 is the amino acid sequence encoding the recombinant bifunctional protein. There are 592 amino acid residues in the whole protein whose molecular weight (MW) is about 65Kd. 1-184 amino acid residues encode TβM1, 185-186 amino acid residues encode restriction enzyme cutting site of EcoRI, 187-418 amino acid residues encode Fc, 419-420 amino acid residues encode restriction enzyme cutting site of Xhol, and 421-592 amino acid residues encode CLEC-2.

**Example 2**

**Expression of TPRII-Fc-CLEC2**

To test the expression capacity of the constructed vector pH-TpRII-Fc-CLEC2, a small scale of transient transfection was performed: 5x10⁴ of CHO cells (Chinese hamster ovary cells) in 0.5ml of DMEM medium were seeded in a 24-well cell culture plate in an incubator (37°C, 5%CO₂) for 24 hours. Transient transfection was performed by mixing vector pH-TpRII-Fc-CLEC2 and transfection reagent (Lipofectamine 2000, Cat#1 1668-027, Invitrogen) and keeping the mixture at room temperature for 20 min. Then the mixture was added in each well and cultivated for 24 hours. The transfection method could follow the instruction of manufacturer. The cell culture supernatant was harvested after 24 hours to analyze the expression of the recombinant bifunctional protein TpRII-Fc-CLEC2 by ELISA binding assay.

When the anti-Fc antibody was used, a hybrid band was detected in both recombinant bifunctional protein TpRII-Fc-CLEC2 and recombinant monofunctional protein TpRII-Fc. When the anti-CLEC2 antibody was used, a hybrid band was detected only in bifunctional protein TpRII-Fc-CLEC2. When the anti-TpRII antibody was used, a hybrid band was detected in both recombinant bifunctional proteins TpRII-Fc-CLEC2 and monofunctional protein TpRII-Fc. (Figure 4A) The
supernatant contained three soluble protein components: the extracellular domain of receptor II TGF-β (TpPJI-ECD), the element Fc of human IgG, and the extracellular domain of CLEC-2 (CLEC-2-ECD).

10 mg of recombinant protein TpRII-Fc-CLEC2 was firstly produced through stable transfection. When loaded on a SDS-PAGE gel, the bifunctional protein appeared as two bands of about 85 kDa and 170 kDa, respectively, corresponding to the respective molecular weight (MW) of the recombinant monomer and dimer protein (Figure 4B).

Example 3

TPRII-Fc-CLEC2 can bind TGF-β1 and podoplanin simultaneously

To confirm the binding activity of TpPJI-Fc-CLEC2 to two corresponding ligands, ELISA binding assay and Flow Cytometer Analysis were performed.

For TGF-β binding capacity, Falcon 96-Well ELISA Micro Plate was coated overnight at room temperature with recombinant human TGF-β1 (R&D Systems) in PBS (25 ng per well). The coated plate was blocked with 3% dry fat milk in PBS-T buffer (PBS containing 0.05% of Tween-20). 100 µl of serially diluted protein solution (from 200 nM to 0.1 nM) was then transferred into the plate. After incubation at room temperature for 1 hour, the plate was washed 5 times with PBS-T solution. HRP-conjugated Fc-specific antibody was then added to the plate and kept at room temperature for 1 hour. The plate was washed for 5 times with the PBS-T buffer and then developed with 100 µl of HRP substrate solution for up to 5 min. Reaction was stopped with IN H₂SO₄, and the absorbance at 450 nM was determined in a standard plate reader.

For podoplanin binding activity assay, OVCAR-8 cells positive for podoplanin were incubated for 30 min with the recombinant TpRII-Fc-CLEC2 (20 µg/ml) in cold PBS. After washing 2 times, cells were incubated with the Fc fragment-specific FITC-conjugated goat F(ab')2 for another 30 min. Cells were then analyzed by flow cytometer.

ELISA results (Figure 5A) indicated that the TpRII-Fc-CLEC2 could interact with TGF-β1. Increased TGF-β1-binding activity was detected following the increased amount of TpRII-Fc-CLEC2 in the reaction, and the value of ED₅₀ was about 3 nM.
The results of flow cytometer confirmed that TpRII-Fc-CLEC2 could bind to the podoplanin-expressing cell line (OVCAR-8), to a similar extent to 18H5, which is a monoclonal antibody against podoplanin (Figure 5B).

Example 4

**TPRII-Fc-CLEC2 inhibits TGF-βI mediated Smad2 phosphorylation**

Cellular effects of TGF-β1 are mediated by the induction of Smad2 phosphorylation that help transduce signals required for initiation of target gene expression and regulation. Therefore status of Smad2 phosphorylation could be used as a parameter for TGF-βI-initiated signaling.

3ml of MDA-MB-231 cells (human breast adenocarcinoma) (1x10^5/ml) cells in DMEM medium containing 10% of FBS were incubated in a 6-well plate at 37°C, 5%CO₂ for 24 hours, cells were starved for overnight in serum-free DMEM medium, and then incubated with 5ng/ml of TGF-βI in the presence of variable amount of the recombinant bifunctional protein or human IgG for 30min. Cells were washed twice with cold PBS and then dissolved in 200µl of lysis buffer (50 mM Tris, pH 7.4, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, pH 8.0, 150 mM NaCl). After centrifugation and quantitation, equal amount of supernatant from each sample was subjected to Western blotting analysis using antibody specific for phosphor Smad2 (Ser465/Ser467) (Cat#40-0800, ZYMED). Alternatively, the phosphorylated Smad2 was detected by ELISA using PathScan® Phospho-Smad2 (Ser465/467) Sandwich ELISA Kit (Cat#7348S, Cell Signaling).

As showed in Figure 6, TGF-βI-induced Smad2 phosphorylation was inhibited by TpRII-Fc-CLEC2, in a dose-dependent manner. These data indicated that TpRII-Fc-CLEC2 could block the interaction of TGF-βI with cell surface TGF-βI receptor, and that TpRII-Fc-CLEC2 could inhibit TGF-βI mediated Smad2 phosphorylation even in the lowest dose (0.78nM).

Example 5

**TβMI-Ec-CEEC2 inhibits tumor cell migration in a dose-dependent manner**

Migration and invasion of tumor cells is one of the most important steps in tumor metastasis. It has been reported that TGF-β promotes tumor cell invasion and migration
through induction of epithelial-mesenchymal transition (EMT), which is able to disrupt cell-cell junction and facilitate the trans-endothelial passage. Blockade of TGF-β signing would result in a significant suppression of tumor metastases.

For cell migration assay, 3ml of PC-3 cells (1x10^6/ml) in DMEM containing 10% FBS were seeded in 6-well plates and incubated for 24 hours. Confluent monolayer cells were starved overnight in serum-free DMEM medium. Beeline or river (1 mm thick) was created using a 200 μι micropipette tip. Cells were washed and incubated in serum free medium containing either TGF-β1 (5ng/ml), or TGF-β1 plus TpRII-Fc-CLEC2 or control human IgG at a variable amount (Figure 7). Cell migration was monitored at 24 hour intervals and photographed.

It was found that no closure of beeline was observed in cells with medium alone, full closure was obtained when TGF-β1 was added. Strikingly, the closure of the beeline was dramatically blocked when TpRII-Fc-CLEC2 was added (Figure 7), complete blockade of the closure was observed when the concentration of TpRII-Fc-CLEC2 reached 100ngM. As expected, the control human IgG had little impact on beeline closure. The recombinant bifunctional protein TpRII-Fc-CLEC2 inhibited tumor cell migration in a dose-dependent manner.

**Example 6**

**TpRII-Fc-CLEC2 inhibits tumor cell invasion in a dose-dependent manner**

Cell invasion assay was performed using BD BioCoat™ Matrigel™ Invasion Chamber(Cat#354480) following the procedures described in the instruction menu by the manufacturer. Briefly, the lower chamber was filled with serum-free DMEM or DMEM containing TGF-β1 (5ng/ml). 2x10^5 of PC-3 cells in DMEM containing 1% of FBS were seeded into the upper chamber. The recombinant TpRII-Fc-CLEC2 or human IgG (control) were added to the upper chamber, at a concentration of 10μg/ml. The invasion chambers were incubated for 24 hours at 37°C, 5% CO_2 atmosphere. After incubation, cells on the upper surface of the membrane were removed by scrubbing with a cotton swab, and the membrane was fixed in 4% paraformaldehyde. Cells on the lower surface of the membrane were stained with 0.5% methylene blue in 50% methanol and counted under the microscope at 40 magnifications. All invasion assays were done in triplicate.
As shown in Figure 8A, PC-3 cells could not invade without TGF-β1 and there were few cells on lower surface of the membrane. The adding of TGF-β1 induced PC-3 cells’ invasion from the upper surface to the lower surface significantly. The adding of TpPJI-Fc-CLEC2 in the upper surface of the membrane completely inhibited TGF-β1 induced PC-3 cell invasion, while there were no similar observations when the control protein (human hlgG) was added.

Figure 8B shows the number of invaded cells in each group.

**Example 7**

**TPRII-Fc-CLEC2 can bind to podoplanin-positive tumor cells**

Podoplanin is a transmembrane glycoprotein which is up-regulated in many types of tumors, including breast cancers, lung cancers, head and neck tumors, mesotheliomas, and testicular seminomas. Tumor cells expressing podoplanin could induce platelet activation through interaction with CLEC-2 expressed on platelets. Activated platelets facilitate tumor metastasis through a number of mechanisms. Therefore inhibition of tumor-mediated activated platelets is expected to block tumor metastasis. For the same reason, if TpPJI-Fc-CLEC2 could bind to podoplanin on the surface of tumor cells, the binding of podoplanin and CLEC-2 could be blocked, and the activated platelets could be inhibited.

The binding capacity of TpPJI-Fc-CLEC2 to podoplanin-expressing tumor cells was evaluated by staining analysis using podoplanin-expressing cell line OVCAR-8 (Figure 5B, left). It was observed that TpRII-Fc-CLEC2 could bind to the cell surface (Figure 5B, right), to a similar extent to 18H5, which is a monoclonal antibody against podoplanin.

**Example 8**

**TPRII-Fc-CLEC2 inhibits the binding of monoclonal antibody 18H5 and podoplanin-positive tumor cells**

18H5 is a monoclonal antibody against podoplanin and could bind to the OVCAR-8 (Figure 5B). OVCAR-8 cells were incubated at 4°C, 5%CO₂ for 30 min with different concentrations of TpRII-Fc-CLEC2. Cells were washed and incubated with monoclonal antibody 18H5 at 4°C, 5%CO₂ for another 30 min. Cells were washed again
and stained with PE-labeled second antibody. Cells were then analyzed by flow cytometer.

Figure 9 shows that TpPJI-Fc-CLEC2 could inhibit the binding of 18H5 to OVCAR-8 cells.

Figure 10 further indicates that TpPJI-Fc-CLEC2 binds podoplanin in a dose-dependent manner.

Example 9

Preparation of pharmaceutical composition

Mixed the components below, and obtained the solution containing recombinant bifunctional fusion protein with the final concentration of 1wt%:

- the recombinant protein 10mg
- saline to 10ml
- adjusted pH to 6.8-7.1.

Inoculated OVCAR-8 cells on the right back of nude mice ($5 \times 10^6$/cells/mouse). The mice were randomly divided into two groups after the tumor grew to 200cm$^3$ (ten mice in each group). One group of mice was injected intraperitoneally with TpRII-Fc-CLEC2 (10ml/kg) for six weeks continuously; another group was injected intraperitoneally with the same dose of human IgG as control. The size of tumor was measured for eight weeks before the medicine was injected every time. The lungs were taken out and metastases were recorded before the end of experiment. Analyzed and compared the size of tumor and number of lung metastases in the two groups after the experiment.

Result showed that TpRII-Fc-CLEC2 significantly inhibited the growth of tumor cells with the value of $T/C$ % reached up to about 40%. The number of lung metastases of mice treated with TpRII-Fc-CLEC2 decreased significantly.

All the documents cited herein are incorporated into the invention as reference, as if each of them is individually incorporated. Further, it would be appreciated that, in the above teaching of the invention, the skilled in the art could make certain changes or modifications to the invention, and these equivalents would still be within the scope of the invention defined by the appended claims of the present application.
What is claimed is:

1. A monomer protein having the structure as shown in formula Ia or lb:
   \[ \text{A-B-C (Ia), or} \]
   \[ \text{C-B-A (lb)} \]

   wherein,
   A is an element of an extracellular domain of a type I transmembrane protein;
   B is an element of IgG;
   C is an element of an extracellular domain of a type II transmembrane protein;
   '-' denotes a peptide bond or a peptide linker that links the elements described above.

2. The monomer protein of claim 1, wherein the monomer protein has one or more features selected from the group consisting of:
   the extracellular domain of the type I transmembrane protein is selected from the group consisting of: TGF-\(\beta\)RI, TGF-pRII, SIRPla, RANK, VEGFR1, VEGFR2, CTLA4, and the combination thereof; and
   the extracellular domain of the type II transmembrane protein is selected from the group consisting of: CLEC-2, DECTIN-1, NKG2D, DC-SIGN, Mincle, and the combination thereof.

3. The monomer protein according to claim 1, wherein the monomer protein has one or more functions selected from the group consisting of:
   a) binding two kinds of ligand simultaneously;
   b) inhibiting TGF-\(\beta\) mediated Smad2 phosphorylation;
   c) inhibiting the binding of monoclonal antibody 18H5 and podoplanin-positive tumor cells; and
   d) inhibiting migration and invasion of tumor cells.

4. A dimer bifunctional protein wherein the dimer protein is composed of the monomer protein of any one of claim 1-3.

5. The dimer protein according to claim 4, wherein the dimer protein has the structure as shown in formula IIa or lib:
   \[ \text{A-B-C (IIa), or} \]
   \[ \text{C-B-A (lib)} \]
wherein,
A is the element of the extracellular domain of the type I transmembrane protein;
B is the element of IgG;
C is the element of the extracellular domain of the type II transmembrane protein;
'-' is the peptide bond or the peptide linker that links the above elements;
'"' is a disulfide bond.
6. An isolated polynucleotide encoding the monomer protein of claim 1.
7. A vector containing the polynucleotide of claim 6.
8. A host cell containing the vector of claim 7 or whose genome is integrated with
the polynucleotide of claim 6.
9. A method for producing a protein comprising the steps of:
culturing the host cells of claim 8 under suitable conditions to express the
monomer protein of claim 1; and
isolating the monomer protein or the dimer protein formed there from.
10. A pharmaceutical composition containing the monomer protein of claim 1
and/or the bifunctional dimer protein of claim 4, and a pharmaceutically acceptable
carrier.
11. A use of the monomer protein according to claim 1 and/or the bifunctional
dimer protein according to claim 4 in the preparation of the pharmaceutical
composition for treating diseases.
Fig. 1

Fig. 2

Fig. 3
**Fig. 4**

**A**

TGF-β1-binding activity

**B**

Con. of soluble protein

**Fig. 5**

B
Fig. 6

Fig. 7
Fig. 10
**INTERNATIONAL SEARCH REPORT**

**A. CLASSIFICATION OF SUBJECT MATTER**

See extra sheet
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC: C07K; C12N; A61K; A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database consulted during the international search (name of data base and, where practicable, search terms used)

WPI; EPODOC; CNKI; CPRS; ISI Web of knowledge; ELSEVIER and keywords : BIFUNCTION; FUSION PROTEIN; TGF; TRANSFORMING GROWTH FACTOR; CLEC; C TYPE LECTIN LIKE RECEPTOR; FC; RECEPTOR; SIRP; RANK; VEGF; CTLA4 etc.

GenBank; EMBL; Retrieving System for Biological Sequence of Chinese Patent and searched sequences: SEQ ID NOs: 7 and 8

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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<td>CN1490335 A (ZHANG, Q. Y.) 21 Apr. 2004 (21.04.2004) See page 9, paragraph 4 - page 16, paragraph 1 of the description and claims</td>
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<td>CN162141 A (INST BASIC MEDICAL SCI CHINESE ACAD MED) 01 Jun. 2005 (01.06.2005) See page 3, line 5 - page 4, line 28 of the description and claims</td>
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* Further documents are listed in the continuation of Box C.

\[T\] later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\[X\] document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\[Y\] document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

\[O\] document of the same patent family

Date of the actual completion of the international search 22 Mar. 2012 (22.03.2012)

Date of mailing of the international search report 05 Apr. 2012 (05.04.2012)

Name and mailing address of the ISA/CN
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Facsimile No. 86-10-62019451

Authorized officer MA, Lan
Telephone No. (86-10) 624 12 18 1

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**DOCUMENTS CONSIDERED TO BE RELEVANT**

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<td>CN1465597 A (LANSHENG SHANGKE CHUANGYE INVESTMENT CO) 07 Jan. 2004 (07.01.2004)</td>
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Form PCT/ISA/210 (continuation of second sheet) (July 2009)
## INTERNATIONAL SEARCH REPORT

**Box No. 1** Nucleotide and/or amino acid sequence(s) (Continuation of item item.l.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of:
   
   a. a sequence listing filed or furnished
      
      - [ ] on paper
      - [x] in electronic form

   b. time of filing or furnishing
      
      - [ ] contained in the application as filed
      - [x] filed together with the application in electronic form
      - [ ] furnished subsequently to this Authority for the purposes of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

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