DESCRIPTION

WT1-ANTIGEN PEPTIDE CONJUGATE VACCINE

TECHNICAL FIELD

[0001]

The present invention belongs to the field of cancer immunotherapy, and relates to a conjugate vaccine that can be subjected to trimming by peptidase ERAP1, is obtained by conjugating peptide precursors derived from WT1 antigen protein via a sulfur-sulfur covalent bond, and efficiently induces cytotoxic T cells.

BACKGROUND ART

[0002]

For eradication of cancer cells in the body, cellular immunity, particularly cytotoxic T cell (cytotoxic T-lymphocyte, Cytotoxic T-cell, hereinafter to be referred to as CTL) mainly plays an important role. CTL is produced by differentiation and proliferation of a precursor T cell that recognized a complex formed by an antigen peptide derived from a cancer antigen protein (cancer antigen peptide) and an MHC class I molecule, and attacks cancer cells.

[0003]

A cancer suppressor gene of Wilms tumor, WT1 (WT1 gene) is considered a new cancer antigen protein for leukemia and solid tumor (see non-patent document 1).

[0004]

As for WT1 protein, for example, the following cancer antigen peptides that are bound to and presented by MHC class I have been reported (see patent documents 1, 2).

$WT1_{126-134}$ peptide: RMFPNAPYL (Arg-Met-Phe-Pro-Asn-Ala-Pro-Tyr-Leu) (SEQ ID NO: 2),

$WT1_{235-243}$ peptide: CMTWNQMNL (Cys-Met-Thr-Trp-Asn-Gln-Met-Asn-Leu)

(SEQ ID NO: 3), 7

$WT1_{10-18}$ peptide: ALLPAVPSL (Ala-Leu-Leu-Pro-Ala-Val-Pro-Ser-Leu) (SEQ ID NO: 5),

$WT1_{187-195}$ peptide: SLGEQQYSV (Ser-Leu-Gly-Glu-Gln-Gln-Tyr-Ser-Val) (SEQ ID NO: 6),

$WT1_{302-310}$ peptide: RVPGVAPTL (Arg-Val-Pro-Gly-Val-Ala-Pro-Thr-Leu) (SEQ ID NO: 7) and the like.

[0005]

In cancer immunotherapy, activation of helper T cell is also important for activating other T cells including CTL. In general, an antigen protein is degraded by intracellular lysosome, a part of peptide fragments constituted by a peptide consisting of about 13 - 17 amino acid residues binds as an antigen peptide to MHC class II molecule and is presented to helper T cell-TCR·CD3 complex to activate helper T cell. As for WT1 protein, for example, the following cancer antigen peptides that are presented by binding to MHC class II have been reported (see patent documents 3 - 5).

$WT1_{332-347}$ peptide: KRYFKLSHLQMHSRKH (Lys-Arg-Tyr-Phe-Lys-Leu-Ser-His-Leu-Gln-Met-His-Ser-Arg-Lys-His) (SEQ ID NO: 8),

$WT1_{328-349}$ peptide: PGCNKRYFKLSHLQMHSRKHTG (Pro-Gly-Cys-Asn-Lys-Arg-Tyr-Phe-Lys-Leu-Ser-His-Leu-Gln-Met-His-Ser-Arg-Lys-His-Thr-Gly) (SEQ ID NO: 10),

$WT1_{122-140}$ peptide: SGQARMFPNAPYLPSCLES (Ser-Gly-Gln-Ala-Arg-Met-Phe-Pro-Asn-Ala-Pro-Tyr-Leu-Pro-Ser-Cys-Leu-Glu-Ser) (SEQ ID NO: 11) and the like.

[0006]

As a vaccine antigen of WT1, an antigen protein itself or the aforementioned antigen protein-derived antigen peptide is mainly used (see non-patent document 2). Since a cancer vaccine using a protein generally contains various cancer antigen peptides, it can simultaneously induce a plurality of CTLs and helper T cells. On the other hand, since the cancer protein vaccine posesses problems in stable supply and quality control,

peptides that facilitate production and quality control are widely used as cancer antigen of WT1. Generally, however, since conventional peptide vaccines are mainly constituted by a single MHC class I-presented peptide antigen, it has been pointed out in recent years that efficient induction of CTL requires further improvement (see non-patent document 3).

[0007]

One of the solving means is a multivalent antigen peptide presenting WT1 peptide cancer vaccine. As such peptide cancer vaccine, a cocktail vaccine containing a mixture of a plurality of peptide antigens presented by MHC class I and class II (see non-patent document 4), a long chain peptide vaccine containing peptide antigens presented by MHC class I and class II, which are bound by an amide bond, and the like have been reported (see non-patent document 5). In the case of a cocktail vaccine, however, since each peptide antigen composed of various amino acids shows various properties, the development of an optimal formulation capable of efficiently inducing CTL corresponding thereto is often problematic. In the case of a long chain peptide vaccine, the production thereof sometimes poses problems, like protein. Furthermore, since peptide antigens presented by class I and class II are bonded via any peptide spacer in a long chain peptide vaccine, control and prediction of the cleavage sites by intracellular enzyme are difficult. In the meantime, a peptide dimer wherein two peptide monomers are mutually bonded by a disulfide bond has been reported (see patent document 6). Different from cocktail vaccine, two single peptides are bonded, and therefore, they have single physical property and can be produced conveniently. On the other hand, to form a conjugate, WT1 cancer antigen peptides are required to contain cysteine in the amino acid sequence thereof, and therefore, applicable ones are liminted. Furthermore, an altered compound obtained by condensing the N-terminal cysteine of cancer antigen peptide with cysteine, glutathione or thioglycolic acid by a disulfide

bond has also been reported (see patent document 7).

[0008]

The process of cancer antigen peptide presentation on MHC class I involves a plurality of peptidases. Of such peptidases, Endoplasmic reticulum aminopeptidase 1 (hereinafter to be referred to as ERAP1) is one of the trimming enzymes in the endoplasmic reticulum (hereinafter to be referred to as ER), and has been reported to recognize a particular antigen peptide sequence and the peptide length, and cleaves the cancer antigen peptide precursor from the N-terminal to control the length to be optimal for binding to MHC class I (see non-patent documents 6-8). However, there is no report to date on a WT1 peptide cancer antigen precursor containing cysteine, that is controlled in its length from the N-terminal, by the trimming function of ERAP1.

[DOCUMENT LIST]

[PATENT DOCUMENTS]

[0009]

Patent Document 1: WO 00/06602

Patent Document 2: WO 00/18795

Patent Document 3: WO 2005/045027

Patent Document 4: WO 2007/047764

Patent Document 5: WO 2007/120673

Patent Document 6: WO 2004/063217

Patent Document 7: WO 2007/063903

[NON-PATENT DOCUMENTS]

[0010]

Non-Patent Document 1: The Journal of Immunology, 2000; 164(4); 1873-1880

Non-Patent Document 2: The Oncologist, 2012; 17(2); 250-259

Non-Patent Document 3: Cancer Journal, 2011; 17(5); 343-350

Non-Patent Document 4: Blood, 2010; 166(2); 171-179

Non-Patent Document 5: Cancers, 2011; 3; 3991-4009

Non-Patent Document 6: Proceedings of the National Academy of

Sciences of United States of America, 2005; 102(47); 17107-17112

Non-Patent Document 7: The Journal of Immunology, 2009; 183; 5526-5536

Non-Patent Document 8: The Journal of Immunology, 2010; 184; 4725-4732

SUMMARY OF THE INVENTION

PROBLEMS TO BE SOLVED BY THE INVENTION

[0011]

The problem to be solved by the present invention is to provide a WT1 conjugate vaccine that induces CTL efficiently.

MEANS OF SOLVING THE PROBLEMS

[0012]

The present inventors have conducted intensive studies in an attempt to solve the aforementioned problem, and conceived, when considering adopting conjugate vaccine, an idea of adding cysteine in WT1 cancer antigen peptide, and further confirmed that the results of pharmacological tests and the like using in vivo animal model strongly suggest that ERAP1 cleaves cysteine from the N-terminal of a WT1 cancer antigen peptide precursor that is generated by intracellular reductive cleavage of disulfide bond, to efficiently convert the same to a cancer antigen peptide, which in turn led to the finding of a polyvalent antigen peptide presenting conjugate vaccine capable of inducing CTL in the body, and the completion of the present invention.

To be specific, during the process of studying the solving means to the above-mentioned problem, they have obtained an idea of a method for introducing cysteine, which is necessary for forming a conjugate of two different WT1 cancer antigen peptides, into any position of the N-terminal or C-terminal, without influencing the antigen presentation by MHC class I. As a result of further study, they have created a peptide by

introducing 0 - 5 amino acids containing cysteine into the N terminal of a WT1 cancer antigen peptide, and a conjugate of the peptides containing a disulfide bond via cysteine. Furthermore, the present inventors have confirmed for the first time that said peptide and the conjugate are susceptible to trimming by ERAP1 in vitro and/or in vivo, which in turn results in the formation of a cancer antigen peptide, and thereby, completed the present invention.

While the development of a novel multivalent WT1 antigen peptide presenting peptide cancer vaccine, which can be produced easily, is applicable to various ones, and induces CTL with high efficiency, has been desired, the conjugate invented by the present inventors has enabled the development of a WT1 conjugate vaccine that induces CTL efficiently, is superior in physicochemical properties, can be produced easily, facilitates production management, and is applicable to various ones.

[0013]

Accordingly, the present invention relates to the following.

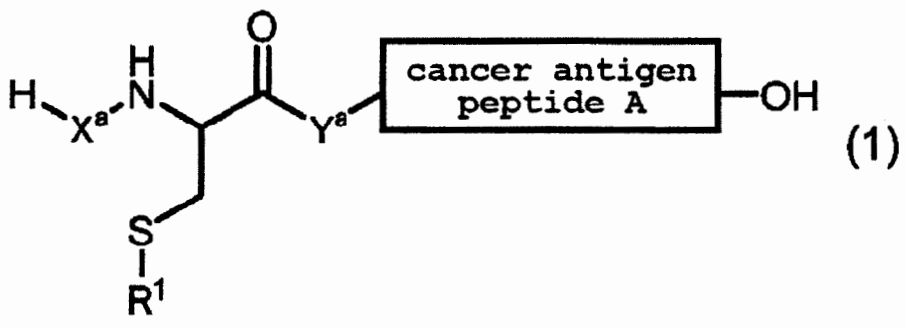
[0014]

1. First embodiment

[0015]

1. A compound represented by the formula (1):

[0016]



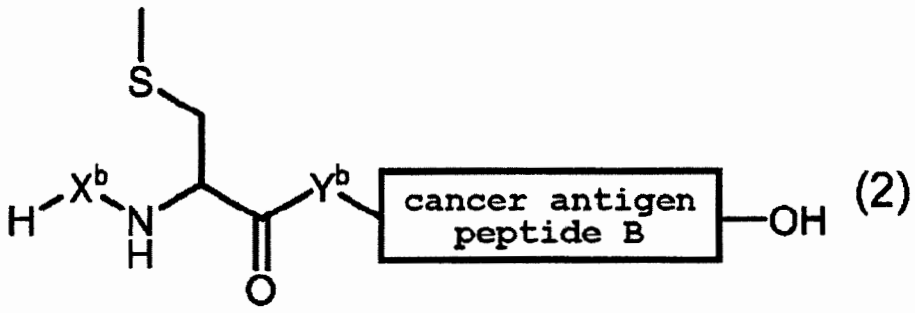
(1)

[0017]

wherein X^a and Y^a are each independently a single bond or a divalent peptide group consisting of 1 - 4 amino acid residues,

and a total of the amino acid residue number for X^a and the amino acid residue number for Y^a is an integer of 0 - 4,
cancer antigen peptide A is an MHC class I-restricted WT1 peptide consisting of 7 - 30 amino acid residues, an amino group of an N-terminal amino acid of the cancer antigen peptide A binds to Y^a in the formula (1), and a carbonyl group of a C-terminal amino acid of the cancer antigen peptide A binds to a hydroxyl group in the formula (1),
R^1 is a hydrogen atom, a group represented by the formula (2):

[0018]



[0019]

wherein X^b and Y^b are each independently a single bond or a divalent peptide group consisting of 1 - 4 amino acid residues, and a total of the amino acid residue number for X^b and the amino acid residue number for Y^b is an integer of 0 - 4,
cancer antigen peptide B has a sequence different from that of the cancer antigen peptide A, and is an MHC class I-restricted WT1 peptide consisting of 7 - 30 amino acid residues, an amino group of an N-terminal amino acid of the cancer antigen peptide B binds to Y^b in the formula (2), and a carbonyl group of a C-terminal amino acid of the cancer antigen peptide B binds to a hydroxyl group in the formula (2), and
a thioether group in the formula (2) binds to a thioether group in the formula (1),
or cancer antigen peptide C,
wherein the cancer antigen peptide C has a sequence different from that of the cancer antigen peptide A, and is an MHC class

I-restricted WT1 peptide consisting of 7 - 30 amino acid residues containing one cysteine residue or an MHC class II-restricted WT1 peptide consisting of 7 - 30 amino acid residues containing one cysteine residue, and a thioether group of the cysteine residue of the cancer antigen peptide C binds to a thioether group in the formula (1),
provided when R^1 is a hydrogen atom, the sequence of a compound represented by the formula (1) is not the same as the partial sequence of a WT1 protein,
or a pharmaceutically acceptable salt thereof;

[0020]

2. the compound according to 1, wherein X^a is a divalent peptide group consisting of 2 amino acid residues and Y^a is a single bond, or X^a and Y^a are each independently a divalent peptide group consisting of 1 amino acid residue, or X^a is a single bond and Y^a is a divalent peptide group consisting of 2 amino acid residues, or X^a is a divalent peptide group consisting of 1 amino acid residue and Y^a is a single bond, or X^a is a single bond and Y^a is a divalent peptide group consisting of 1 amino acid residue, or X^a and Y^a are each a single bond, or a pharmaceutically acceptable salt thereof;

[0021]

3. the compound according to 1 or 2, wherein X^a is a single bond, and Y^a is a single bond, an alanine residue, a leucine residue or a methionine residue, or a pharmaceutically acceptable salt thereof;

[0022]

4. the compound according to 1 or 2, wherein X^a is a single bond or a divalent peptide group consisting of 1 amino acid residue, and Y^a is a single bond, or a pharmaceutically acceptable salt thereof;

[0023]

5. the compound according to any one of 1 - 4, wherein X^a and Y^a are each a single bond, or a pharmaceutically acceptable salt

thereof;

[0024]

6. the compound according to any one of 1 - 5, wherein the cancer antigen peptide A is an MHC class I-restricted WT1 peptide consisting of 7 - 15 amino acid residues, or a pharmaceutically acceptable salt thereof;

[0025]

7. the compound according to any one of 1 - 6, wherein the cancer antigen peptide A is a peptide comprising any amino acid sequence selected from the following amino acid sequences:

RMFPNAPYL (SEQ ID NO: 2),

CMTWNQMNL (SEQ ID NO: 3),

ALLPAVPSL (SEQ ID NO: 5),

SLGEQQYSV (SEQ ID NO: 6) and

RVPGVAPTL (SEQ ID NO: 7), or

a peptide comprising an altered amino acid sequence, which is any amino acid sequence selected from SEQ ID NOs: 2, 3, 5, 6 and 7 but containing alteration of amino acid residue(s), and having a CTL induction activity, or a pharmaceutically acceptable salt thereof;

[0026]

8. the compound according to any one of 1 - 7, wherein the cancer antigen peptide A is a peptide consisting of any amino acid sequence selected from the following amino acid sequences:

RMFPNAPYL (SEQ ID NO: 2),

CMTWNQMNL (SEQ ID NO: 3),

CYTWNQMNL (SEQ ID NO: 4),

ALLPAVPSL (SEQ ID NO: 5),

SLGEQQYSV (SEQ ID NO: 6) and

RVPGVAPTL (SEQ ID NO: 7),

or a pharmaceutically acceptable salt thereof;

[0027]

9. the compound according to any one of 1 - 8, wherein R^1 is a hydrogen atom, or a pharmaceutically acceptable salt thereof;

[0028]

10. the compound according to any one of 1 - 9, wherein the compound represented by the formula (1) is a peptide consisting of any amino acid sequence selected from the following amino acid sequences:

CRMFPNAPYL (SEQ ID NO: 13),

CCMTWNQMNL (SEQ ID NO: 14),

CCYTWNQMNL (SEQ ID NO: 15),

CALLPAVPSL (SEQ ID NO: 16),

CSLGEQQYSV (SEQ ID NO: 17) and

CRVPGVAPTL (SEQ ID NO: 18),

or a pharmaceutically acceptable salt thereof;

[0029]

11. the compound according to any one of 1 - 8, wherein R^1 is a group represented by the formula (2), or a pharmaceutically acceptable salt thereof;

[0030]

12. the compound according to any one of 1 - 8 and 11, wherein X^b is a divalent peptide group consisting of 2 amino acid residues and Y^b is a single bond, or X^b and Y^b are each independently a divalent peptide group consisting of 1 amino acid residue, or X^b is a single bond and Y^b is a divalent peptide group consisting of 2 amino acid residues, or X^b is a divalent peptide group consisting of 1 amino acid residue and Y^b is a single bond, or X^b is a single bond and Y^b is a divalent peptide group consisting of 1 amino acid residue, or X^b and Y^b are each a single bond, or a pharmaceutically acceptable salt thereof;

[0031]

13. the compound according to any one of 1 - 8 and 11 - 12, wherein X^b is a single bond, and Y^b is a single bond, an alanine residue, a leucine residue or a methionine residue, or a pharmaceutically acceptable salt thereof;

[0032]

14. the compound according to any one of 1 - 8 and 11 - 12,

wherein X^b is a single bond or a divalent peptide group consisting of 1 amino acid residue, and Y^b is a single bond, or a pharmaceutically acceptable salt thereof;

[0033]

15. the compound according to any one of 1 - 8 and 11 - 14, wherein X^b and Y^b are each a single bond, or a pharmaceutically acceptable salt thereof;

[0034]

16. the compound according to any one of 1 - 8 and 11 - 15, wherein the cancer antigen peptide B is an MHC class I-restricted WT1 peptide consisting of 7 - 15 amino acid residues, or a pharmaceutically acceptable salt thereof;

[0035]

17. the compound according to any one of 1 - 8 and 11 - 16, wherein the cancer antigen peptide B is a peptide comprising any amino acid sequence selected from the following amino acid sequences:

RMFPNAPYL (SEQ ID NO: 2),

CMTWNQMNL (SEQ ID NO: 3),

ALLPAVPSL (SEQ ID NO: 5),

SLGEQQYSV (SEQ ID NO: 6) and

RVPGVAPTL (SEQ ID NO: 7), or

a peptide comprising an altered amino acid sequence, which is any amino acid sequence selected from SEQ ID NOs: 2, 3, 5, 6 and 7 but containing alteration of amino acid residue(s), and having a CTL induction activity, or a pharmaceutically acceptable salt thereof;

[0036]

18. the compound according to any one of 1 - 8 and 11 - 17, wherein the cancer antigen peptide B is a peptide consisting of any amino acid sequence selected from the following amino acid sequences:

RMFPNAPYL (SEQ ID NO: 2),

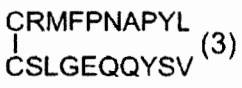
CMTWNQMNL (SEQ ID NO: 3),

CYTWNQMNL (SEQ ID NO: 4),
ALLPAVPSL (SEQ ID NO: 5),
SLGEQQYSV (SEQ ID NO: 6) and
RVPGVAPTL (SEQ ID NO: 7),
or a pharmaceutically acceptable salt thereof;

[0037]

19. the compound according to any one of 1 - 8 and 11 - 18, wherein the compound represented by the formula (1) is a compound represented by the formula (3):

[0038]



(3)

[0039]

wherein the bond between C and C is a disulfide bond, or a pharmaceutically acceptable salt thereof;

[0040]

20. the compound according to any one of 1 - 8, wherein R^1 is cancer antigen peptide C, or a pharmaceutically acceptable salt thereof;

[0041]

21. the compound according to any one of 1 - 8 and 20, wherein the cancer antigen peptide C is an MHC class I-restricted WT1 peptide consisting of 7 - 15 amino acid residues, or a pharmaceutically acceptable salt thereof;

[0042]

22. the compound according to any one of 1 - 8 and 20 - 21, wherein the cancer antigen peptide C is a peptide comprising the following amino acid sequence:
CMTWNQMNL (SEQ ID NO: 3), or
a peptide comprising an altered amino acid sequence, which is the amino acid sequence of SEQ ID NO: 3 but containing alteration of amino acid residue(s), and having a CTL induction activity, or a pharmaceutically acceptable salt thereof;

[0043]

23. the compound according to any one of 1 - 8 and 20 - 22, wherein the cancer antigen peptide C is a peptide consisting of any amino acid sequence selected from the following amino acid sequences:

CMTWNQMNL (SEQ ID NO: 3) and

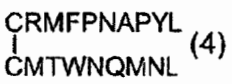
CYTWNQMNL (SEQ ID NO: 4),

or a pharmaceutically acceptable salt thereof;

[0044]

24. the compound according to any one of 1 - 8 and 20 - 23, wherein the compound represented by the formula (1) is a compound represented by the formula (4):

[0045]



(4)

[0046]

wherein the bond between C and C is a disulfide bond, or a compound represented by the formula (5):

[0047]



(5)

[0048]

wherein the bond between C and C is a disulfide bond, or a pharmaceutically acceptable salt thereof;

[0049]

25. the compound according to any one of 1 - 8 and 20, wherein the cancer antigen peptide C is an MHC class II-restricted WT1 peptide consisting of 14 - 30 amino acid residues, or a pharmaceutically acceptable salt thereof;

[0050]

26. the compound according to any one of 1 - 8, 20 and 25, wherein the cancer antigen peptide C is a peptide comprising any amino acid sequence selected from the following amino acid sequences:

SGQARMFPNAPYLPSC (SEQ ID NO: 19),

SGQARMFPNAPYLPSCLES (SEQ ID NO: 11),
PGCNKRYFKLSHLQMHSRK (SEQ ID NO: 20),
PGCNKRYFKLSHLQMHSRKH (SEQ ID NO: 21),
PGCNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 10),
CNKRYFKLSHLQMHSRK (SEQ ID NO: 22),
CNKRYFKLSHLQMHSRKH (SEQ ID NO: 23) and
CNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 24), or
a peptide comprising an altered amino acid sequence, which is any amino acid sequence selected from SEQ ID NOs: 10 - 11 and 19 - 24 but containing alteration of amino acid residue(s), and having a helper T cell induction activity, or a pharmaceutically acceptable salt thereof;

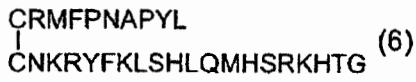
[0051]

27. the compound according to any one of 1 - 8, 20 and 25 - 26, wherein the cancer antigen peptide C is a peptide consisting of an amino acid sequence selected from the following amino acid sequences:
SGQARMFPNAPYLPSC (SEQ ID NO: 19),
SGQAYMFPNAPYLPSC (SEQ ID NO: 25),
SGQARMFPNAPYLPSCLES (SEQ ID NO: 11),
SGQAYMFPNAPYLPSCLES (SEQ ID NO: 12),
PGCNKRYFKLSHLQMHSRK (SEQ ID NO: 20),
PGCNKRYFKLSHLQMHSRKH (SEQ ID NO: 21),
PGCNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 10),
CNKRYFKLSHLQMHSRK (SEQ ID NO: 22),
CNKRYFKLSHLQMHSRKH (SEQ ID NO: 23) and
CNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 24),
or a pharmaceutically acceptable salt thereof;

[0052]

28. the compound according to any one of 1 - 8, 20 and 25 - 27, wherein the compound represented by the formula (1) is a compound represented by the formula (6):

[0053]



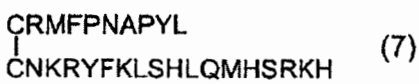
(6)

[0054]

wherein the bond between C and C is a disulfide bond,

a compound represented by the formula (7):

[0055]



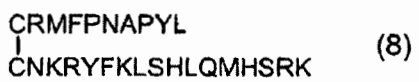
(7)

[0056]

wherein the bond between C and C is a disulfide bond,

a compound represented by the formula (8):

[0057]



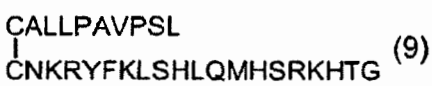
(8)

[0058]

wherein the bond between C and C is a disulfide bond, or

a compound represented by the formula (9):

[0059]



(9)

[0060]

wherein the bond between C and C is a disulfide bond,

or a pharmaceutically acceptable salt thereof;

[0061]

29. a pharmaceutical composition comprising the compound according to any one of 1 - 28, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier;

[0062]

30. the pharmaceutical composition according to 29, which is used as a cancer vaccine;

[0063]

31. use of the compound according to any one of 1 - 28 or a pharmaceutically acceptable salt thereof for the production of a cancer vaccine;

[0064]

32. a method of treating or preventing cancer, comprising administering a therapeutically or prophylactically effective amount of the compound according to any one of 1 - 28 or a pharmaceutically acceptable salt thereof to a WT1 positive cancer patient in need thereof; and

[0065]

33. a method of obtaining two different MHC class I-restricted epitopes, or an MHC class I-restricted epitope and an MHC class II-restricted epitope, comprising reacting the compound according to any one of 1 - 28 or a pharmaceutically acceptable salt thereof with ERAP1.

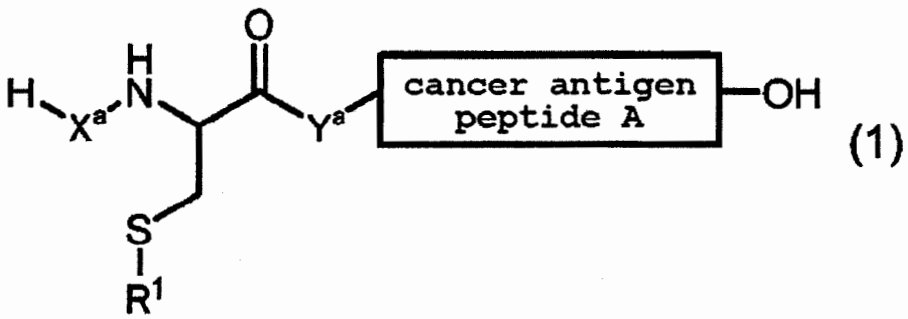
[0066]

2. Second embodiment

[0067]

1. A compound represented by the formula (1):

[0068]



(1)

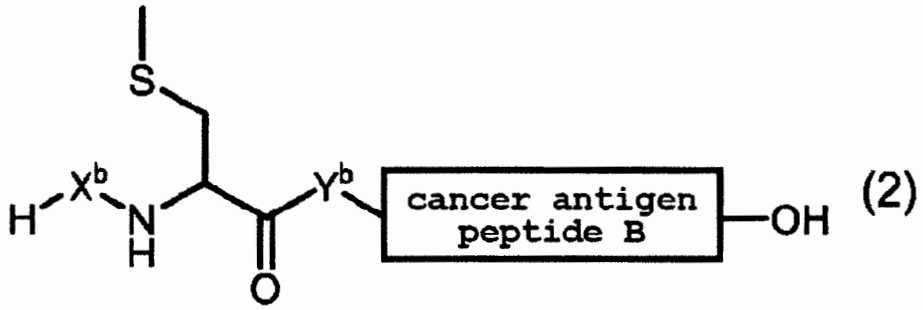
[0069]

wherein X^a and Y^a are each independently a single bond or a divalent peptide group consisting of 1 - 4 amino acid residues, and a total of the amino acid residue number for X^a and the amino acid residue number for Y^a is an integer of 0 - 4, cancer antigen peptide A is an MHC class I-restricted WT1 peptide consisting of 7 - 30 amino acid residues, an amino group of an N-terminal amino acid of the cancer antigen peptide A binds to Y^a in the formula (1), and a carbonyl group of a C-terminal amino acid of the cancer antigen peptide A binds to a

hydroxyl group in the formula (1),

R^1 is a hydrogen atom, a group represented by the formula (2):

[0070]



[0071]

wherein X^b and Y^b are each independently a single bond or a divalent peptide group consisting of 1 - 4 amino acid residues, and a total of the amino acid residue number for X^b and the amino acid residue number for Y^b is an integer of 0 - 4,

cancer antigen peptide B has a sequence different from that of the cancer antigen peptide A in the sequence and is an MHC class I-restricted WT1 peptide consisting of 7 - 30 amino acid residues, an amino group of an N-terminal amino acid of the cancer antigen peptide B binds to Y^b in the formula (2), and a carbonyl group of a C-terminal amino acid of the cancer antigen peptide B binds to a hydroxyl group in the formula (2), and a thioether group in the formula (2) binds to a thioether group in the formula (1),

or cancer antigen peptide C,

wherein the cancer antigen peptide C is an MHC class I-restricted WT1 peptide different from the cancer antigen peptide A in the sequence and consisting of 7 - 30 amino acid residues containing one cysteine residue or an MHC class II-restricted WT1 peptide consisting of 7 - 30 amino acid residues containing one cysteine residue, and a thioether group of the cysteine residue of the cancer antigen peptide C binds to a thioether group in the formula (1),

provided when R^1 is a hydrogen atom, the sequence of the

compound represented by the formula (1) is not the same as the partial sequence of a WT1 protein,
or a pharmaceutically acceptable salt thereof;

[0072]

2. the compound according to 1, wherein X^a is a divalent peptide group consisting of 2 amino acid residues and Y^a is a single bond, or X^a and Y^a are each independently a divalent peptide group consisting of 1 amino acid residue, or X^a is a single bond and Y^a is a divalent peptide group consisting of 2 amino acid residues, or X^a is a divalent peptide group consisting of 1 amino acid residue and Y^a is a single bond, or X^a is a single bond and Y^a is a divalent peptide group consisting of 1 amino acid residue, or X^a and Y^a are each a single bond, or a pharmaceutically acceptable salt thereof;

[0073]

3. the compound according to 1 or 2, wherein X^a is a single bond, and Y^a is a single bond, an alanine residue, a leucine residue or a methionine residue, or a pharmaceutically acceptable salt thereof;

[0074]

4. the compound according to 1 or 2, wherein X^a is a single bond or a divalent peptide group consisting of 1 amino acid residue, and Y^a is a single bond, or a pharmaceutically acceptable salt thereof;

[0075]

5. the compound according to any one of 1 - 4, wherein X^a and Y^a are each a single bond, or a pharmaceutically acceptable salt thereof;

[0076]

6. the compound according to any one of 1 - 5, wherein the cancer antigen peptide A is an MHC class I-restricted WT1 peptide consisting of 7 - 15 amino acid residues, or a pharmaceutically acceptable salt thereof;

[0077]

7. the compound according to any one of 1 - 6, wherein the cancer antigen peptide A is a peptide comprising any amino acid sequence selected from the following amino acid sequences:
RMFPNAPYL (SEQ ID NO: 2),
CMTWNQMNL (SEQ ID NO: 3),
ALLPAVPSL (SEQ ID NO: 5),
SLGEQQYSV (SEQ ID NO: 6) and
RVPGVAPTL (SEQ ID NO: 7), or
a peptide comprising an altered amino acid sequence, which is any amino acid sequence selected from SEQ ID NOs: 2, 3, 5, 6 and 7 but containing alteration of amino acid residue(s), and having a CTL induction activity, or a pharmaceutically acceptable salt thereof;

[0078]

8. the compound according to any one of 1 - 7, wherein the cancer antigen peptide A is a peptide consisting of any amino acid sequence selected from the following amino acid sequences:
RMFPNAPYL (SEQ ID NO: 2),
CMTWNQMNL (SEQ ID NO: 3),
CYTWNQMNL (SEQ ID NO: 4),
ALLPAVPSL (SEQ ID NO: 5),
SLGEQQYSV (SEQ ID NO: 6) and
RVPGVAPTL (SEQ ID NO: 7),
or a pharmaceutically acceptable salt thereof;

[0079]

9. the compound according to any one of 1 - 8, wherein R^1 is a hydrogen atom, or a pharmaceutically acceptable salt thereof;

[0080]

10. the compound according to any one of 1 - 9, wherein the compound represented by the formula (1) is a peptide consisting of any amino acid sequence selected from the following amino acid sequences:
CRMFPNAPYL (SEQ ID NO: 13),
CCMTWNQMNL (SEQ ID NO: 14),

CCYTWNQMNL (SEQ ID NO: 15),
CALLPAVPSL (SEQ ID NO: 16),
CSLGEQQYSV (SEQ ID NO: 17) and
CRVPGVAPTL (SEQ ID NO: 18),
or a pharmaceutically acceptable salt thereof;

[0081]

11. the compound according to any one of 1 - 8, wherein R^1 is a group represented by the formula (2), or a pharmaceutically acceptable salt thereof;

[0082]

12. the compound according to any one of 1 - 8 and 11, wherein X^b is a divalent peptide group consisting of 2 amino acid residues and Y^b is a single bond, or X^b and Y^b are each independently a divalent peptide group consisting of 1 amino acid residue, or X^b is a single bond and Y^b is a divalent peptide group consisting of 2 amino acid residues, or X^b is a divalent peptide group consisting of 1 amino acid residue and Y^b is a single bond, or X^b is a single bond and Y^b is a divalent peptide group consisting of 1 amino acid residue, or X^b and Y^b are each a single bond, or a pharmaceutically acceptable salt thereof;

[0083]

13. the compound according to any one of 1 - 8 and 11 - 12, wherein X^b is a single bond, and Y^b is a single bond, an alanine residue, a leucine residue or a methionine residue, or a pharmaceutically acceptable salt thereof;

[0084]

14. the compound according to any one of 1 - 8 and 11 - 12, wherein X^b is a single bond or a divalent peptide group consisting of 1 amino acid residue, and Y^b is a single bond, or a pharmaceutically acceptable salt thereof;

[0085]

15. the compound according to any one of 1 - 8 and 11 - 14, wherein X^b and Y^b are each a single bond, or a pharmaceutically acceptable salt thereof;

[0086]

16. the compound according to any one of 1 - 8 and 11 - 15, wherein the cancer antigen peptide B is an MHC class I-restricted WT1 peptide consisting of 7 - 15 amino acid residues, or a pharmaceutically acceptable salt thereof;

[0087]

17. the compound according to any one of 1 - 8 and 11 - 16, wherein the cancer antigen peptide B is a peptide comprising any amino acid sequence selected from the following amino acid sequences:

RMFPNAPYL (SEQ ID NO: 2),

CMTWNQMNL (SEQ ID NO: 3),

ALLPAVPSL (SEQ ID NO: 5),

SLGEQQYSV (SEQ ID NO: 6) and

RVPGVAPTL (SEQ ID NO: 7), or

a peptide comprising an altered amino acid sequence, which is any amino acid sequence selected from SEQ ID NOs: 2, 3, 5, 6 and 7 but containing alteration of amino acid residue(s), and having a CTL induction activity, or a pharmaceutically acceptable salt thereof;

[0088]

18. the compound according to any one of 1 - 8 and 11 - 17, wherein the cancer antigen peptide B is a peptide consisting of any amino acid sequence selected from the following amino acid sequences:

RMFPNAPYL (SEQ ID NO: 2),

CMTWNQMNL (SEQ ID NO: 3),

CYTWNQMNL (SEQ ID NO: 4),

ALLPAVPSL (SEQ ID NO: 5),

SLGEQQYSV (SEQ ID NO: 6) and

RVPGVAPTL (SEQ ID NO: 7),

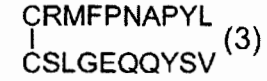
or a pharmaceutically acceptable salt thereof;

[0089]

19. the compound according to any one of 1 - 8 and 11 - 18,

wherein the compound represented by the formula (1) is a compound represented by the formula (3):

[0090]



(3)

[0091]

wherein the bond between C and C is a disulfide bond, or a pharmaceutically acceptable salt thereof;

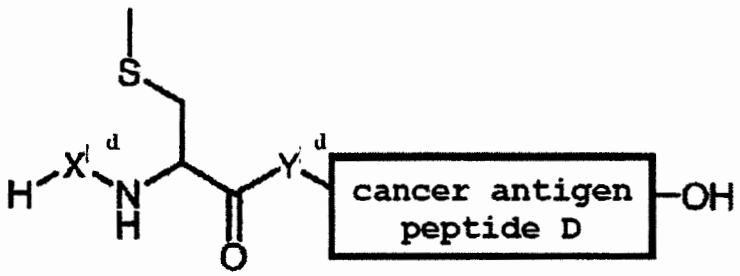
[0092]

20. the compound according to 13, wherein Y^b is an alanine residue, or a pharmaceutically acceptable salt thereof;

[0093]

21. the compound according to any one of 1 - 8 and 11 - 13 and 20, wherein, when the cancer antigen peptide B is an MHC class I-restricted WT1 peptide containing one cysteine residue, the thioether group in the cancer antigen peptide B is bonded to the thioether group in the formula (16):

[0094]



(16)

[0095]

wherein X^d and Y^d are each independently a single bond or a divalent peptide group consisting of 1 - 4 amino acid residues, and a total of the amino acid residue number for X^d and the amino acid residue number for Y^d is an integer of 0 - 4, cancer antigen peptide D is an MHC class II-restricted WT1 peptide consisting of 7 - 30 amino acid residues, an amino group of an N-terminal amino acid of the cancer antigen peptide D binds to Y^d in the formula (16), and a carbonyl group of a C-terminal amino acid of the cancer antigen peptide D binds to a

hydroxyl group in the formula (16), or to the thioether group of the cysteine residue of the cancer antigen peptide E, which is an MHC class II-restricted WT1 peptide consisting of 7 - 30 amino acid residues containing one cysteine residue, or a pharmaceutically acceptable salt thereof;

[0096]

22. the compound according to 21, wherein the cancer antigen peptide B is an MHC class I-restricted WT1 peptide consisting of 7 - 15 amino acid residues, or a pharmaceutically acceptable salt thereof;

[0097]

23. the compound according to any one of 21 - 22, wherein the cancer antigen peptide B is a peptide consisting of any amino acid sequence selected from the following amino acid sequences:
CMTWNQMNL (SEQ ID NO: 3) and
CYTWNQMNL (SEQ ID NO: 4),
or a pharmaceutically acceptable salt thereof;

[0098]

24. the compound according to any one of 21 - 23, wherein X^d is a divalent peptide group consisting of 2 amino acid residues and Y^d is a single bond, or X^d and Y^d are each independently a divalent peptide group consisting of 1 amino acid residue, or X^d is a single bond and Y^d is a divalent peptide group consisting of 2 amino acid residues, or X^d is a divalent peptide group consisting of 1 amino acid residue and Y^d is a single bond, or X^d is a single bond and Y^d is a divalent peptide group consisting of 1 amino acid residue, or X^d and Y^d are each a single bond, or a pharmaceutically acceptable salt thereof;

[0099]

25. the compound according to any one of 21 - 24, wherein X^d is a single bond, Y^d is a single bond, an alanine residue, a leucine residue or a methionine residue, or a pharmaceutically acceptable salt thereof;

[0100]

26. the compound according to any one of 21 - 24, wherein X^d is a single bond or a divalent peptide group consisting of one amino acid residue, and Y^d is a single bond, or a pharmaceutically acceptable salt thereof;

[0101]

27. the compound according to any one of 21 - 26, wherein X^d and Y^d are each a single bond, or a pharmaceutically acceptable salt thereof;

[0102]

28. the compound according to any one of 21 - 27, wherein the cancer antigen peptide D is an MHC class II-restricted WT1 peptide consisting of 14 - 30 amino acid residues, or a pharmaceutically acceptable salt thereof;

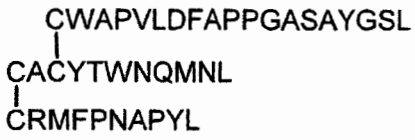
[0103]

29. the compound according to any one of 21 - 28, wherein the cancer antigen peptide D is a peptide consisting of any amino acid sequence selected from the following amino acid sequences:
SGQARMFPNAPYLPSC (SEQ ID NO: 19),
SGQAYMFPNAPYLPSC (SEQ ID NO: 25),
SGQARMFPNAPYLPSCLES (SEQ ID NO: 11),
SGQAYMFPNAPYLPSCLES (SEQ ID NO: 12),
PGCNKRYFKLSHLQMHSRK (SEQ ID NO: 20),
PGCNKRYFKLSHLQMHSRKH (SEQ ID NO: 21),
PGCNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 10),
CNKRYFKLSHLQMHSRK (SEQ ID NO: 22),
CNKRYFKLSHLQMHSRKH (SEQ ID NO: 23),
CNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 24) and
WAPVLDFAPPGASAYGSL (SEQ ID NO: 244), or a pharmaceutically acceptable salt thereof;

[0104]

30. the compound according to any one of 1 - 8, 11 - 13 and 20 - 29, wherein the compound represented by the formula (1) is a compound represented by the formula (15):

[0105]



(15)

[0106]

wherein the bond between C and C is a disulfide bond, or a pharmaceutically acceptable salt thereof;

[0107]

31. the compound according to any one of 21 - 23, wherein the cancer antigen peptide E is an MHC class II-restricted WT1 peptide consisting of 14 - 30 amino acid residues, or a pharmaceutically acceptable salt thereof;

[0108]

32. the compound according to any one of 21 - 23 and 31, wherein the cancer antigen peptide E is a peptide consisting of any amino acid sequence selected from the following amino acid sequences:

SGQARMFPNAPYLPSC (SEQ ID NO: 19),
SGQAYMFPNAPYLPSC (SEQ ID NO: 25),
SGQARMFPNAPYLPSCLES (SEQ ID NO: 11),
SGQAYMFPNAPYLPSCLES (SEQ ID NO: 12),
PGCNKRYFKLSHLQMHSRK (SEQ ID NO: 20),
PGCNKRYFKLSHLQMHSRKH (SEQ ID NO: 21),
PGCNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 10),
CNKRYFKLSHLQMHSRK (SEQ ID NO: 22),
CNKRYFKLSHLQMHSRKH (SEQ ID NO: 23) and
CNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 24), or a pharmaceutically acceptable salt thereof;

[0109]

33. the compound according to any one of 1 - 8, wherein R^1 is cancer antigen peptide C, or a pharmaceutically acceptable salt thereof;

[0110]

34. the compound according to any one of 1 - 8 and 33, wherein the cancer antigen peptide C is an MHC class I-restricted WT1

peptide consisting of 7 - 15 amino acid residues, or a pharmaceutically acceptable salt thereof;

[0111]

35. the compound according to any one of 1 - 8 and 33 - 34, wherein the cancer antigen peptide C is a peptide comprising the following amino acid sequence:

CMTWNQMNL (SEQ ID NO: 3), or

a peptide comprising an altered amino acid sequence, which is the amino acid sequence of SEQ ID NO: 3 but containing alteration of amino acid residue(s), and having a CTL induction activity, or a pharmaceutically acceptable salt thereof;

[0112]

36. the compound according to any one of 1 - 8 and 33 - 35, wherein the cancer antigen peptide C is a peptide consisting of any amino acid sequence selected from the following amino acid sequences:

CMTWNQMNL (SEQ ID NO: 3) and

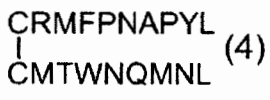
CYTWNQMNL (SEQ ID NO: 4),

or a pharmaceutically acceptable salt thereof;

[0113]

37. the compound according to any one of 1 - 8 and 33 - 36, wherein the compound represented by the formula (1) is a compound represented by the formula (4):

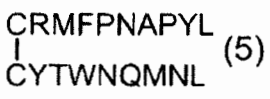
[0114]



[0115]

wherein the bond between C and C is a disulfide bond, or a compound represented by the formula (5):

[0116]



[0117]

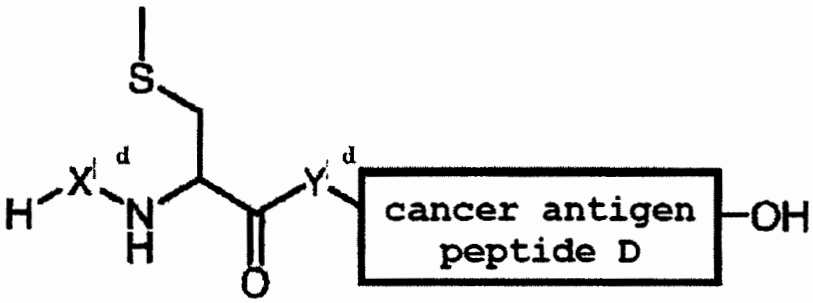
wherein the bond between C and C is a disulfide bond,

or a pharmaceutically acceptable salt thereof;

[0118]

38. the compound according to any one of 1 - 8 and 33, wherein, when the peptide consisting of 1 - 4 amino acid residues containing one cysteine residue is further bonded to the N-terminal of the cancer antigen peptide C, the thioether group of the cysteine residue of the peptide bonded to the N-terminal of the cancer antigen peptide C is bonded to the thioether group in the formula (16):

[0119]



(16)

[0120]

wherein X^d and Y^d are each independently a single bond or a divalent peptide group consisting of 1 - 4 amino acid residues, and a total of the amino acid residue number for X^d and the amino acid residue number for Y^d is an integer of 0 - 4, cancer antigen peptide D is an MHC class II-restricted WT1 peptide consisting of 7 - 30 amino acid residues, an amino group of an N-terminal amino acid of the cancer antigen peptide D binds to Y^d in the formula (16), and a carbonyl group of a C-terminal amino acid of the cancer antigen peptide D binds to a hydroxyl group in the formula (16), or to the thioether group of the cysteine residue of the cancer antigen peptide E, which is an MHC class II-restricted WT1 peptide consisting of 7 - 30 amino acid residues containing one cysteine residue, or a pharmaceutically acceptable salt thereof;

[0121]

39. the compound according to 38, wherein the peptide consisting

of 1 - 4 amino acid residues containing one cysteine residue bonded to the N-terminal of the cancer antigen peptide C is a dipeptide consisting of CA, or a pharmaceutically acceptable salt thereof;

[0122]

40. the compound according to any one of 38 - 39, wherein the cancer antigen peptide C is an MHC class I-restricted WT1 peptide consisting of 7 - 15 amino acid residues, or a pharmaceutically acceptable salt thereof;

[0123]

41. the compound according to any one of 38 - 40, wherein the cancer antigen peptide C is a peptide consisting of any amino acid sequence selected from the following amino acid sequences:
CMTWNQMNL (SEQ ID NO: 3) and
CYTWNQMNL (SEQ ID NO: 4),
or a pharmaceutically acceptable salt thereof;

[0124]

42. the compound according to any one of 38 - 41, wherein X^d is a divalent peptide group consisting of 2 amino acid residues and Y^d is a single bond, or X^d and Y^d are each independently a divalent peptide group consisting of 1 amino acid residue, or X^d is a single bond and Y^d is a divalent peptide group consisting of 2 amino acid residues, or X^d is a divalent peptide group consisting of 1 amino acid residue and Y^d is a single bond, or X^d is a single bond and Y^d is a divalent peptide group consisting of 1 amino acid residue, or X^d and Y^d are each a single bond, or a pharmaceutically acceptable salt thereof;

[0125]

43. the compound according to any one of 38 - 42, wherein X^d is a single bond, and Y^d is a single bond, an alanine residue, a leucine residue or a methionine residue, or a pharmaceutically acceptable salt thereof;

[0126]

44. the compound according to any one of 38 - 42, wherein X^d is

a single bond or a divalent peptide group consisting of 1 amino acid residue, and Y^d is a single bond, or a pharmaceutically acceptable salt thereof;

[0127]

45. the compound according to any one of 38 - 44, wherein X^d and Y^d are each a single bond, or a pharmaceutically acceptable salt thereof;

[0128]

46. the compound according to any one of 38 - 45, wherein the cancer antigen peptide D is an MHC class II-restricted WT1 peptide consisting of 14 - 30 amino acid residues, or a pharmaceutically acceptable salt thereof;

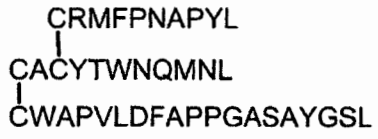
[0129]

47. the compound according to any one of 38 - 46, wherein the cancer antigen peptide D is a peptide consisting of any amino acid sequence selected from the following amino acid sequences:
SGQARMFPNAPYLPSC (SEQ ID NO: 19),
SGQAYMFPNAPYLPSC (SEQ ID NO: 25),
SGQARMFPNAPYLPSCLES (SEQ ID NO: 11),
SGQAYMFPNAPYLPSCLES (SEQ ID NO: 12),
PGCNKRYFKLSHLQMHSRK (SEQ ID NO: 20),
PGCNKRYFKLSHLQMHSRKH (SEQ ID NO: 21),
PGCNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 10),
CNKRYFKLSHLQMHSRK (SEQ ID NO: 22),
CNKRYFKLSHLQMHSRKH (SEQ ID NO: 23),
CNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 24) and
WAPVLDFAPPGASAYGSL (SEQ ID NO: 244)
or a pharmaceutically acceptable salt thereof;

[0130]

48. the compound according to any one of 38 - 47, wherein the compound represented by the formula (1) is a compound represented by the formula (14):

[0131]



(14)

[0132]

wherein the bond between C and C is a disulfide bond,

or a pharmaceutically acceptable salt thereof;

[0133]

49. the compound according to any one of 38 - 41 and 44, wherein the cancer antigen peptide E is a peptide consisting of any amino acid sequence selected from the following amino acid sequences:

SGQARMFPNAPYLPSC (SEQ ID NO: 19),

SGQAYMFPNAPYLPSC (SEQ ID NO: 25),

SGQARMFPNAPYLPSCLES (SEQ ID NO: 11),

SGQAYMFPNAPYLPSCLES (SEQ ID NO: 12),

PGCNKRYFKLSHLQMHSRK (SEQ ID NO: 20),

PGCNKRYFKLSHLQMHSRKH (SEQ ID NO: 21),

PGCNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 10),

CNKRYFKLSHLQMHSRK (SEQ ID NO: 22),

CNKRYFKLSHLQMHSRKH (SEQ ID NO: 23) and

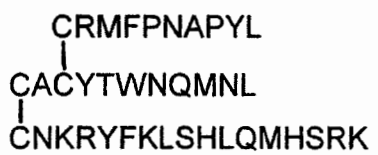
CNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 24)

or a pharmaceutically acceptable salt thereof;

[0134]

50. the compound according to any one of 38 - 41 and 49, wherein the compound represented by the formula (1) is a compound represented by the formula (12):

[0135]



(12)

[0136]

wherein the bond between C and C is a disulfide bond, or a pharmaceutically acceptable salt thereof;

[0137]

51. the compound according to any one of 1 - 8 and 33, wherein the cancer antigen peptide C is an MHC class II-restricted WT1 peptide consisting of 14 - 30 amino acid residues, or a pharmaceutically acceptable salt thereof;

[0138]

52. the compound according to any one of 1 - 8, 33 and 51, wherein the cancer antigen peptide C is a peptide comprising any amino acid sequence selected from the following amino acid sequences:

SGQARMFPNAPYLPSC (SEQ ID NO: 19),

SGQARMFPNAPYLPSCLES (SEQ ID NO: 11),

PGCNKRYFKLSHLQMHSRK (SEQ ID NO: 20),

PGCNKRYFKLSHLQMHSRKH (SEQ ID NO: 21),

PGCNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 10),

CNKRYFKLSHLQMHSRK (SEQ ID NO: 22),

CNKRYFKLSHLQMHSRKH (SEQ ID NO: 23) and

CNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 24), or

a peptide comprising an altered amino acid sequence, which is any amino acid sequence selected from SEQ ID NOs: 10 - 11 and 19 - 24 but containing alteration of amino acid residue(s), and having a helper T cell induction activity, or a pharmaceutically acceptable salt thereof;

[0139]

53. the compound according to any one of 1 - 8, 33 and 51 - 52, wherein the cancer antigen peptide C is a peptide consisting of an amino acid sequence selected from the following amino acid sequences:

SGQARMFPNAPYLPSC (SEQ ID NO: 19),

SGQAYMFPNAPYLPSC (SEQ ID NO: 25),

SGQARMFPNAPYLPSCLES (SEQ ID NO: 11),

SGQAYMFPNAPYLPSCLES (SEQ ID NO: 12),

PGCNKRYFKLSHLQMHSRK (SEQ ID NO: 20),

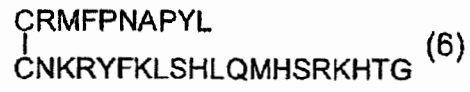
PGCNKRYFKLSHLQMHSRKH (SEQ ID NO: 21),

PGCNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 10),
CNKRYFKLSHLQMHSRK (SEQ ID NO: 22),
CNKRYFKLSHLQMHSRKH (SEQ ID NO: 23) and
CNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 24),
or a pharmaceutically acceptable salt thereof;

[0140]

54. the compound according to any one of 1 - 8, 33 and 51 - 53, wherein the compound represented by the formula (1) is a compound represented by the formula (6):

[0141]

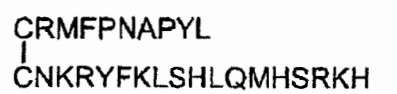


(6)

[0142]

wherein the bond between C and C is a disulfide bond, a compound represented by the formula (7):

[0143]

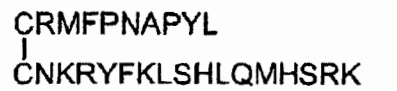


(7)

[0144]

wherein the bond between C and C is a disulfide bond, a compound represented by the formula (8):

[0145]



(8)

[0146]

wherein the bond between C and C is a disulfide bond, or a compound represented by the formula (9):

[0147]



(9)

[0148]

wherein the bond between C and C is a disulfide bond, or a pharmaceutically acceptable salt thereof;

[0149]

55. an altered form of an MHC class II-restricted WT1 peptide consisting of 7 - 30 amino acid residues;

[0150]

56. the altered form according to 55, which is the following amino acid sequence:

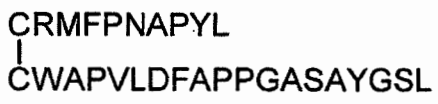
CWAPVLDFAPPGASAYGSL (SEQ ID NO: 242) or

WAPVLDFAPPGASAYGSLC (SEQ ID NO: 243);

[0151]

57. a compound represented by the formula (10):

[0152]



(10)

[0153]

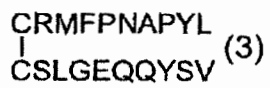
wherein the bond between C and C is a disulfide bond,

or a pharmaceutically acceptable salt thereof;

[0154]

58. a composition comprising a compound selected from the group consisting of a compound represented by the formula (3):

[0155]



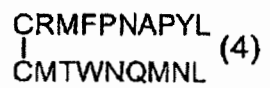
(3)

[0156]

wherein the bond between C and C is a disulfide bond,

a compound represented by the formula (4):

[0157]



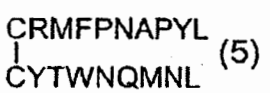
(4)

[0158]

wherein the bond between C and C is a disulfide bond,

and a compound represented by the formula (5):

[0159]



(5)

[0160]

wherein the bond between C and C is a disulfide bond, and a peptide consisting of an amino acid sequence selected from the group consisting of the following amino acid sequences:
CNKRYFKLSHLQMHSRK (SEQ ID NO: 22),
CNKRYFKLSHLQMHSRKH (SEQ ID NO: 23),
CNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 24),
WAPVLDFAPPGASAYGSL (SEQ ID NO: 244),
CWAPVLDFAPPGASAYGSL (SEQ ID NO: 242) and
WAPVLDFAPPGASAYGSLC (SEQ ID NO: 243);

[0161]

59. a pharmaceutical composition comprising the compound according to any one of 1 - 54 and 57, or a pharmaceutically acceptable salt thereof, or the composition according to 58 and a pharmaceutically acceptable carrier;

[0162]

60. the pharmaceutical composition according to 59, which is used as a cancer vaccine;

[0163]

61. use of the compound according to any one of 1 - 54 and 57, or a pharmaceutically acceptable salt thereof, or the composition according to 58 for the production of a cancer vaccine;

[0164]

62. a method of treating or preventing cancer, comprising administering a therapeutically or prophylactically effective amount of the compound according to any one of 1 - 54 and 57 or a pharmaceutically acceptable salt thereof or the composition according to 58 to a WT1 positive cancer patient in need thereof;

[0165]

63. a method of obtaining two different MHC class I-restricted epitopes, or an MHC class I-restricted epitope and an MHC class II-restricted epitope, comprising reacting the compound according to any one of 1 - 54 and 57 or a pharmaceutically

acceptable salt thereof with ERAP1; and

[0166]

64. a method of synthesizing a compound, comprising the following steps:

(1) a step of synthesizing, by using Fmoc-C(Mmt)A-SBn and cancer antigen peptide C, a peptide wherein a carbonyl group of the C-terminal amino acid of C(Mmt)A and the N-terminal amino group of the cancer antigen peptide C are bonded, wherein the antigen peptide C is an MHC class I-restricted WT1 peptide consisting of 7 - 30 amino acid residues containing one cysteine residue or an MHC class II-restricted WT1 peptide consisting of 7 - 30 amino acid residues containing one cysteine residue,

(2) a step of synthesizing, by using the peptide obtained in the aforementioned step (1) and cancer antigen peptide A wherein one cysteine residue protected by Npys group is bonded to the N-terminal, a peptide wherein a thioether group of the cysteine residue of the cancer antigen peptide C in the peptide obtained in the aforementioned step (1) and a thioether group of the cysteine residue bonded to the N-terminal of cancer antigen peptide A are bonded, wherein the cancer antigen peptide A is an MHC class I-restricted WT1 peptide consisting of 7 - 30 amino acid residues, and

(3) a step of synthesizing, by using the peptide obtained in the aforementioned step (2) and cancer antigen peptide D containing a cysteine residue protected by Spy group, a peptide wherein a thioether group of the cysteine residue bonded to the N-terminal of the cancer antigen peptide A in the peptide obtained in the aforementioned step (2), and a thioether group of the cysteine residue of the cancer antigen peptide D are bonded, wherein the cancer antigen peptide D is an MHC class II-restricted WT1 peptide consisting of 7 - 30 amino acid residues containing one cysteine residue bonded to the N terminal or an MHC class II-restricted WT1 peptide consisting of 7 - 30 amino acid residues containing one cysteine residue,

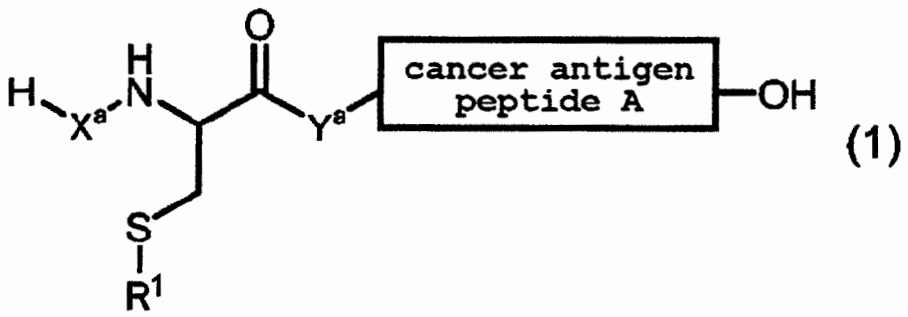
[0167]

3. Third embodiment

[0168]

1. A compound represented by the formula (1):

[0169]



(1)

[0170]

wherein X^a and Y^a are each a single bond,

cancer antigen peptide A is a peptide consisting of any amino acid sequence selected from the following amino acid sequences:

RMFPNAPYL (SEQ ID NO: 2),

ALLPAVPSL (SEQ ID NO: 5),

SLGEQQYSV (SEQ ID NO: 6) and

RVPGVAPTL (SEQ ID NO: 7),

an amino group of an N-terminal amino acid of the cancer antigen peptide A binds to Y^a in the formula (1), and a carbonyl group of a C-terminal amino acid of the cancer antigen peptide A binds to a hydroxyl group in the formula (1),

R^1 is a cancer antigen peptide C,

the cancer antigen peptide C has a sequence different from that of the cancer antigen peptide A, which is a peptide consisting of any amino acid sequence selected from the following amino acid sequences:

CMTWNQMNL (SEQ ID NO: 3) and

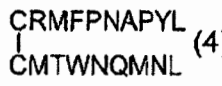
CYTWNQMNL (SEQ ID NO: 4), and a thioether group of the cysteine residue of the cancer antigen peptide C is bonded to the thioether group in the formula (1),

or a pharmaceutically acceptable salt thereof;

[0171]

2. the compound according to 1, wherein the compound represented by the formula (1) is a compound represented by the formula (4):

[0172]



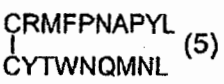
[0173]

wherein the bond between C and C is a disulfide bond, or a pharmaceutically acceptable salt thereof;

[0174]

3. the compound according to 1, wherein the compound represented by the formula (1) is a compound represented by the formula (5):

[0175]



[0176]

wherein the bond between C and C is a disulfide bond, or a pharmaceutically acceptable salt thereof;

[0177]

4. a pharmaceutical composition comprising the compound according to any one of 1 - 3, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier;

[0178]

5. the pharmaceutical composition according to 4, comprising at least one peptide consisting of an amino acid sequence selected from the group consisting of the following amino acid sequences:

CNKRYFKLSHLQMHSRK (SEQ ID NO: 22),

CNKRYFKLSHLQMHSRKH (SEQ ID NO: 23),

CNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 24),

WAPVLDFAPPGASAYGSL (SEQ ID NO: 244),

CWAPVLDFAPPGASAYGSL (SEQ ID NO: 242) and

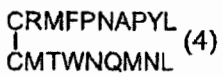
WAPVLDFAPPGASAYGSLC (SEQ ID NO: 243); and

[0179]

6. a composition comprising a compound selected from the group

consisting of a compound represented by the formula (4):

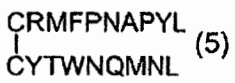
[0180]



wherein the bond between C and C is a disulfide bond,

and a compound represented by the formula (5):

[0181]



[0182]

wherein the bond between C and C is a disulfide bond, and at least one peptide consisting of an amino acid sequence selected from the group consisting of the followingamino acid sequences:

CNKRYFKLSHLQMHSRK (SEQ ID NO: 22),

CNKRYFKLSHLQMHSRKH (SEQ ID NO: 23),

CNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 24),

WAPVLDFAPPGASAYGSL (SEQ ID NO: 244),

CWAPVLDFAPPGASAYGSL (SEQ ID NO: 242) and

WAPVLDFAPPGASAYGSLC (SEQ ID NO: 243).

EFFECT OF THE INVENTION

[0183]

According to the present invention, the aforementioned compound represented by the formula (1) useful as a cancer immunotherapeutic agent (hereinafter sometimes to be referred to as the compound of the present invention) can be provided. According to the compound of the present invention, cancer vaccines and cancer immunotherapeutic agents that efficiently induce CTL in vivo and in vitro can be provided. To be specific, according to the compound of the present invention, it is now possible to produce two MHC class I-restricted WT1 peptides having different sequences or two MHC class I-restricted WT1 eptopes having different sequences, an MHC class I-restricted

WT1 peptide and an MHC class II-restricted WT1 peptide, an MHC class I-restricted WT1 epitope and an MHC class II-restricted WT1 epitope, two MHC class I-restricted WT1 peptide and MHC class II-restricted WT1 peptide having different sequences, or two MHC class I-restricted WT1 epitope and MHC class II-restricted WT1 epitope having different sequences in vivo and in vitro and efficiently induce CTL.

As for the HLA subtypes of two MHC class I-restricted WT1 peptides having different sequence, the compound of the present invention (conjugate) obtained by combining A02 type (A-0201, A0206 and the like) peptide and A24 type (A-2402 and the like) peptide is particularly preferable. In Europeans and Americans (Caucasian), the population of HLA-A0201 subtype or HLA-A0206 subtype is the highest and about 47%, then HLA-A2402 subtype is about 13%, and the total of these subtypes occupies about 56%, excluding duplicates (i.e., duplicate calculation of humans having both subtypes) (Human Immunol. 62:1009;2001). In Japanese people and the like, the population of HLA-A2402 is the highest and about 60%, then HLA-A0201 or HLA-A0206 is about 39%, and the total of these subtypes occupies about 81%, excluding duplicates (i.e., duplicate calculation of humans having both subtypes) (www.bmdc.irc.or.jp/GF-A.htm). Therefore, the advantages of the compound of the present invention are, specifically, that a larger population is covered by a single compound of the present invention, and selection of the HLA subtype of the patients before administration is not always essential and the like. In view of such advantages of the compound of the present invention, a compound represented by the formula (3), the formula (4) or the formula (5) is preferable, and a compound represented by the formula (5) is more preferable.

Moreover, according to the compound of the present invention, an active ingredient of a cancer vaccine superior in physicochemical properties and stability, and easily produced and easily controlled can be provided. As a result, formulation

of cancer vaccine has been facilitated.

Specifically, examples of the physicochemical properties include solubility, viscosity of solution, easiness of purification resulting therefrom, easy handling after freeze-drying, easiness of purification resulting therefrom and the like. The stability includes stability after salt substitution, hygroscopicity, thermal stability, stability after emulsion formation and the like. The pharmacological activity includes efficacy as cancer vaccine, difference caused by API (Active Pharmaceutical Ingredient), interaction with additive in preparation and the like. Of these, the difference caused by API is a difference as a cancer vaccine due to API. Specifically, in two APIs having vastly different solubilities, API with smaller solubility is prone to precipitate, and it is easily expected that a sterilization treatment by filtration with a membrane filter, which is an essential requirement for pharmaceutical products, cannot be performed. Even if a sterilization treatment by filtration of API with small solubility is barely possible, it is considered that the amount of API contained in the filtrate markedly decreases and CTL induction ability essential for a cancer vaccine markedly decreases. Therefore, a demerit of markedly decreased production efficiency of API with small solubility is easily predictable.

BRIEF DESCRIPTION OF THE DRAWINGS

[0184]

Fig. 1 is a Figure showing the test results of Experimental Example 1 as to the time-dependent change of N-terminal amino acid trimming by ERAP1 of each peptide of SEQ ID NOs: 13, 16, 17 and 18 synthesized in Examples 2 - 5.

Fig. 2 is a Figure showing the test results of Experimental Example 2 as to the in vivo CTL induction ability of a compound represented by the formula (5) synthesized in

Example 1, by IFNγ ELISPOT assay using HLA-A0201 transgenic mouse.

Fig. 3 is a Figure showing the test results of Experimental Example 2 as to the in vivo CTL induction ability of a compound represented by the formula (5) synthesized in Example 1, by IFNγ ELISPOT assay using HLA-A2402 transgenic mouse.

Fig. 4 is a Figure showing the test results of Experimental Example 4 as to the in vivo CTL induction ability of a compound represented by the formula (3) synthesized in Example 6, by IFNγ ELISPOT assay using HLA-A0201 transgenic mouse.

Fig. 5 is a Figure showing the test results of Experimental Example 5 as to the ability of a compound represented by the formula (6) synthesized in Example 7 to induce cells reactive with peptide shown by SEQ ID NO: 24 in vivo, by IFNγ ELISPOT assay using HLA-A0201 transgenic mouse in the pulsed or non-pulsed state with peptide of SEQ ID NO: 24.

Fig. 6 is a Figure showing the test results of Experimental Example 5 as to the ability of a compound represented by the formula (6) synthesized in Example 7 to induce cells reactive with peptide shown by SEQ ID NO: 24 in vivo, by IFNγ ELISPOT assay using HLA-A0201 transgenic mouse in the pulsed or non-pulsed state with peptide of SEQ ID NO: 24.

Fig. 7 is a Figure showing the test results of Experimental Example 6 as to the in vivo CTL induction ability of a compound represented by the formula (8) synthesized in Example 9 in the pulsed or non-pulsed state with peptide of SEQ ID NO: 2, by IFNγ ELISPOT assay using HLA-A0201 transgenic mouse.

Fig. 8 is a Figure showing the test results of Experimental Example 6 as to the ability of a compound represented by the formula (8) synthesized in Example 9 to induce cells reactive with peptide shown by SEQ ID NO: 22 in vivo, by IFNγ ELISPOT assay using HLA-A0201 transgenic mouse in

the pulsed or non-pulsed state with peptide of SEQ ID NO: 22.

Fig. 9 is a Figure showing the test results of Experimental Example 8 as to the in vivo CTL induction ability of a compound represented by the formula (7) synthesized in Example 8 in the pulsed or non-pulsed state with peptide of SEQ ID NO: 2, by IFNγ ELISPOT assay using HLA-A0201 transgenic mouse.

Fig. 10 is a Figure showing the test results of Experimental Example 8 as to the ability of a compound represented by the formula (7) synthesized in Example 8 to induce cells reactive with peptide shown by SEQ ID NO: 23 in vivo, by IFNγ ELISPOT assay using HLA-A0201 transgenic mouse in the pulsed or non-pulsed state with peptide of SEQ ID NO: 23.

Fig. 11 is a Figure showing the test results of Experimental Example 9 as to the in vivo CTL induction ability of a compound represented by the formula (9) synthesized in Example 10 in the pulsed or non-pulsed state with peptide of SEQ ID NO: 5, by IFNγ ELISPOT assay using HLA-A0201 transgenic mouse.

Fig. 12 is a Figure showing the test results of Experimental Example 9 as to the ability of a compound represented by the formula (9) synthesized in Example 10 to induce cells reactive with peptide shown by SEQ ID NO: 24 in vivo, by IFNγ ELISPOT assay using HLA-A0201 transgenic mouse in the pulsed or non-pulsed state with peptide of SEQ ID NO: 24.

Fig. 13 is a Figure showing the test results of Comparative Example 1 as to the in vivo CTL induction ability of peptides shown by SEQ ID NO: 238 and 239 synthesized in Reference Examples 8 and 9 in the pulsed or non-pulsed state with peptide of SEQ ID NO: 2, by IFNγ ELISPOT assay using HLA-A0201 transgenic mouse.

Fig. 14 is a Figure showing the test results of Comparative Example 1 as to the in vivo CTL induction ability of peptides shown by SEQ ID NO: 238 and 239 synthesized in Reference Examples 8 and 9 in the pulsed or non-pulsed state with peptide of SEQ ID NO: 4, by IFNγ ELISPOT assay using HLA-

A2402 transgenic mouse.

Fig. 15 is a Figure showing the test results of Comparative Example 2 as to the in vivo CTL induction ability of peptides shown by SEQ ID NO: 240 and 241 synthesized in Reference Examples 10 and 11 in the pulsed or non-pulsed state with peptide of SEQ ID NO: 2, by IFNγ ELISPOT assay using HLA-A0201 transgenic mouse.

Fig. 16 is a Figure showing the test results of Comparative Example 2 as to the in vivo CTL induction ability of peptides shown by SEQ ID NO: 240 and 241 synthesized in Reference Examples 10 and 11 in the pulsed or non-pulsed state with peptide of SEQ ID NO: 4, by IFNγ ELISPOT assay using HLA-A2402 transgenic mouse.

Fig. 17 is a Figure showing the test results of Experimental Example 11 as to the in vivo CTL induction ability of a compound represented by the formula (10) synthesized in Example 13 in the pulsed or non-pulsed state with peptide of SEQ ID NO: 2, by IFNγ ELISPOT assay using HLA-A0201 transgenic mouse.

Fig. 18 is a Figure showing the test results of Comparative Example 3 as to the in vivo CTL induction ability of a compound represented by the formula (11) synthesized in Reference Example 12 in the pulsed or non-pulsed state with peptide of SEQ ID NO: 2, by IFNγ ELISPOT assay using HLA-A0201 transgenic mouse.

Fig. 19 is a Figure showing the test results of Experimental Example 12 as to the in vivo CTL induction ability of a compound represented by the formula (12) synthesized in Example 14 in the pulsed or non-pulsed state with peptide of SEQ ID NO: 2, by IFNγ ELISPOT assay using HLA-A0201 transgenic mouse.

Fig. 20 is a Figure showing the test results of Experimental Example 12 as to the in vivo CTL induction ability of a compound represented by the formula (12) synthesized in Example 14 in the pulsed or non-pulsed state with peptide of SEQ ID NO: 4, by IFNγ ELISPOT assay using HLA-A2402 transgenic mouse.

Fig. 21 is a Figure showing the test results of Experimental Example 13 as to the in vivo CTL induction ability of a compound represented by the formula (14) synthesized in Example 15 in the pulsed or non-pulsed state with peptide of SEQ ID NO: 2, by IFNγ ELISPOT assay using HLA-A0201 transgenic mouse.

Fig. 22 is a Figure showing the test results of Experimental Example 13 as to the in vivo CTL induction ability of a compound represented by the formula (14) synthesized in Example 15 in the pulsed or non-pulsed state with peptide of SEQ ID NO: 4, by IFNγ ELISPOT assay using HLA-A2402 transgenic mouse.

Fig. 23 is a Figure showing the test results of Experimental Example 14 as to the in vivo CTL induction ability of a cocktail vaccine of a compound represented by the formula (5) synthesized in Example 1 and the peptide shown by SEQ ID NO: 22 synthesized in Reference Example 1 in the pulsed or non-pulsed state with peptide of SEQ ID NO: 2, by IFNγ ELISPOT assay using HLA-A0201 transgenic mouse.

Fig. 24 is a Figure showing the test results of Experimental Example 15 as to the in vivo CTL induction ability of a cocktail vaccine of a compound represented by the formula (5) synthesized in Example 1 and the peptide shown by SEQ ID NO: 244 synthesized in Reference Example 13 in the pulsed or non-pulsed state with peptide of SEQ ID NO: 2, by IFNγ ELISPOT assay using HLA-A0201 transgenic mouse.

Fig. 25 is a Figure showing the test results of Experimental Example 16 as to the in vivo CTL induction ability of a cocktail vaccine of a compound represented by the formula (5) synthesized in Example 1 and the peptide shown by SEQ ID NO: 24 synthesized in Reference Example 2 in the pulsed or non-pulsed state with peptide of SEQ ID NO: 2, by IFNγ ELISPOT assay using HLA-A0201 transgenic mouse.

Fig. 26 is a Figure showing the test results of Experimental Example 17 as to the in vivo CTL induction ability of a cocktail vaccine of a compound represented by the formula

(5) synthesized in Example 1 and the peptide shown by SEQ ID NO: 242 synthesized in Example 11 in the pulsed or non-pulsed state with peptide of SEQ ID NO: 2, by IFNγ ELISPOT assay using HLA-A0201 transgenic mouse.

Fig. 27 is a Figure showing the test results of Experimental Example 18 as to the in vivo CTL induction ability of a cocktail vaccine of a compound represented by the formula (5) synthesized in Example 1 and the peptide shown by SEQ ID NO: 243 synthesized in Example 11 in the pulsed or non-pulsed state with peptide of SEQ ID NO: 2, by IFNγ ELISPOT assay using HLA-A0201 transgenic mouse.

DESCRIPTION OF EMBODIMENTS

[0185]

The embodiment of the present invention is explained in detail in the following.

[0186]

The "amino acid residue" in the present invention means a region corresponding to one unit of amino acid constituting a peptide or protein in a peptide or protein molecule. Examples of the "amino acid residue" include natural or non-natural μ-amino acid residue, β-amino acid residue, γ-amino acid residue or δ-amino acid residue. Specific examples thereof include natural α-amino acid residue, ornithine residue, homoserine residue, homocysteine residue, β-alanine, γ-aminobutanoic acid or δ-aminopentanoic acid and the like. When the "amino acid residue" can be an optically active substance, it may be any of an L-form and a D-form, and an L-form is preferable.

[0187]

When the "amino acid residue" in the present invention is shown in abbreviation, the following abbreviations are used.

Ala or A: alanine residue

Arg or R: arginine residue

Asn or N: asparagine residue

Asp or D: aspartic acid residue
Cys or C: cysteine residue
Gln or Q: glutamine residue
Glu or E: glutamic acid residue
Gly or G: glycine residue
His or H: histidine residue
Ile or I: isoleucine residue
Leu or L: leucine residue
Lys or K: lysine residue
Met or M: methionine residue
Phe or F: phenylalanine residue
Pro or P: proline residue
Ser or S: serine residue
Thr or T: threonine residue
Trp or W: tryptophan residue
Tyr or Y: tyrosine residue
Val or V: valine residue
Abu: 2-aminobutyric acid residue (to be also referred to as α-aminobutyric acid residue)
Orn: ornithine residue
Cit: citrulline residue

[0188]

The amino acid sequence of the "peptide" in the present invention is described according to the conventional method, wherein the amino acid residue of the N-terminal amino acid is positioned on the left side, and the amino acid residue of the C-terminal amino acid is positioned on the right side. In the "peptide", unless particularly indicated, the amino group of the amino acid residue of the N-terminal amino acid is bonded to hydrogen atom, and the carbonyl group of the amino acid residue of the C-terminal amino acid is bonded to hydroxyl group. The divalent group of peptide means a group bonding via the amino group of the amino acid residue of the N-terminal amino acid and the carbonyl group of the amino acid residue of the C-terminal

amino acid.

In the compound of the present invention, for example, in the compounds represented by the formulae (3) - (9) and as regards peptide, which is a partial structure thereof, unless particularly indicated, the amino group of the amino acid residue of the N-terminal amino acid is bonded to hydrogen atom, and the carbonyl group of the amino acid residue of the C-terminal amino acid is bonded to hydroxyl group.

[0189]

"X^a" and "Y^a" in the present invention mean, independently, a single bond or a divalent group of peptides consisting of 1 - 4 amino acid residues. The sum of the amino acid residue number of X^a and that of Y^a is an integer of 0 - 4. For example, an integer of said sum being 0 means that X^a and Y^a are each a single bond. When the sum is an integer of 4, examples thereof include X^a and Y^a independently being divalent groups of peptide consisting of 2 amino acid residues, X^a being a divalent group of peptide consisting of 3 amino acid residues and Y^a being a divalent group of peptide consisting of 1 amino acid residue, X^a being a divalent group of peptide consisting of 4 amino acid residues and Y^a being a single bond and the like.

The integer of said sum is preferably 0 - 2, more preferably 0 - 1, most preferably 0. That is, X^a and Y^a are most preferably single bonds.

When the sum is an integer of 2, examples thereof include X^a being a divalent group of peptide consisting of 2 amino acid residues and Y^a being a single bond, X^a and Y^a independently being divalent groups of peptide consisting of 1 amino acid residue, or X^a being a single bond and Y^a being a divalent group of peptide consisting of 2 amino acid residues.

When the sum is an integer of 1, examples thereof include X^a being a divalent group of peptide consisting of 1 amino acid residue and Y^a being a single bond, and X^a being a single bond and Y^a being a divalent group of peptide consisting of 1 amino

acid residue. Of these, preferred is X^a being a single bond and Y^a being an alanine residue, leucine residues or methionine residue.

[0190]

The "cancer antigen peptide A" in the present invention is an MHC class I-restricted WT1 peptide consisting of 7 - 30 amino acid residues. In cancer antigen peptide A in the formula (1), the amino group of the N-terminal amino acid is bonded to Y^a in the formula (1) and the carbonyl group of the C-terminal amino acid is bonded to the hydroxyl group in the formula (1).

[0191]

The "MHC class I-restricted" in the present invention means the property to induce CTL by binding to an MHC class I molecule which is class I of the major histocompatibility complex (MHC).

[0192]

MHC in human is called human leukocyte-type antigen (HLA). HLA corresponding to the MHC class I-molecule is classified into subtypes of HLA-A, B, Cw, F and G and the like. Preferable examples of the "MHC class I-restricted" include HLA-A-restricted, HLA-B-restricted and HLA-Cw-restricted.

Polymorphism (allele) of each subtype of HLA is known. Examples of the polymorphism of HLA-A include not less than 27 kinds such as HLA-A1, HLA-A0201, HLA-A24 and the like, examples of the polymorphism of HLA-B include not less than 59 kinds such as HLA-B7, HLA-B40, HLA-B4403 and the like, and examples of the polymorphism of HLA-Cw include not less than 10 kinds such as HLA-Cw0301, HLA-Cw0401, HLA-Cw0602 and the like. Among these polymorphisms, HLA-A0201 and HLA-A24 are preferable.

[0193]

The "WT1 peptide" in the present invention is a partial peptide consisting of continuous 7 - 30 amino acid residues in the amino acid sequence of human WT1 described in SEQ ID NO: 1.

[0194]

Therefore, the "MHC class I-restricted WT1 peptide" in the present invention is a peptide that binds to an MHC class I antigen in vitro and/or in vivo and is presented as a complex, and induces CTL as a result of recognition of the complex by precursor T cells. The number of the amino acid residues of the "MHC class I-restricted WT1 peptide" is 7 - 30, preferably 7 - 15, more preferably 8 - 12, further preferably 8 - 11, most preferably 8 or 9.

[0195]

The "MHC class I-restricted WT1 peptide" consisting of 7 - 12 or preferably 9 amino acid residues is also called "an MHC class I-restricted WT1 epitope". The "MHC class I-restricted WT1 epitope" in the present invention means a peptide per se that binds to an MHC class I antigen and is presented as a complex. That is, "MHC class I-restricted WT1 peptide" produces "MHC class I-restricted WT1 epitope" in vitro and/or in vivo, by intracellular decomposition of the compound of the present invention by proteosome and/or protease such as Gamma-Interferon-inducible Lysosomal Thiol Reductase (GILT, GLT) and the like (proteolysis, reductive cleavage of disulfide bond), and/or cleavage into the optimal residue number (also called trimming) by Endoplasmic reticulum aminopeptidase 1 (ERAP1, ER-aminopeptidase 1). In this production, a production process wherein the C-terminal amino acid of the "MHC class I-restricted WT1 epitope" first results from the degradation by proteosome and/or protease, after which N-terminal amino acid of the "MHC class I-restricted WT1 epitope" results from trimming (cleavage) by ERAP1 is mainly considered. In this production, however, a process other than this production process is also possible. At present, ERAP1 is also referred to as ERAAP (ER aminopeptidase associated with antigen presentation), and used to be also called A-LAP, PILS-AP or ARTS-1.

[0196]

Therefore, the "MHC class I-restricted WT1 peptide" is

preferably a peptide consisting of 7 - 30 amino acid residues produced by adding 1 - 23 amino acid residues to the carbonyl group of the C-terminal amino acid of the "MHC class I-restricted WT1 epitope" consisting of 7 - 12 amino acid residues.

[0197]

Examples of the "MHC class I-restricted WT1 peptide" include peptides described in Tables 1 - 44. In each Table, the "position" means a position in the amino acid sequence of human WT1 described in SEQ ID NO: 1.

[0198]

Table 1

position	amino acid sequence	sequence No.	HLA subtype
2-10	GSDVRDLNA	26	A1
3-11	SDVRDLNAL	27	B40, B60, B61, B3701, B4403, Cw0301, Cw0602
4-12	DVRDLNALL	28	A24, A68.1, A3302, B7, B8, B3501, B3701, Cw0401, Cw0602
6-14	RDLNALLPA	29	B40, B61, B3701
7-15	DLNALLPAV	30	A0201, B62, B5201
10-18	ALLPAVPSL	5	A0201, A0205, A24, A3, B14, B7, B8, B3801, B3901, B3902, Cw0301, Cw0401, Cw0602

[0199]

Table 2

position	amino acid sequence	sequence No.	HLA subtype
17-25	SLGGGGGCA	31	B62
18-26	LGGGGGCAL	32	B60, B7, B3801, B5101, B5102
20-28	GGGGCALPV	33	B61, B5101, B5102, B5201

[0200]

Table 3

position	amino acid sequence	sequence No.	HLA subtype
23-31	GCALPVSGA	34	B40, B61
24-32	CALPVSGAA	35	B40, B5102, Cw0301
26-34	LPVSGAAQW	36	B40, B3501, B5801
29-37	SGAAQWAPV	37	B5101, B5102, B5201, B61
30-38	GAAQWAPVL	38	B40, B60, B7, B8, B3902, B5101, B5102, Cw0301, Cw0602

[0201]

Table 4

position	amino acid sequence	sequence No.	HLA subtype
32-40	AQWAPVLDF	39	A3, A3101, B62, B2702, B2705, B3902, B5201
33-41	QWAPVLDFA	40	Cw0702
37-45	VLDFAPPGA	41	A1, A0201
38-46	LDFAPPGAS	42	B40, B3701
39-47	DFAPPGASA	43	Cw0401
40-48	FAPPGASAY	44	A1, B62, B3501, B4403, B5801, Cw0702

[0202]

Table 5

position	amino acid sequence	sequence No.	HLA subtype
47-55	AYGSLGGPA	45	A24

[0203]

Table 6

position	amino acid sequence	sequence No.	HLA subtype
63-71	PPPPPPHSF	46	Cw0401
64-72	PPPPPHSFI	47	B5101, B5102, B5201
65-73	PPPPHSFIK	48	A1101
70-78	SFIKQEPSW	49	Cw0401

[0204]

Table 7

position	amino acid sequence	sequence No.	HLA subtype
73-81	KQEPSWGGA	50	A1, A0205
80-88	GAEPHEEQC	51	A1

[0205]

Table 8

position	amino acid sequence	sequence No.	HLA subtype
81-89	AEPHEEQCL	52	A0205, B40, B60, B61, B3701, B4403
82-90	EPHEEQCLS	53	B3501, B5101
83-91	PHEEQCLSA	54	B3801
84-92	HEEQCLSAF	55	B40, B3701, B4403, Cw0702
85-93	EEQCLSAFT	56	B40, B60, B61, B3701, B4403
86-94	EQCLSAFTV	57	A0201, B62, B5201
88-96	CLSAFTVHF	58	A3, B62

[0206]

Table 9

position	amino acid sequence	sequence No.	HLA subtype
92-100	FTVHFSGQF	59	B62, B5801, Cw0301
93-101	TVHFSGQFT	60	A0201, A0205
96-104	FSGQFTGTA	61	B5801, B4403
98-106	GQFTGTAGA	62	A0205, B40, B62, B2702, B5201
99-107	QFTGTAGAC	63	Cw0401
100-108	FTGTAGACR	64	A68.1, A1101, A3101, A3302

[0207]

Table 10

position	amino acid sequence	sequence No.	HLA subtype
101-109	TGTAGACRY	65	B62, B4403, Cw0702
104-112	AGACRYGPF	66	B4403, B5201
107-115	CRYGPFGPP	67	B2702
110-118	GPFGPPPPS	68	B5101, B5102

[0208]

Table 11

position	amino acid sequence	sequence No.	HLA subtype
118-126	SQASSGQAR	69	A68.1, A1101, A3101, A3302
119-127	QASSGQARM	70	B3501, B5101, B5102
120-128	ASSGQARMF	71	B3501, B3801, B4403, B5801

[0209]

Table 12

position	amino acid sequence	sequence No.	HLA subtype
123-131	GQARMFPNA	72	B62
125-133	ARMFPNAPY	73	B14, B2702, B2705, Cw0702
126-134	RMFPNAPYL	2	A0201, A0205, A24, A3, B14, B7, B2702, B2705, B3901, B3902, Cw0301
128-136	FPNAPYLPS	74	B5101
130-138	NAPYLPSCL	75	A24, B60, B7, B8, B3902, B5101, B5102, Cw0301, Cw0602, Cw0702

[0210]

Table 13

position	amino acid sequence	sequence No.	HLA subtype
136-144	SCLESQPAI	76	B8, B3901, B5102, Cw0301
137-145	CLESQPAIR	77	A1, A3, A68.1, A1101, A3101, A3302
138-146	LESQPAIRN	78	B60, B61
139-147	ESQPAIRNQ	79	A3302

[0211]

Table 14

position	amino acid sequence	sequence No.	HLA subtype
141-149	QPAIRNQGY	80	B8, B3501, B4403, Cw0401, Cw0702
143-151	AIRNQGYST	81	B7
144-152	IRNQGYSTV	82	B14, B2702, B2705, B3901
146-154	NQGYSTVTF	83	B62, B2702, B3902, B5201

[0212]

Table 15

position	amino acid sequence	sequence No.	HLA subtype
152-160	VTFDGTPSY	84	A1, A3, B62, B3501, B4403, B5801, Cw0702

[0213]

Table 16

position	amino acid sequence	sequence No.	HLA subtype
161-169	GHTPSHHAA	85	B3801
163-171	TPSHHAAQF	86	B3501, B3801, Cw0401, Cw0702
165-173	SHHAAQFPN	87	B3801
168-176	AAQFPNHSF	88	B5801
169-177	AQFPNHSFK	89	A3, A68.1, A1101, A3101, B2705

[0214]

Table 17

position	amino acid sequence	sequence No.	HLA subtype
174-182	HSFKHEDPM	90	B14, B3501, B5801
177-185	KHEDPMGQQ	91	B3801
179-187	EDPMGQQGS	92	B3701
180-188	DPMGQQGSL	93	A24, B14, B60, B7, B8, B3501, B3801, B3901, B3902, B5101, B5102, Cw0301, Cw0401, Cw0602

[0215]

Table 18

position	amino acid sequence	sequence No.	HLA subtype
185-193	QGSLGEQQY	94	B4403, Cw0702
187-195	SLGEQQYSV	6	A0201, A0205, A3, B62

[0216]

Table 19

position	amino acid sequence	sequence No.	HLA subtype
191-199	QQYSVPPPV	95	A0201, A0205, B61, B62, B2702, B2705, B5201
192-200	QYSVPPPVY	96	A24, Cw0401, Cw0702
194-202	SVPPPVYGC	97	A0205, A3

[0217]

Table 20

position	amino acid sequence	sequence No.	HLA subtype
202-210	CHTPTDSCT	98	B3801
204-212	TPTDSCTGS	99	B5101
206-214	TDSCTGSQA	100	B40, B61, B3701
207-215	DSCTGSQAL	101	A24, A3302, B60, B7, B8, B3501, B3901, B3902, Cw0602
208-216	SCTGSQALL	102	B60, B7, B8, B3701, B3801, B3901, B3902
209-217	CTGSQALLL	103	B60, B7, B3701, B3902
210-218	TGSQALLLR	104	A3302

[0218]

Table 21

position	amino acid sequence	sequence No.	HLA subtype
211-219	GSQALLLRT	105	B5801
213-221	QALLLRTPY	106	A1, B3501, B4403, B5801, Cw0602, Cw0702
217-225	LRTPYSSDN	107	B2702
218-226	RTPYSSDNL	108	A24, B60, B7, B3902, B5801
219-227	TPYSSDNLY	109	B3501, B5101, B5102, Cw0401, Cw0702

[0219]

Table 22

position	amino acid sequence	sequence No.	HLA subtype
221-229	YSSDNLYQM	110	B60, B3501
222-230	SSDNLYQMT	111	A1, B5801
223-231	SDNLYQMTS	112	B3701
225-233	NLYQMTSQL	113	A0201, A0205, A24, B14, B7, B8, B3801, B3901, B3902, Cw0301, Cw0602
227-235	YQMTSQLEC	114	A0201, A0205, B62
228-236	QMTSQLECM	115	A0201
230-238	TSQLECMTW	116	B5801

[0220]

Table 23

position	amino acid sequence	sequence No.	HLA subtype
232-240	QLECMTWNQ	117	A1
233-241	LECMTWNQM	118	B40, B60, B61, B3701, B4403
235-243	CMTWNQMNL	3	A0201, A0205, A24, A3, B7
239-247	NQMNLGATL	119	A0201, A0205, A24, B14, B60, B62, B7, B2702, B2705, B3901, B3902, B5201, Cw0301, Cw0602
240-248	QMNLGATLK	120	A24, A3, A1101, A3101

[0221]

Table 24

position	amino acid sequence	sequence No.	HLA subtype
242-250	NLGATLKGV	121	A0201, A0205, B62, Cw0602
243-251	LGATLKGVA	122	B5201
244-252	GATLKGVAA	123	B61, B8
250-258	VAAGSSSSV	124	B61, B5101, B5102

[0222]

Table 25

position	amino acid sequence	sequence No.	HLA subtype
251-259	AAGSSSSVK	125	A68.1, A1101
252-260	AGSSSSVKW	126	B5801
260-268	WTEGQSNHS	127	A1

[0223]

Table 26

position	amino acid sequence	sequence No.	HLA subtype
261-269	TEGQSNHST	128	B40, B60, B61, B4403
263-271	GQSNHSTGY	129	A3, B62, B2702, Cw0702
269-277	TGYESDNHT	130	B5102, B5201
270-278	GYESDNHTT	131	A24

[0224]

Table 27

position	amino acid sequence	sequence No.	HLA subtype
272-280	ESDNHTTPI	132	A1, A3302, B5101
273-281	SDNHTTPIL	133	B40, B60, B3701, B5201
276-284	HTTPILCGA	134	B5801
278-286	TPILCGAQY	135	B3501, B4403, Cw0401, Cw0702
279-287	PILCGAQYR	136	A3101
280-288	ILCGAQYRI	137	A0201, A0205, A3, B62, B5101

[0225]

Table 28

position	amino acid sequence	sequence No.	HLA subtype
285-293	QYRIHTHGV	138	A24, Cw0401
286-294	YRIHTHGVF	139	B14, B2702, B2705, B5201, Cw0301
287-295	RIHTHGVFR	140	A3, A1101, A3101, A3302
289-297	HTHGVFRGI	141	B5801

[0226]

Table 29

position	amino acid sequence	sequence No.	HLA subtype
292-300	GVFRGIQDV	142	A0201, A0205, A3, A68.1, A1101, B3901, B5102, B5201, Cw0602
293-301	VFRGIQDVR	143	A3101
294-302	FRGIQDVRR	144	B2705
295-303	RGIQDVRRV	145	B61, B5101, B5102, B5201, Cw0602
298-306	QDVRRVPGV	146	B61, B3701
299-307	DVRRVPGVA	147	A68.1, A3302, B7, B8

[0227]

Table 30

position	amino acid sequence	sequence No.	HLA subtype
301-309	RRVPGVAPT	148	B14, B2702, B2705, Cw0301
302-310	RVPGVAPTL	7	A0205, A24, B7, B3701, B3801, B3901, B3902
303-311	VPGVAPTLV	149	B7, B3501, B5102, B5201, Cw0401
306-314	VAPTLVRSA	150	B4403
309-317	TLVRSASET	151	A0201, A0205

[0228]

Table 31

position	amino acid sequence	sequence No.	HLA subtype
312-320	RSASETSEK	152	A68.1, A1101
313-321	SASETSEKR	153	A3101, A3302
315-323	SETSEKRPF	154	B40, B3701, B4403
316-324	ETSEKRPFM	155	B8, B3501
317-325	TSEKRPFMC	156	A1, B5801
318-326	SEKRPFMCA	157	B40, B60, B61, B4403
319-327	EKRPFMCAY	158	Cw0602, Cw0702

[0229]

Table 32

position	amino acid sequence	sequence No.	HLA subtype
324-332	MCAYPGCNK	159	A68.1, A1101
325-333	CAYPGCNKR	160	A1, A68.1, A1101, A3101, A3302
326-334	AYPGCNKRY	161	A24, Cw0401, Cw0702
327-335	YPGCNKRYF	162	B3501, B3801, B5201, Cw0401, Cw0702
329-337	GCNKRYFKL	163	A24, B14, B60, B7, B8, B3902, Cw0301

[0230]

Table 33

position	amino acid sequence	sequence No.	HLA subtype
332-340	KRYFKLSHL	164	B14, B8, B2702, B2705, B3901, B3902, Cw0301, Cw0602
334-342	YFKLSHLQM	165	Cw0401
337-345	LSHLQMHSR	166	A68.1, A3302
340-348	LQMHSRKHT	167	A0201, A0205

[0231]

Table 34

position	amino acid sequence	sequence No.	HLA subtype
343-351	HSRKHTGEK	168	A68.1
345-353	RKHTGEKPY	169	Cw0702
347-355	HTGEKPYQC	170	B8, B5801
349-357	GEKPYQCDF	171	B40, B3701, B4403

[0232]

Table 35

position	amino acid sequence	sequence No.	HLA subtype
351-359	KPYQCDFKD	172	B5102
354-362	QCDFKDCER	173	A1, A68.1, A3101, A3302
356-364	DFKDCERRF	174	A24, Cw0401
358-366	KDCERRFSR	175	A3101

[0233]

Table 36

position	amino acid sequence	sequence No.	HLA subtype
362-370	RRFSRSDQL	176	B2702, B2705, B3901, Cw0301, Cw0602
363-371	RFSRSDQLK	177	A1101
364-372	FSRSDQLKR	178	A68.1, A3302
366-374	RSDQLKRHQ	179	A1
368-376	DQLKRHQRR	180	A68.1, A3101, A3302

[0234]

Table 37

position	amino acid sequence	sequence No.	HLA subtype
371-379	KRHQRRHTG	181	B14
372-380	RHQRRHTGV	182	B14, B3801, B3901
373-381	HQRRHTGVK	183	A1101, A3101, B2705
375-383	RRHTGVKPF	184	B2702, B2705, Cw0702
379-387	GVKPFQCKT	185	A68.1

[0235]

Table 38

position	amino acid sequence	sequence No.	HLA subtype
383-391	FQCKTCQRK	186	A3, A1101, A3101, B2705
384-392	QCKTCQRKF	187	B62, B8
386-394	KTCQRKFSR	188	A1, A3, A68.1, A1101, A3101
387-395	TCQRKFSRS	189	B8
389-397	QRKFSRSDH	190	B2702, B2705
390-398	RKFSRSDHL	191	B14, B3901, B3902, Cw0301

[0236]

Table 39

position	amino acid sequence	sequence No.	HLA subtype
391-399	KFSRSDHLK	192	A1101, A3101
394-402	RSDHLKTHT	193	A1, B5801
396-404	DHLKTHTRT	194	B3801, B3901

[0237]

Table 40

position	amino acid sequence	sequence No.	HLA subtype
401-409	HTRTHTGKT	195	B8
406-414	TGKTSEKPF	196	B5201
408-416	KTSEKPFSC	197	A0201, B5801
409-417	TSEKPFSCR	198	A1, A68.1, A3302
410-418	SEKPFSCRW	199	B40, B4403

[0238]

Table 41

position	amino acid sequence	sequence No.	HLA subtype
412-420	KPFSCRWPS	200	B3501, B5102
415-423	SCRWPSCQK	201	A1101
416-424	CRWPSCQKK	202	B2702, B2705
417-425	RWPSCQKKF	203	A24, B3801, Cw0401
418-426	WPSCQKKFA	204	B5102
419-427	PSCQKKFAR	205	A3302
420-428	SCQKKFARS	206	B8

[0239]

Table 42

position	amino acid sequence	sequence No.	HLA subtype
423-431	KKFARSDEL	207	B14, B3901, B3902, Cw0301, Cw0602
424-432	KFARSDELV	208	Cw0401
425-433	FARSDELVR	209	A68.1, A1101, A3101, A3302
426-434	ARSDELVRH	210	B2702, B2705
427-435	RSDELVRHH	211	A1
428-436	SDELVRHHN	212	B3701
429-437	DELVRHHNM	213	B14, B40, B60, B61, B3701, B4403, Cw0301

[0240]

Table 43

position	amino acid sequence	sequence No.	HLA subtype
432-440	VRHHNMHQR	214	B2705
433-441	RHHNMHQRN	215	B3801
434-442	HHNMHQRNM	216	B3901
436-444	NMHQRNMTK	217	A3, A1101, A3101
437-445	MHQRNMTKL	218	B14, B3701, B3901, B3902, Cw0301
439-447	QRNMTKLQL	220	B14, B2702, B2705, B3901, Cw0602
440-448	RNMTKLQLA	221	B61

[0241]

Table 44

position	amino acid sequence	sequence No.	HLA subtype
441-449	NMTKLQLAL	222	A0201, A0205, A24, A3, B7, B3902, Cw0602

[0242]

Preferable examples of the "MHC class I-restricted WT1 peptide" include a peptide comprising any amino acid sequence selected from the following amino acid sequences:

RMFPNAPYL (SEQ ID NO: 2),
CMTWNQMNL (SEQ ID NO: 3),
ALLPAVPSL (SEQ ID NO: 5),
SLGEQQYSV (SEQ ID NO: 6) and
RVPGVAPTL (SEQ ID NO: 7), and
a peptide comprising an altered amino acid sequence, which is any amino acid sequence selected from SEQ ID NOs: 2, 3, 5, 6 and 7 but containing alteration of amino acid residue(s), and having a CTL induction activity, more preferably a peptide of any of the amino acid sequences selected from SEQ ID NOs: 2, 3, 5, 6 and 7.

[0243]

The "peptide comprising an amino acid sequence" in the present invention means, as usual, a peptide wherein a further amino acid is added to the N-terminal amino acid and/or C-terminal amino acid of the amino acid sequence. When the "MHC class I-restricted WT1 peptide" in the "cancer antigen peptide A" and "cancer antigen peptide B" is added, a peptide with addition to the C-terminal side is preferable. When the "MHC class I-restricted WT1 epitope" is added, addition to the C-terminal side is preferable.

[0244]

The "peptide comprising an altered amino acid sequence that contains alteration of amino acid residue(s) in the amino acid sequence, and having a CTL induction activity" in the present invention is also called an "altered killer peptide". The altered killer peptide means a peptide that consists of an amino acid sequence wherein 1 to 3 amino acids are deleted, substituted and/or added and binds to MHC class I to induce CTL. The substitution position of the substituted amino acid includes the 1st-position (N-terminal), the 2nd-position, the 3rd-position or the 9th-position for a peptide consisting of 9 amino acid residues. The number of the amino acids to be added (also including insertion) is preferably 1 or 2, more preferably 1. A

preferable addition position is the C-terminal. The number of the amino acids to be deleted is preferably 1. In the alteration, the amino acid to be added or amino acid to be substituted may be a non-natural amino acid other than the 20 kinds of amino acids encoded by the gene.

[0245]

It is known that the amino acid sequence of a peptide bindable to HLA antigen has regularity (binding motif) for each polymorphism of HLA subtype. For example, as a binding motif of HLA-A24, a peptide consisting of 8 - 11 amino acid residues, wherein the 2nd-position amino acid is Tyr, Phe, Met or Trp, and the C-terminal amino acid is Phe, Leu, Ile, Trp or Met, is known (J. Immunol., 152, p3913, 1994, J. Immunol., 155, p4307, 1994, Immunogenetics, 41, p178, 1995). Therefore, for example, in the case of a peptide consisting of 9 amino acid residues, the 2nd-position can be substituted by Tyr, Phe, Met or Trp and/or the 9th-position can be substituted by Phe, Leu, Ile, Trp or Met, and a peptide having such substitutions is preferable as an altered killer peptide. Similarly, a peptide consisting of 8 - 11 amino acid residues, wherein the 2nd-position amino acid is Leu or Met and the C-terminal amino acid is Val or Leu, is known as a binding motif of HLA-A0201. Therefore, for example, in the case of a peptide consisting of 9 amino acid residues, the 2nd-position can be substituted by Leu or Met and/or the 9th-position can be substituted by Val or Leu, and a peptide having such substitutions is preferable as an altered killer peptide.

[0246]

Examples of the altered killer peptide include the following peptides.

RYFPNAPYL (SEQ ID NO: 223) (see WO03/106682),

FMFPNAPYL (SEQ ID NO: 224),

RLFPNAPYL (SEQ ID NO: 225),

RMMPNAPYL (SEQ ID NO: 226),

RMFPNAPYV (SEQ ID NO: 227) and

YMFPNAPYL (SEQ ID NO: 228) (see WO2009/072610), which are altered killer peptides of RMFPNAPYL (SEQ ID NO: 2);

[0247]

CYTWNQMNL (SEQ ID NO: 4) (see WO02/79253);

Xaa-Met-Thr-Trp-Asn-Gln-Met-Asn-Leu (SEQ ID NO: 229) (wherein Xaa is Ser or Ala) and

Xaa-Tyr-Thr-Trp-Asn-Gln-Met-Asn-Leu (SEQ ID NO: 230) (wherein Xaa is Ser, Ala, Abu, Arg, Lys, Orn, Cit, Leu, Phe or Asn) (see WO2004/026897), which are altered killer peptides of CMTWNQMNL (SEQ ID NO: 3);

[0248]

AYLPAVPSL (SEQ ID NO: 231) (see WO2003/106682), which is an altered killer peptide of ALLPAVPSL (SEQ ID NO: 5);

[0249]

FLGEQQYSV (SEQ ID NO: 232),

SMGEQQYSV (SEQ ID NO: 233) and

SLMEQQYSV (SEQ ID NO: 234) (WO2009/072610), which are altered killer peptides of SLGEQQYSV (SEQ ID NO: 6); and

[0250]

RYPGVAPTL (SEQ ID NO: 235) (WO2003/106682), which is an altered killer peptide of RVPGVAPTL (SEQ ID NO: 7).

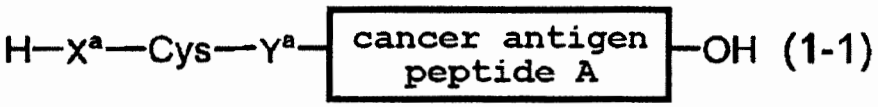
[0251]

"R^1" in the present invention is a hydrogen atom, a group represented by the aforementioned formula (2) or cancer antigen peptide C, preferably a group represented by the aforementioned formula (2) or cancer antigen peptide C.

[0252]

When R^1 is a hydrogen atom, the compound of the formula (1) is a compound represented by the formula (1-1):

[0253]



[0254]

wherein X^a, Y^a and cancer antigen peptide A are as defined in the

above for the formula (1), and Cys is a cysteine residue, namely, a peptide.

[0255]

The compound of the formula (1), wherein R^1 is a hydrogen atom, namely, a peptide represented by the formula (1-1), has a sequence different from a partial sequence of WT1 protein. The requirement of the formula (1), "has a sequence different from a partial sequence of WT1 protein" means that a peptide represented by the formula (1-1) is not a partial peptide consisting of continuous 8 - 35 amino acid residues in the amino acid sequence of human WT1 described in SEQ ID NO: 1.

That is, the compound of the formula (1), wherein R^1 is a hydrogen atom, is not a partial peptide consisting of continuous 8 - 35 amino acid residues in the amino acid sequence of human WT1 described in SEQ ID NO: 1. A specific explanation is given by taking a case when the cancer antigen peptide A is a $WT1_{138-146}$ peptide as an example. $WT1_{138-146}$ peptide is a partial peptide consisting of continuous 9 amino acid residues at the 138th-position - 146th-position of the amino acid sequence of human WT1 described in SEQ ID NO: 1, and has an amino acid sequence of LESQPAIRN (SEQ ID NO: 78). In SEQ ID NO: 1, the 137th-position continuing from the N-terminal side of $WT1_{138-146}$ peptide is C. Therefore, $WT1_{137-146}$ peptide (CLESQPAIRN) (SEQ ID NO: 236) corresponds to a partial peptide consisting of continuous 10 amino acid residues of the amino acid sequence of human WT1 described in SEQ ID NO: 1. On the other hand, based on the requirement of the present invention, "the compound of the formula (1), wherein R^1 is a hydrogen atom, is not a partial peptide consisting of continuous 8 - 35 amino acid residues in the amino acid sequence of human WT1 described in SEQ ID NO: 1", in the compound of the formula (1) wherein R^1 is a hydrogen atom, when the cancer antigen peptide A is $WT1_{138-146}$ peptide (LESQPAIRN) (SEQ ID NO: 78), $WT1_{137-146}$ peptide (CLESQPAIRN) (SEQ ID NO: 236) is excluded from the compound of the present invention, and

therefore, X^a and Y^a are not simultaneously a single bond.

[0256]

As a compound of the formula (1) wherein R^1 is a hydrogen atom, a peptide consisting of any amino acid sequence selected from the following amino acid sequences:

CRMFPNAPYL (SEQ ID NO: 13),

CCMTWNQMNL (SEQ ID NO: 14),

CCYTWNQMNL (SEQ ID NO: 15),

CALLPAVPSL (SEQ ID NO: 16),

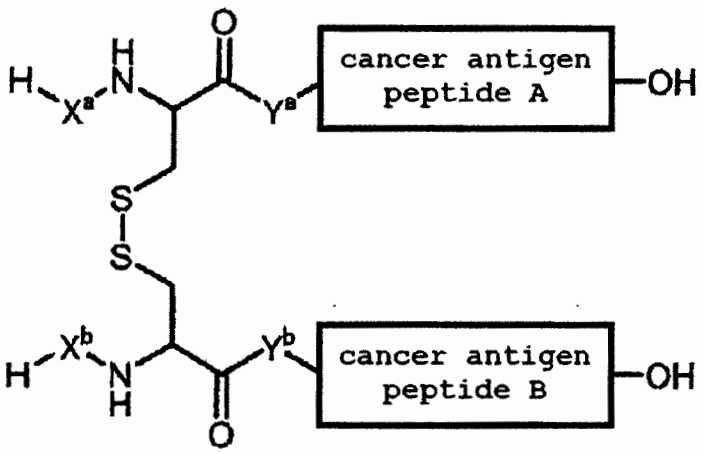
CSLGEQQYSV (SEQ ID NO: 17) and

CRVPGVAPTL (SEQ ID NO: 18) is preferable.

[0257]

When "R^1" is a group represented by the aforementioned formula (2), a compound of the formula (1) is a compound represented by the formula (1-2):

[0258]



(1-2)

[0259]

wherein X^a, Y^a and cancer antigen peptide A are as defined in the above for the formula (1), and X^b, Y^b and cancer antigen peptide B are as defined in the above for the formula (2).

[0260]

"X^b" and "Y^b" in the present invention mean, independently, a single bond or a divalent group of peptides consisting of 1 - 4 amino acid residues. The sum of the amino acid residue number of X^b and that of Y^b is an integer of 0 - 4. For example, an

integer of said sum being 0 means that X^b and Y^b are each a single bond. When the sum is an integer of 4, examples thereof include X^b and Y^b independently being divalent groups of peptide consisting of 2 amino acid residues, X^b being a divalent group of peptide consisting of 3 amino acid residues and Y^b being a divalent group of peptide consisting of 1 amino acid residue, X^b being a divalent group of peptide consisting of 4 amino acid residues and Y^b being a single bond and the like.

The integer of said sum is preferably 0 - 2, more preferably 0 - 1, most preferably 0. That is, X^b and Y^b are most preferably single bonds.

When the sum is an integer of 2, examples thereof include X^b being a divalent group of peptide consisting of 2 amino acid residues and Y^b being a single bond, X^b and Y^b independently being divalent groups of peptide consisting of 1 amino acid residue, and X^b being a single bond and Y^b being a divalent group of peptide consisting of 2 amino acid residues.

When the sum is an integer of 1, examples thereof include X^b being a divalent group of peptide consisting of 1 amino acid residue and Y^b being a single bond, and X^b being a single bond and Y^b being a divalent group of peptide consisting of 1 amino acid residue. Of these, preferred is X^b being a single bond and Y^b being an alanine residue, leucine residues or methionine residue.

[0261]

The "cancer antigen peptide B" in the present invention is an MHC class I-restricted WT1 peptide consisting of 7 - 30 amino acid residues. The "MHC class I-restricted WT1 peptide" is as defined above. However, in the compound represented by the formula (1), cancer antigen peptide A and cancer antigen peptide B are not simultaneously the same peptide. That is, cancer antigen peptide B is limited by the requirement, "different from cancer antigen peptide A".

Since cancer antigen peptide A and cancer antigen peptide

B are not simultaneously the same peptide, the compound of the formula (1), wherein R^1 is a group represented by the aforementioned formula (2), is not a homodimer but a heterodimer, even when X^a and X^b are the same and Y^a and Y^b are the same. Homodimer means a dimer wherein the same peptide monomers are dimerized, and heterodimer means a dimer wherein different peptide monomers are dimerized.

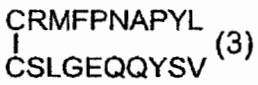
[0262]

In cancer antigen peptide B, the amino group of the N-terminal amino acid is bonded to Y^b in the formula (2) (i.e., also bonded to Y^b in the formula (1-2)), and the carbonyl group of the C-terminal amino acid is bonded to the hydroxyl group in the formula (2).

[0263]

As a compound of the formula (1) wherein R^1 is a group represented by the formula (2), i.e., a compound of the formula (1-2), a compound represented by the formula (3):

[0264]



(3)

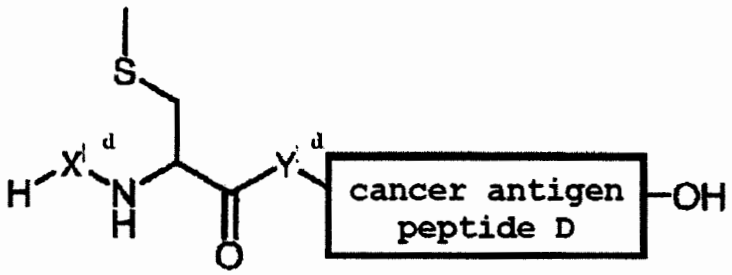
[0265]

wherein the bond between C and C is a disulfide bond, is preferable.

[0266]

In the present invention, moreover, when the "cancer antigen peptide B" is an MHC class I-restricted WT1 peptide containing one cysteine residue, the compound of the formula (1) may be a compound wherein the thioether group in the cancer antigen peptide B is bonded to a thioether group in the formula (16):

[0267]



(16)

[0268]

or to a thioether group of the cysteine residue of the cancer antigen peptide E.

[0269]

"X^d" and "Y^d" in the present invention mean, independently, a single bond or a divalent group of peptides consisting of 1 - 4 amino acid residues. The sum of the amino acid residue number of X^d and that of Y^d is an integer of 0 - 4. For example, an integer of said sum being 0 means that X^d and Y^d are each a single bond. When the sum is an integer of 4, examples thereof include X^d and Y^d independently being divalent groups of peptide consisting of 2 amino acid residues, X^d being a divalent group of peptide consisting of 3 amino acid residues and Y^d being a divalent group of peptide consisting of 1 amino acid residue, X^d being a divalent group of peptide consisting of 4 amino acid residues and Y^d being a single bond and the like.

The integer of said sum is preferably 0 - 2, more preferably 0 - 1, most preferably 0. That is, X^d and Y^d are most preferably single bonds.

When the sum is an integer of 2, examples thereof include X^d being a divalent group of peptide consisting of 2 amino acid residues and Y^b being a single bond, X^d and Y^d independently being divalent groups of peptide consisting of 1 amino acid residue, or X^d being a single bond and Y^d being a divalent group of peptide consisting of 2 amino acid residues.

When the sum is an integer of 1, examples thereof include X^d being a divalent group of peptide consisting of 1 amino acid residue and Y^d being a single bond, and X^d being a single bond

and Y^d being a divalent group of peptide consisting of 1 amino acid residue. Of these, preferred is X^d being a single bond and Y^d being an alanine residue, leucine residues or methionine residue.

[0270]

The "cancer antigen peptide D" in the present invention is an MHC class II-restricted WT1 peptide consisting of 7 - 30 amino acid residues. In the formula (16), an amino group of the N-terminal amino acid of the cancer antigen peptide D binds to Y^d in the formula (16), and a carbonyl group of the C-terminal amino acid binds to a hydroxyl group in the formula (16).

[0271]

In the present invention, "MHC class II-restricted" means the property to induce helper T cell by binding to an MHC class II molecule, and is as defined for the below-mentioned "cancer antigen peptide C".

[0272]

HLA corresponding to the MHC class II-molecule is classified into subtypes of HLA-DR, DQ and DP and the like. Preferable examples of the "MHC class II-restricted" include HLA-DR-restricted, HLA-DQ-restricted and HLA-DP-restricted.

[0273]

Therefore, the "MHC class II-restricted WT1 peptide" in the present invention is a peptide that binds to an MHC class II antigen in vitro and/or in vivo and induces helper T cells. The number of the amino acid residues of the "MHC class II-restricted WT1 peptide" is 7 - 30, preferably 14 - 30.

[0274]

As the "cancer antigen peptide D", like the amino acid sequence recited in the below-mentioned "cancer antigen peptide C", a peptide consisting of any amino acid sequence selected from the following amino acid sequences:

SGQARMFPNAPYLPSC (SEQ ID NO: 19),

SGQAYMFPNAPYLPSC (SEQ ID NO: 25),

SGQARMFPNAPYLPSCLES (SEQ ID NO: 11),
SGQAYMFPNAPYLPSCLES (SEQ ID NO: 12),
PGCNKRYFKLSHLQMHSRK (SEQ ID NO: 20),
PGCNKRYFKLSHLQMHSRKH (SEQ ID NO: 21),
PGCNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 10),
CNKRYFKLSHLQMHSRK (SEQ ID NO: 22),
CNKRYFKLSHLQMHSRKH (SEQ ID NO: 23),
CNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 24) and
WAPVLDFAPPGASAYGSL (SEQ ID NO: 244)
can be mentioned.

[0275]

In the compound of the formula (1), moreover, when the "cancer antigen peptide B" is an MHC class I-restricted WT1 peptide containing one cysteine residue, as a compound wherein the thioether group in the cancer antigen peptide B is bonded to a thioether group in the formula (16), preferably, a compound represented by the formula (15):

[0276]



(15)

[0277]

wherein the bond between C and C is a disulfide bond, can be mentioned.

[0278]

In the present invention, the "cancer antigen peptide E" is an MHC class II-restricted WT1 peptide consisting of 7 - 30 amino acid residues containing one cysteine residue, and is as defined for the "MHC class II-restricted WT1 peptide consisting of 7 - 30 amino acid residues containing one cysteine residue" in the below-mentioned "cancer antigen peptide C".

[0279]

As the "cancer antigen peptide E", like the amino acid sequence recited in the below-mentioned "cancer antigen peptide

C", a peptide consisting of any amino acid sequence selected from the following amino acid sequences:
SGQARMFPNAPYLPSC (SEQ ID NO: 19),
SGQAYMFPNAPYLPSC (SEQ ID NO: 25),
SGQARMFPNAPYLPSCLES (SEQ ID NO: 11),
SGQAYMFPNAPYLPSCLES (SEQ ID NO: 12),
PGCNKRYFKLSHLQMHSRK (SEQ ID NO: 20),
PGCNKRYFKLSHLQMHSRKH (SEQ ID NO: 21),
PGCNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 10),
CNKRYFKLSHLQMHSRK (SEQ ID NO: 22),
CNKRYFKLSHLQMHSRKH (SEQ ID NO: 23) and
CNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 24)
can be mentioned.

[0280]

When R^1 is cancer antigen peptide C, the thioether group of the cysteine residue of cancer antigen peptide C is bonded to the thioether group in the formula (1).

The cancer antigen peptide C is an MHC class I-restricted WT1 peptide consisting of 7 - 30 amino acid residues containing one cysteine residue or an MHC class II-restricted WT1 peptide consisting of 7 - 30 amino acid residues containing one cysteine residue.

[0281]

In the "MHC class I-restricted WT1 peptide consisting of 7 - 30 amino acid residues containing one cysteine residue" in the present invention, the amino acid sequence of the peptide only needs to contain at least one cysteine residue. The number of the cysteine residues to be contained is preferably 1 - 3, more preferably 1 - 2, most preferably 1. The "MHC class I-restricted WT1 peptide" is as defined above. Also, the compound of the formula (1) wherein R^1 is "an MHC class I-restricted WT1 peptide consisting of 7 - 30 amino acid residues containing one cysteine residue" is not a homodimer but a heterodimer.

[0282]

Preferable examples of the "MHC class I-restricted WT1 peptide consisting of 7 - 30 amino acid residues containing one cysteine residue" include peptides described in Tables 45 - 52. In each Table, the "position" means the position in the amino acid sequence of human WT1 described in SEQ ID NO: 1.

[0283]

Table 45

position	amino acid sequence	sequence No.	HLA subtype
17-25	SLGGGGGCA	31	B62
18-26	LGGGGGCAL	32	B60, B7, B3801, B5101, B5102
20-28	GGGGCALPV	33	B61, B5101, B5102, B5201
23-31	GCALPVSGA	34	B40, B61
24-32	CALPVSGAA	35	B40, B5102, Cw0301

[0284]

Table 46

position	amino acid sequence	sequence No.	HLA subtype
80-88	GAEPHEEQC	51	A1
81-89	AEPHEEQCL	52	A0205, B40, B60, B61, B3701, B4403
82-90	EPHEEQCLS	53	B3501, B5101
83-91	PHEEQCLSA	54	B3801
84-92	HEEQCLSAF	55	B40, B3701, B4403, Cw0702
85-93	EEQCLSAFT	56	B40, B60, B61, B3701, B4403
86-94	EQCLSAFTV	57	A0201, B62, B5201
88-96	CLSAFTVHF	58	A3, B62
99-107	QFTGTAGAC	63	Cw0401
100-108	FTGTAGACR	64	A68.1, A1101, A3101, A3302

[0285]

Table 47

position	amino acid sequence	sequence No.	HLA subtype
101-109	TGTAGACRY	65	B62, B4403, Cw0702
104-112	AGACRYGPF	66	B4403, B5201
107-115	CRYGPFGPP	67	B2702
130-138	NAPYLPSCL	75	A24, B60, B7, B8, B3902, B5101, B5102, Cw0301, Cw0602, Cw0702
136-144	SCLESQPAI	76	B8, B3901, B5102, Cw0301
137-145	CLESQPAIR	77	A1, A3, A68.1, A1101, A3101, A3302
194-202	SVPPPVYGC	97	A0205, A3

[0286]

Table 48

position	amino acid sequence	sequence No.	HLA subtype
202-210	CHTPTDSCT	98	B3801
204-212	TPTDSCTGS	99	B5101
206-214	TDSCTGSQA	100	B40, B61, B3701
207-215	DSCTGSQAL	101	A24, A3302, B60, B7, B8, B3501, B3901, B3902, Cw0602
208-216	SCTGSQALL	102	B60, B7, B8, B3701, B3801, B3901, B3902
209-217	CTGSQALLL	103	B60, B7, B3701, B3902
227-235	YQMTSQLEC	114	A0201, A0205, B62
228-236	QMTSQLECM	115	A0201

[0287]

Table 49

position	amino acid sequence	sequence No.	HLA subtype
230-238	TSQLECMTW	116	B5801
232-240	QLECMTWNQ	117	A1
233-241	LECMTWNQM	118	B40, B60, B61, B3701, B4403
235-243	CMTWNQMNL	3	A0201, A0205, A24, A3, B7
276-284	HTTPILCGA	134	B5801
278-286	TPILCGAQY	135	B3501, B4403, Cw0401, Cw0702
279-287	PILCGAQYR	136	A3101
280-288	ILCGAQYRI	137	A0201, A0205, A3, B62, B5101

[0288]

Table 50

position	amino acid sequence	sequence No.	HLA subtype
317-325	TSEKRPFMC	156	A1, B5801
318-326	SEKRPFMCA	157	B40, B60, B61, B4403
319-327	EKRPFMCAY	158	Cw0602, Cw0702
324-332	MCAYPGCNK	159	A68.1, A1101
325-333	CAYPGCNKR	160	A1, A68.1, A1101, A3101, A3302
326-334	AYPGCNKRY	161	A24, Cw0401, Cw0702
327-335	YPGCNKRYF	162	B3501, B3801, B5201, Cw0401, Cw0702
329-337	GCNKRYFKL	163	A24, B14, B60, B7, B8, B3902, Cw0301
347-355	HTGEKPYQC	170	B8, B5801
349-357	GEKPYQCDF	171	B40, B3701, B4403

[0289]

Table 51

position	amino acid sequence	sequence No.	HLA subtype
351-359	KPYQCDFKD	172	B5102
354-362	QCDFKDCER	173	A1, A68.1, A3101, A3302
356-364	DFKDCERRF	174	A24, Cw0401
358-366	KDCERRFSR	175	A3101
379-387	GVKPFQCKT	185	A68.1
383-391	FQCKTCQRK	186	A3, A1101, A3101, B2705
384-392	QCKTCQRKF	187	B62, B8
386-394	KTCQRKFSR	188	A1, A3, A68.1, A1101, A3101
387-395	TCQRKFSRS	189	B8

[0290]

Table 52

position	amino acid sequence	sequence No.	HLA subtype
408-416	KTSEKPFSC	197	A0201, B5801
409-417	TSEKPFSCR	198	A1, A68.1, A3302
410-418	SEKPFSCRW	199	B40, B4403
412-420	KPFSCRWPS	200	B3501, B5102
415-423	SCRWPSCQK	201	A1101
416-424	CRWPSCQKK	202	B2702, B2705
417-425	RWPSCQKKF	203	A24, B3801, Cw0401
418-426	WPSCQKKFA	204	B5102
419-427	PSCQKKFAR	205	A3302
420-428	SCQKKFARS	206	B8

[0291]

More preferable examples of the "MHC class I-restricted WT1 peptide consisting of 7 - 30 amino acid residues containing one cysteine residue" include a peptide comprising the following amino acid sequence:

CMTWNQMNL (SEQ ID NO: 3)

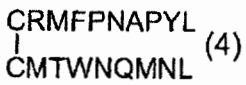
and a peptide comprising an altered amino acid sequence, which is the amino acid sequence of SEQ ID NO: 3 but containing alteration of amino acid residue(s), and having a CTL induction

activity. Said "containing the amino acid sequence" and "peptide comprising an altered amino acid sequence containing alteration of amino acid residue(s) in an amino acid sequence, and having a CTL induction activity" are as defined above. Most preferably, a peptide consisting of any amino acid sequence selected from the following amino acid sequences:
CMTWNQMNL (SEQ ID NO: 3) and
CYTWNQMNL (SEQ ID NO: 4),
can be mentioned.

[0292]

As the compound of the formula (1) wherein R^1 is "an MHC class I-restricted WT1 peptide consisting of 7 - 30 amino acid residues containing one cysteine residue", a compound represented by the formula (4):

[0293]

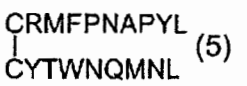


(4)

[0294]

wherein the bond between C and C is a disulfide bond, or a compound represented by the formula (5):

[0295]



(5)

[0296]

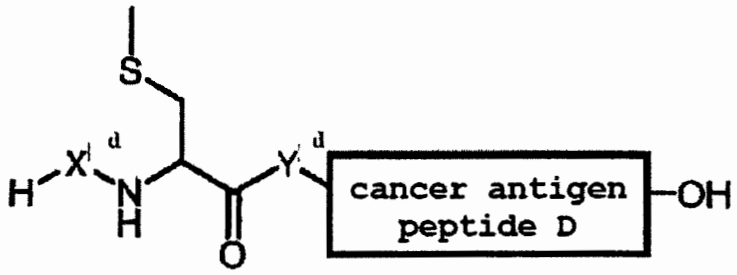
wherein the bond between C and C is a disulfide bond, is preferable. Of these, a compound represented by the formula (5) is more preferable.

[0297]

In the present invention, moreover, when a peptide consisting of 1 - 4 amino acid residues containing one cysteine residue is further bonded to the N-terminal of the "cancer antigen peptide C" which is an MHC class I-restricted WT1 peptide, the compound of the formula (1) may be a compound wherein the thioether group of the cysteine residue of the

peptide bonded to the N-terminal of the cancer antigen peptide C is bonded to a thioether group in the formula (16):

[0298]



(16)

[0299]

or to a thioether group of the cysteine residue of the cancer antigen peptide E.

[0300]

The "X^d", "Y^d", "cancer antigen peptide D" and "cancer antigen peptide E" in the present invention are as defined for the aforementioned "X^d", "Y^d", "cancer antigen peptide D" and "cancer antigen peptide E".

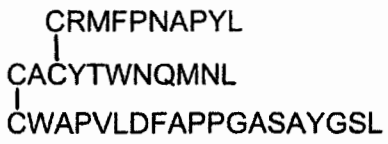
[0301]

In the present invention, the peptide consisting of 1 - 4 amino acid residues containing one cysteine residue, which is bonded to the N-terminal of the "cancer antigen peptide C" which is an MHC class I-restricted WT1 peptide, is preferably a dipeptide consisting of CA.

[0302]

In the compound of the formula (1), when a peptide consisting of 1 - 4 amino acid residues containing one cysteine residue is further bonded to the N-terminal of the "cancer antigen peptide C" which is an MHC class I-restricted WT1 peptide, the compound wherein the thioether group of the cysteine residue of the peptide bonded to the N-terminal of the cancer antigen peptide C is bonded to a thioether group in the formula (16) is preferably a compound represented by the formula (14):

[0303]



(14)

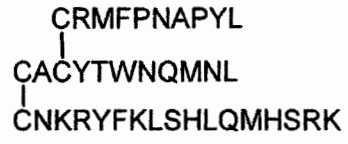
[0304]

wherein the bond between C and C is a disulfide bond.

[0305]

In addition, in the compound of the formula (1), when a peptide consisting of 1 - 4 amino acid residues containing one cysteine residue is further bonded to the N-terminal of the "cancer antigen peptide C" which is an MHC class I-restricted WT1 peptide, the compound wherein the thioether group of the cysteine residue of the peptide bonded to the N-terminal of the cancer antigen peptide C is bonded to a thioether group in the "cancer antigen peptide E" is preferably a compound represented by the formula (12):

[0306]



(12)

[0307]

wherein the bond between C and C is a disulfide bond.

[0308]

In the "MHC class II-restricted WT1 peptide consisting of 7 - 30 amino acid residues containing one cysteine residue" in the present invention, the amino acid sequence of the peptide only needs to contain at least one cysteine residue. The number of the cysteine residues to be contained is preferably 1 - 3, more preferably 1 - 2, most preferably 1.

[0309]

In the present invention, "MHC class II-restricted" means the property to induce helper T cell by binding to an MHC class II molecule.

[0310]

HLA corresponding to the MHC class II-molecule is

classified into subtypes of HLA-DR, DQ and DP and the like. Preferable examples of the "MHC class II-restricted" include HLA-DR-restricted, HLA-DQ-restricted and HLA-DP-restricted.

[0311]

Therefore, the "MHC class II-restricted WT1 peptide" in the present invention is a peptide that binds to an MHC class II antigen in vitro and/or in vivo and induces helper T cells. The number of the amino acid residues of the "MHC class II-restricted WT1 peptide" is 7 - 30, preferably 14 - 30.

[0312]

Examples of the "MHC class II-restricted WT1 peptide consisting of 7 - 30 amino acid residues containing one cysteine residue" include the peptides described in Table 53. In each Table, the "position" means a position in the amino acid sequence of human WT1 described in SEQ ID NO: 1.

[0313]

Table 53

position	amino acid sequence	sequence No.
117-139	PSQASSGQARMFPNAPYLPSCLE	237
122-140	SGQARMFPNAPYLPSCLES	11
202-233	CHTPTDSCTGSQALLLRTPYSSDNLYQMTSQL	9
328-349	PGCNKRYFKLSHLQMHSRKHTG	10
421-441	CQKKFARSDELVRHHNMHQRN	219

[0314]

As the "MHC class II-restricted WT1 peptide consisting of 7 - 30 amino acid residues containing one cysteine residue", a peptide comprising any amino acid sequence selected from the following amino acid sequences:

SGQARMFPNAPYLPSCLES (SEQ ID NO: 11) and

PGCNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 10),

or

a peptide comprising an altered amino acid sequence, which is any amino acid sequence selected from SEQ ID NOs: 10 - 11 but containing alteration of amino acid residue(s), and having a helper T cell induction activity is preferable.

[0315]

The "peptide comprising an amino acid sequence" means, as mentioned above, a peptide wherein a further amino acid is added to the N-terminal amino acid and/or C-terminal amino acid of the amino acid sequence. When added to the "MHC class II-restricted WT1 peptide containing one cysteine residue", the addition may be made to the N-terminal side and/or C-terminal side.

[0316]

The "peptide comprising an altered amino acid sequence containing alteration of amino acid residue(s) in the amino acid sequence, and having a helper T cell induction activity" in the present invention is also called an "altered helper peptide". The altered helper peptide means a peptide that consists of an amino acid sequence wherein 1 to 3 amino acids are deleted, substituted and/or added and binds to MHC class II to induce helper T cell. The number of the amino acids to be added (also including insertion) is preferably 1 - 3. The number of the amino acids to be deleted is preferably 1 - 5. In the alteration, the amino acid to be added or amino acid to be substituted may be a non-natural amino acid other than the 20 kinds of amino acids encoded by the gene.

[0317]

As the altered helper peptide, for example, the following peptides can be mentioned:

SGQAYMFPNAPYLPSCLES (SEQ ID NO: 12) (see patent document 6),

SGQARMFPNAPYLPSC (SEQ ID NO: 19) and

SGQAYMFPNAPYLPSC (SEQ ID NO: 25), which are altered helper peptide of SGQARMFPNAPYLPSCLES (SEQ ID NO: 11); and

[0318]

GCNKRYFKLSHLQMHSRK (SEQ ID NO: 20),

PGCNKRYFKLSHLQMHSRKH (SEQ ID NO: 21),

CNKRYFKLSHLQMHSRK (SEQ ID NO: 22),

CNKRYFKLSHLQMHSRKH (SEQ ID NO: 23) and

CNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 24), which are altered helper peptide of PGCNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 10).

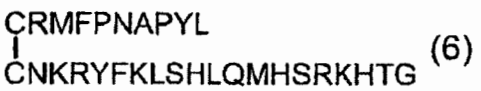
[0319]

As the "MHC class II-restricted WT1 peptide consisting of 7 - 30 amino acid residues containing one cysteine residue", a peptide consisting of any amino acid sequence selected from the following amino acid sequences:
SGQARMFPNAPYLPSC (SEQ ID NO: 19),
SGQAYMFPNAPYLPSC (SEQ ID NO: 25),
SGQARMFPNAPYLPSCLES (SEQ ID NO: 11),
SGQAYMFPNAPYLPSCLES (SEQ ID NO: 12),
PGCNKRYFKLSHLQMHSRK (SEQ ID NO: 20),
PGCNKRYFKLSHLQMHSRKH (SEQ ID NO: 21),
PGCNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 10),
CNKRYFKLSHLQMHSRK (SEQ ID NO: 22),
CNKRYFKLSHLQMHSRKH (SEQ ID NO: 23) and
CNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 24) is more preferable.

[0320]

As the compound of the formula (1) wherein R^1 is "an MHC class II-restricted WT1 peptide consisting of 7 - 30 amino acid residues containing one cysteine residue", a compound represented by the formula (6):

[0321]

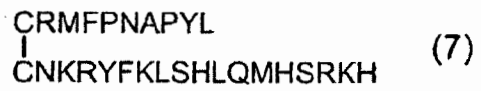


(6)

[0322]

wherein the bond between C and C is a disulfide bond,
a compound represented by the formula (7):

[0323]



(7)

[0324]

wherein the bond between C and C is a disulfide bond,
a compound represented by the formula (8):

[0325]

CRMFPNAPYL
|
CNKRYFKLSHLQMHSRK (8)

[0326]

wherein the bond between C and C is a disulfide bond, and a compound represented by the formula (9):

[0327]

CALLPAVPSL
|
CNKRYFKLSHLQMHSRKHTG (9)

[0328]

wherein the bond between C and C is a disulfide bond, are preferable.

[0329]

The present invention also provides a composition comprising the compound of the present invention and one or more MHC class II-restricted WT1 peptides.

[0330]

Examples of the compound of the present invention to be contained in the composition of the present invention include

[0331]

a compound represented by the formula (3):

[0332]

CRMFPNAPYL
|
CSLGEQQYSV (3)

[0333]

wherein the bond between C and C is a disulfide bond, a compound represented by the formula (4):

[0334]

CRMFPNAPYL
|
CMTWNQMNL (4)

[0335]

wherein the bond between C and C is a disulfide bond, and a compound represented by the formula (5):

[0336]

CRMFPNAPYL
|
CYTWNQMNL (5)

[0337]

wherein the bond between C and C is a disulfide bond.

[0338]

Examples of the MHC class II-restricted WT1 peptide to be contained in the composition of the present invention include the following amino acid sequences:

CNKRYFKLSHLQMHSRK (SEQ ID NO: 22),

CNKRYFKLSHLQMHSRKH (SEQ ID NO: 23),

CNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 24),

WAPVLDFAPPGASAYGSL (SEQ ID NO: 244),

CWAPVLDFAPPGASAYGSL (SEQ ID NO: 242) and

WAPVLDFAPPGASAYGSLC (SEQ ID NO: 243).

[0339]

The present invention also provides a synthesis method of a compound wherein two different MHC class I-restricted WT1 peptide and MHC class II-restricted WT1 peptide, or two different MHC class I-restricted WT1 epitope and MHC class II-restricted WT1 epitope are each bonded via a disulfide bond. The method of the present invention includes the following steps (1) - (3).

[0340]

In step (1) of the present invention, a peptide wherein a carbonyl group of the C-terminal amino acid of C(Mmt)A and the N-terminal amino group of the cancer antigen peptide C are bonded is synthesized by using Fmoc-C(Mmt)A-SBn and cancer antigen peptide C.

[0341]

The "cancer antigen peptide C" is as defined for the aforementioned "cancer antigen peptide C". "Fmoc" is a 9-fluorenylmethoxycarbonyl group. "Mmt" is a monomethoxytrityl group. "SBn" is a thiobenzyl group.

[0342]

In step (2) of the present invention, a peptide wherein a thioether group of the cysteine residue of the cancer antigen peptide C in the peptide obtained in the aforementioned step (1) and a thioether group of the cysteine residue bonded to the N-terminal of cancer antigen peptide A are bonded is synthesized by using the peptide obtained in the aforementioned step (1) and cancer antigen peptide A wherein one cysteine residue protected by Npys group is bonded to the N-terminal.

[0343]

The "cancer antigen peptide A" is as defined for the aforementioned "cancer antigen peptide A". "Npys" is a 3-nitro-2-pyridylthio group.

[0344]

In step (3) of the present invention, a peptide wherein a thioether group of the cysteine residue bonded to the N-terminal of the cancer antigen peptide A in the peptide obtained in the aforementioned step (2), and a thioether group of the cysteine residue of the cancer antigen peptide D are bonded, is synthesized by using the peptide obtained in the aforementioned step (2) and cancer antigen peptide D containing a cysteine residue protected by Spy group.

[0345]

The "cancer antigen peptide D" is as defined for the aforementioned "cancer antigen peptide D". "SPy" is a 2-pyridylsulfide group.

[0346]

The compound and peptide of the present invention, and peptides to be intermediates therefor can be produced according to the method described in the Examples of the present specification or a method to be generally used for the peptide synthesis. Examples of the production method include the methods described in the documents (Peptide Synthesis, Interscience, New York, 1966; The Proteins, Vol. 2, Academic Press Inc., New York, 1976; peptide synthesis, Maruzen Co., LTD.,

1975; Basics and Experiment of Peptide Synthesis, Maruzen Co., LTD., 1985; Development of Pharmaceutical Product subsequent vol. 14, Peptide Synthesis, Hirokawa Shoten, 1991) and the like.

Examples thereof include a production method by a solid phase synthesizer using Fmoc method or Boc method, and a production method by sequential condensation of Boc-amino acid or Z-amino acid by liquid phase synthesis process (Fmoc is a 9-fluorenylmethoxycarbonyl group, Boc is a t-butoxycarbonyl group, and Z is a benzyloxycarbonyl group).

In the intermediate for the production of the compound of the present invention, a functional group such as an amino group, a carboxy group, a mercapto group and the like can be protected by a suitable protecting group or deprotected as necessary using protection and deprotection techniques. As preferable protecting groups, protection method and deprotection method are described in detail in "Protective Groups in Organic Synthesis 2nd Edition (John Wiley & Sons, Inc.; 1990)" and the like. Examples of the mercapto-protecting group include an acetamidomethyl group, a trityl group and the like.

[0347]

When the compound of the present invention has a disulfide bond, the disulfide bond can be formed between two different peptides containing a cysteine residue or between peptide containing a cysteine residue and cysteine according to a method generally used for peptide chemistry. Examples of the formation method of the disulfide bond include the methods described in the documents (Peptide Synthesis, Interscience, New York, 1966; The Proteins, Vol. 2, Academic Press Inc., New York, 1976; peptide synthesis, Maruzen Co., LTD., 1975; Basics and Experiment of peptide synthesis, Maruzen Co., LTD., 1985; Development of Pharmaceutical Product sequential vol. 14, Peptide Synthesis, Hirokawa Shoten, 1991) and the like.

[0348]

Specifically, when a peptide contains one cysteine residue,

a compound having a disulfide bond (disulfide compound) can be produced by removing all protecting groups including the mercapto-protecting group on the cysteine side chain and oxidizing in an inert solvent. In addition, it can be produced by mixing two intermediates having a mercapto group in a suitable solvent to allow oxidation. As a method for the oxidation, a known method for forming a disulfide bond in general peptide synthesis can be selected as appropriate. For example, iodine oxidation, a method including air oxidation reaction under alkali conditions, a method for forming a disulfide bond by adding an oxidant under alkaline or acidic conditions and the like can be mentioned. Here, as the oxidant, iodine, dimethyl sulfoxide (DMSO), potassium ferricyanide and the like can be mentioned. As the solvent, water, acetic acid, methanol, chloroform, DMF, DMSO and the like, or a mixture thereof can be used. An oxidation reaction often affords a mixture of symmetric, asymmetric disulfide compounds. The object asymmetric disulfide compound can be obtained by purifying by various chromatography, recrystallization and the like. Alternatively, an intermediate having an activated mercapto group and an intermediate having a mercapto group are mixed to form a selective disulfide bond. As the intermediate having an activated mercapto group, a mercapto group bonded with an Npys group (3-nitro-2-pyridinesulphenyl group) and the like can be mentioned. Alternatively, one intermediate and, for example, 2,2'-dithiobis(5-nitropyridine) are mixed in advance to activate the mercapto group, and then the other intermediate is added, whereby a selective disulfide bond can be formed (Tetrahedron Letters. Vol. 37. No. 9, pp. 1347-1350).

[0349]

Also, when two or more cysteine residues are contained in the peptide, a method similar to the aforementioned method can be used. In this case, an isomer with a different manner of disulfide bond is obtained. A dimer wherein a disulfide bond is

formed between the object cysteine residues can be obtained by using a particular combination of the cysteine side chain-protecting groups. As the aforementioned combination of protecting groups, MeBzl (methylbenzyl) group and Acm (acetamidomethyl) group, Trt (trityl) group and Acm group, Npys (3-nitro-2-pyridylthio) group and Acm group, S-Bu-t (S-tert-butyl) group and Acm group and the like can be mentioned. For example, in the case of a combination of MeBzl group and Acm group, a method of forming a disulfide bond between cysteine residues protected Acm group, which includes removing protecting group other than MeBzl group and cysteine side chain, subjecting a solution containing a peptide monomer to air oxidation reaction to form a disulfide bond between the deprotected cysteine residues, and then performing deprotection with iodine and oxidation and the like can be mentioned.

[0350]

The obtained compound, peptide and intermediate of the present invention can be purified according to a method known to those of ordinary skill in the art and a method generally used for peptide chemistry. For example, they can be purified by various chromatography (e.g., silica gel column chromatography, ion exchange column chromatography, gel filtration, reversed-phase chromatography), recrystallization and the like. For example, as the recrystallization solvent, alcohol solvents such as methanol, ethanol, 2-propanol and the like, ether solvents such as diethyl ether and the like, ester solvents such as ethyl acetate and the like, aromatic hydrocarbon solvents such as benzene, toluene and the like, ketone solvents such as acetone and the like, hydrocarbon solvents such as hexane and the like, aprotonic solvents such as dimethylformamide, acetonitrile and the like, water, a mixed solvent thereof and the like can be used. As other purified by methods, the methods described in Jikken Kagaku Kouza (The Chemical Society of Japan ed., Maruzen) vol. 1 etc., and the like can be used.

The purified by methods of the disulfide compound are described in the documents (Peptide Synthesis, Interscience, New York, 1966; The Proteins, Vol. 2, Academic Press Inc., New York, 1976; peptide synthesis, Maruzen Co., LTD., 1975; Basics and Experiment of Peptide Synthesis, Maruzen Co., LTD., 1985; Development of Pharmaceutical Product sequential vol. 14·peptide synthesis, Hirokawa Shoten, 1991) and the like. Among these, HPLC is preferable.

[0351]

When the compound of the present invention has one or more asymmetric points, it can be produced according to a general method and using a starting material having the asymmetric points (amino acid). To increase the optical purity of the compound of the present invention, moreover, optical resolution and the like may be performed at a suitable stage of the production step. As the optical resolution method, for example, a diastereomer method including forming a salt of the compound of the present invention or an intermediate thereof with an optically active acid (e.g., monocarboxylic acids such as mandelic acid, N-benzyloxyalanine, lactic acid and the like, dicarboxylic acids such as tartaric acid, o-diisopropylidenetartaric acid, malic acid and the like, or sulfonic acids such as camphorsulfonic acid, bromocamphorsulfonic acid and the like) in an inert solvent (e.g., alcohol solvents such as methanol, ethanol, 2-propanol and the like, ether solvents such as diethyl ether and the like, ester solvents such as ethyl acetate and the like, hydrocarbon solvents such as toluene and the like, aprotonic solvents such as acetonitrile and the like, and a mixed solvent thereof) can be used. When the compound of the present invention or intermediate has an acidic functional group such as carboxy group and the like, optical resolution can also be performed by forming a salt with an optically active amine (e.g., organic amine such as α-phenethylamine, kinin, quinidine, cinchonidine,

cinchonine, strychnine and the like).

[0352]

The temperature for forming a salt is selected from the range of room temperature to the boiling point of the solvent. To improve the optical purity, it is desirable to once raise the temperature to around the boiling point of the solvent. When the precipitated salt is collected by filtration, it can be cooled as necessary to increase the yield. A suitable amount of the optically active acid, or amine to be used is within the range of about 0.5 - about 2.0 equivalents, preferably about 1 equivalent, relative to the substrate. Where necessary, the crystals may be recrystallized in an inert solvent (e.g., alcohol solvents such as methanol, ethanol, or 2-propanol and the like, ether solvents such as diethyl ether and the like, ester solvents such as ethyl acetate and the like, hydrocarbon solvents such as toluene and the like, aprotonic solvents such as acetonitrile and the like, and a mixed solvent thereof) to also afford an optically active salt with high purity. Where necessary, optically resolved salt may be treated with an acid or base by a general method to give a free form.

[0353]

Examples of the "pharmaceutically acceptable salt" in the present invention include acid addition salt and base addition salt. Examples of the acid addition salt include inorganic acid salts such as hydrochloride, hydrobromide, sulfate, hydroiodide, nitrate, phosphate and the like, and organic acid salts such as citrate, oxalate, acetate, formate, propionate, benzoate, trifluoroacetate, maleate, tartrate, methanesulfonate, benzenesulfonate, p-toluenesulfonate and the like. Examples of the base addition salt include salts with inorganic base such as sodium salt, potassium salt, calcium salt, magnesium salt, ammonium salt and the like, salts with organic base such as triethylammonium salt, triethanolammonium salt, pyridinium salt, diisopropylammonium salt etc., and the like, furthermore, amino

acid salts of basic or acidic amino acids such as arginine, aspartic acid, glutamic acid and the like.

[0354]

The present invention also encompasses hydrates, solvates such as ethanol solvate and the like of the compound of the present invention or a pharmaceutically acceptable salt thereof. Furthermore, the present invention encompasses any stereoisomers such as any diastereomer, enantiomer and the like and any crystals in any embodiments, of the compound represented by the formula (1), that can be present.

[0355]

In general, in the production of peptide, various byproducts such as amino acid-defective peptide, peptide degraded by hydrolysis, oxidation and the like, peptide with racemized amino acid and the like occur in a step of condensing optically active α-amino acid, a step of removing various protecting groups, a step of cleaving peptide from a resin and the like. At a laboratory scale, various chromatographys (e.g., silica gel column chromatography, ion exchange column chromatography, gel filtration, and reversed-phase chromatography) are combined to remove such impurities, whereby peptide and a compound with high purity can be obtained. However, it is not easy to obtain peptide and a compound with high purity at an industrial scale to provide pharmaceutical products.

The compound of the present invention has physicochemical properties to allow mass production of a drug substance for pharmaceutical products. Specifically, it has high solubility, is superior in the stability in a solution, is hard to become gelled when concentrated and the like, and the compound can be produced easily as a drug substance with high purity at a large scale by a purified by step using column chromatography such as reversed-phase HPLC and the like.

[0356]

The thus-produced compound of the present invention is superior in the stability to oxidant and the like in a solution, since the cysteine residues form a disulfide bond and the like, and retains given quality as a drug substance of medicaments and efficient CTL induction activity.

The compound of the present invention is useful as an active ingredient of a CTL induction agent for cancer immunotherapy, an active ingredient of a cancer vaccine, or an active ingredient of a pharmaceutical composition. That is, the compound of the present invention has, as shown in the Examples of the present specification, superior immunogenicity and can efficiently show a superior CTL induction activity. In addition, CTL induced by the compound of the present invention can surprisingly recognize natural type partial peptide of WT1 inherently present in cancer cells.

The CTL induction activity can be detected by measuring the number of CTL by the HLA tetramer method (Int. J. Cancer: 100, 565-570 (2002)) or limiting dilution method (Nat. Med.: 4, 321-327 (1998)). Alternatively, for example, HLA-A24-restricted CTL induction activity can be examined by using the HLA-A24 model mouse described in WO 02/47474 and Int. J. Cancer: 100, 565-570 (2002) and the like.

[0357]

Therefore, the compound of the present invention can be used as a therapeutic drug or prophylactic drug (recurrence preventive drug) for cancer expressing WT1 gene or cancer associated with an increase in the WT1 gene expression level. Examples of the cancer include hematologic cancer such as leukemia, myelodysplastic syndrome, multiple myeloma, malignant lymphoma and the like, and solid tumor such as gastric cancer, colorectal cancer, lung cancer, breast cancer, germ cell cancer, liver cancer, skin cancer, urinary bladder cancer, prostate cancer, uterine cancer, cervical cancer, ovarian cancer, brain tumor and the like.

[0358]

The compound of the present invention or a pharmaceutically acceptable salt thereof can be an active ingredient of a CTL induction agent for cellular immunotherapy of cancer, an active ingredient of a cancer vaccine or/and an active ingredient of a pharmaceutical composition, by formulating each compound or salt in a suitable form.

[0359]

The compound of the present invention can be administered together with a carrier acceptable as a medicament such as a suitable adjuvant so that its cellular immunity will be established effectively. As the adjuvant, those described in a document (Clin. Microbiol. Rev., 7: 277-289, 1994) and the like are applicable. Specifically, fungus-derived components, GM-CSF, cytokines such as interleukin-2, interleukin-7, interleukin-12 and the like, plant-derived components, marine organism-derived components, mineral gel such as aluminum hydroxide, lysolecithin, surfactants such as pluronic polyol, polyanion, peptide, oil emulsion (emulsion preparation) and the like can be mentioned. As the fungus-derived components, lipid A, monophosphoryl lipid A, which is a derivative thereof, dead bacteria (Mycobacterium bacteria such as BCG bacteria and the like), bacterium-derived proteins, polynucleotides, Freund's Incomplete Adjuvant, Freund's Complete Adjuvant, cell wall skeleton components (e.g., BCG-CWS and the like), trehalose dimycolate (TDM) and the like can be mentioned.

In addition, the compound of the present invention can also be administered in the form of a liposome preparation, a particulate preparation including binding to beads with a diameter of several μm, a preparation including binding to a lipid and the like.

Furthermore, the compound of the present invention (conjugate) can be administered together with an MHC class II-restricted WT1 peptide (namely, helper peptide). As a method

for co-administration, a conjugate and a helper peptide may be individually administered. A cocktail preparation (cocktail agent, cocktail) containing a conjugate and a helper peptide in a single pharmaceutical composition is more preferable. The cocktail preparation contains a conjugate capable of producing MHC class I-restricted WT1 peptide (i.e., killer peptide) and MHC class II-restricted WT1 peptide (namely, helper peptide). Therefore, by administering the cocktail preparation containing a helper peptide, as a cancer vaccine for cancer immunotherapy, helper T cells important for functional promotion of other T cells including CTL can also be activated, and function and efficacy (cellular immunocompetence and the like) of the conjugate can be improved.

The MHC class II-restricted WT1 peptide (namely, helper peptide) is as described in the DESCRIPTION. Examples of the helper peptide for the cocktail preparation include the following amino acid sequences:
CNKRYFKLSHLQMHSRK (SEQ ID NO: 22),
CNKRYFKLSHLQMHSRKH (SEQ ID NO: 23),
CNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 24),
WAPVLDFAPPGASAYGSL (SEQ ID NO: 244),
CWAPVLDFAPPGASAYGSL (SEQ ID NO: 242) and
WAPVLDFAPPGASAYGSLC (SEQ ID NO: 243). Of these,
WAPVLDFAPPGASAYGSL (SEQ ID NO: 244) is preferable.

It could be confirmed that the cocktail preparation shows improved efficacy as a cancer vaccine such as cellular immunocompetence and the like, as shown in, for example, Examples and Experimental Examples in the DESCRIPTION.
[0360]

While the dose of the compound of the present invention in the preparation can be appropriately controlled according to the treatment object disease, age and body weight of the patients and the like, it is generally 0.0001 mg - 1000 mg, preferably 0.001 mg - 1000 mg, more preferably 0.1 mg - 10 mg.

As the administration method, intradermal administration, subcutaneous administration, intramuscular administration, intravenous administration, transdermal administration and the like can be mentioned. Intradermal administration and subcutaneous administration are preferable since they efficiently induce CTL. While the administration frequency and administration intervals can be appropriately controlled according to the prophylaxis or treatment of object disease, and individual difference in patients, it is generally multiple times, and administration once per several days to several months is preferable.

By administering a pharmaceutical composition containing such compound of the present invention as an active ingredient to WT1 positive patients, a method for the prophylaxis or treatment of of cancer can be provided.

Examples

[0361]

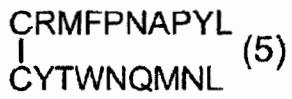
The present invention is specifically explained in the following by referring to Examples, to which, however, the invention is not limited.

[0362]

Example 1

Synthesis of the compound represented by the formula (5):

[0363]



[0364]

wherein the bond between C and C is a disulfide bond.

[0365]

step 1. Synthesis of H-Cys(Npys)-Arg-Met-Phe-Pro-Asn-Ala-Pro-Tyr-Leu-OH

(synthesis of C(Npys)RMFPNAPYL)

Using Fmoc-Leu-Alko-resin (Alko is p-alkoxybenzylalcohol), 282 mg, (manufactured by Watanabe Chemical; 0.71 mmol/g, 0.2

mmol) as a starting material, the peptide chain was assembled by solid phase synthesis according to Fmoc/tBu method. Solid phase synthesis was performed using CS336X peptide synthesizer manufactured by CS Bio, and deprotection of Fmoc group was performed by treatment with a DMF solution of 20% piperidine for 5 min and for 20 min. Coupling of protected amino acid was performed by reaction with a DMF solution of 1.05 mmol of protected amino acid, 1 mmol HBTU and 2 mmol DIPEA for 1 hr. The obtained resin was washed with DMF and ether, and dried under reduced pressure to give Boc-Cys(Npys)-Arg(Pmc)-Met-Phe-Pro-Asn(Trt)-Ala-Pro-Tyr(tBu)-Leu-Alko-resin (630 mg). To this peptide resin was added a mixture of TFA/H_2O/TIS=95/2.5/2.5 (10 ml), and the mixture was shaken at room temperature for 2 hr. The resin was filtered off, and the reaction mixture was concentrated under reduced pressure. The reaction mixture was ice-cooled and diethyl ether (50 ml) was added. The resulting precipitate was collected by filtration, washed with ether and dried under reduced pressure to give crude peptide (217 mg). The obtained crude peptide solution was dissolved in a mixture of 20% aqueous acetic acid (7 ml) and acetonitrile (1 ml) and purified by reversed-phase HPLC.

pump: manufactured by Shimadzu; LC-8A

column: YMC ODS-A 3 cmφ×25 cmL, 10 μm

eluate 1: H_2O/0.1%TFA

eluate 2: CH_3CN/0.1%TFA

flow rate: 20 ml/min

detection: UV220 nm

The crude peptide solution was injected to a column equilibrated with 15% of eluate 2. Thereafter, the concentration of eluate 2 was raised to 37% over 10 min, and thereafter raised at a rate of 0.24% per minute. Fractions containing the object product were collected and freeze dried to give H-Cys(Npys)-Arg-Met-Phe-Pro-Asn-Ala-Pro-Tyr-Leu-OH (53 mg).

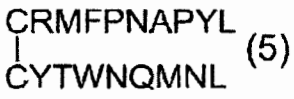
mass spectrometry: LC-ESI/MS m/z=1366.1 $[M+1]^+$

(Calculated=1366.6)

[0366]

step 2. synthesis of (H-Cys-Tyr-Thr-Trp-Asn-Gln-Met-Asn-Leu-OH)(H-Cys-Arg-Met-Phe-Pro-Asn-Ala-Pro-Tyr-Leu-OH) disulfide bond [That is, synthesis of a compound represented by the formula (5):

[0367]



[0368]

wherein the bond between C and C is a disulfide bond.]

H-Cys(Npys)-Arg-Met-Phe-Pro-Asn-Ala-Pro-Tyr-Leu-OH (50 mg) obtained in step 1 and H-Cys-Tyr-Thr-Trp-Asn-Gln-Met-Asn-Leu-OH (i.e., CYTWNQMNL (SEQ ID NO: 4)) (43 mg) synthesized by a known method (e.g., WO07/063903) were mixed, DMSO (1 mL) was added, and the mixture was stirred at room temperature for 20 min. The reaction mixture was diluted with 0.1% TFA water (5 ml) and purified by reversed-phase HPLC.

pump: manufactured by Shimadzu; LC-8A

column: YMC ODS-A 3 cmφ×25 cmL, 10 μm

eluate 1: H_2O/0.1% TFA

eluate 2: CH_3CN/0.1% TFA

flow rate: 20 ml/min

detection: UV220 nm

The reaction solution was injected to a column equilibrated with 25% of eluate 2. Thereafter, the concentration of eluate 2 was raised at a rate of 0.25% per minute. Fractions containing the object product were collected, freeze dried, re-purified by reversed-phase HPLC, and freeze dried to give (H-Cys-Tyr-Thr-Trp-Asn-Gln-Met-Asn-Leu-OH)(H-Cys-Arg-Met-Phe-Pro-Asn-Ala-Pro-Tyr-Leu-OH) disulfide bond (i.e., a compound represented by the formula (5), 21 mg).

mass spectrometry: LC-ESI/MS m/z =1191.8 $[M+2]^{2+}$ (Calculated=1191.9)

[0369]

Example 2

Synthesis of peptide consisting of the following amino acid sequence:

CRMFPNAPYL (SEQ ID NO: 13)

[0370]

step 1. Using Fmoc-Leu-Alko-resin (Alko is p-alkoxybenzylalcohol) (338 mg, manufactured by Watanabe Chemical; 0.74 mmol/g, 0.25 mmol) as a starting material,and solid phase synthesis as in the method described in Example 1 was performed twice to give H-Cys(Trt)-Arg(Pmc)-Met-Phe-Pro-Asn(Trt)-Ala-Pro-Tyr(tBu)-Leu-Alko-resin (1.54 g). To this peptide resin was added a mixture of TFA/H_2O/TIS=95/2.5/2.5 (15 ml), and the mixture was shaken at room temperature for 3 hr. The resin was filtered off, and the reaction mixture was concentrated under reduced pressure. The reaction mixture was ice-cooled and diethyl ether (50 ml) was added. The resulting precipitate was collected by filtration, washed with ether and dried under reduced pressure to give crude peptide (637 mg).

mass spectrometry: LC-ESI/MS m/z=1211.9 $[M+1]^+$ (Calculated=1212.5)

[0371]

tep 2. The crude peptide (321 mg) obtained in step 1 was dissolved in TFA (10 ml), and charged, by a pump of HPLC, into a YMC-PACK ODS-A 3 cmφ×25 cmL column equilibrated with HPLC (manufactured by Shimadzu; LC6AD) eluate 1 =H_2O/0.1% TFA. This state was maintained for about 20 min and, after 20 min, the concentration of eluate 2=CH_3CN/0.1% TFA was raised to 27%. Thereafter, while monitoring the eluate of the object peptide by 220 nm UV, the concentration of eluate 2 was raised at a rate of 0.25% per minute and the fractions containing the object product were collected. The peptide (100 mg) obtained after freeze dry was purified again by reversed-phase under the same conditions, and acetonitrile was evaporated under reduced pressure and the

residue was freeze dried to give the object peptide (CRMFPNAPYL (SEQ ID NO: 13), 37.2 mg).

pump: manufactured by Shimadzu; LC-6A

column: YMC ODS-A 3 cmφ×25 cmL, 10 μm

eluate 1: H_2O/0.1%TFA

eluate 2: CH_3CN/0.1%TFA

flow rate: 20 ml/min

detection: UV220 nm

mass spectrometry: LC-ESI/MS m/z =1212.0 $[M+1]^+$

(Calculated=1211.6)

[0372]

Examples 3 - 5

By a method similar to that in Example 2, peptides consisting of the amino acid sequence of SEQ ID NO: 16, 18 or 17 were synthesized. Table 54 shows the synthesized amount and the results of mass spectrometry.

[0373]

Table 54

Ex. No.	amino acid sequence	sequence No.	synthesized amount (mg)	mass spectrometry: LC-ESI/ MS m/z	mass spectro-metry: Calculated
3	CALLPAVPSL	16	42	983.8 $[M+1]^+$	983.2
4	CRVPGVAPTL	18	53	1012.7 $[M+1H]^+$	1012.2
5	CSLGEQQYSV	17	31	1113.7 $[M+1]^+$	1113.2

[0374]

Experimental Example 1

Time-course changes of trimming of N-terminal amino acid by ERAP1

The peptides of SEQ ID NOs: 13, 16, 18 and 17 synthesized in Examples 2 - 5 were evaluated for the trimming of the N-terminal amino acid by ERAP1 (PLoS One November 2008, vol.3, Issue 11, e3658).

30 μl of ERAP1 (2.0 mg/ml) in PBS buffer solution was

added to 258 μl of Tris·HCl buffer. DMSO solution (12.0 μl) of 10 mM each peptide was added to the aforementioned ERAP1 solution, and the mixture was blended well and stood at room temperature. 1.0, 2.0, 4.0, 8.0 hr later, 50 μl of a sample was added to 150 μl of MeOH to terminate the reaction, 25 μl was injected into UFLC (analysis conditions shown below), and AUC of the object peptide was determined. Peptide obtained by trimming was chemically synthesized separately, and analyzed under similar conditions free of enzyme. The formation ratio of peptide obtained by trimming was determined based on the obtained AUC.

analysis conditions

pump: UFLC manufactured by Shimadzu

column: Shim-pack XR-ODS 3.0 mmi.d.x75 mm

solution: 0.1% TFA H_2O(A) – 0.1% TFA CH_3CN(B)

oven temperature: 40°C

flow rate: 1.0 ml/min

detection wavelength: λ=220 nm

gradient:

1. Concentration of SOLUTION B was raised from 1.0% to 70% from 0.0 min to 5.0 min
2. Concentration of SOLUTION B was raised from 1.0% to 50% from 0.0 min to 5.0 min

object peptide:

As for the peptides synthesized in Examples 2 - 5, the amino acid sequences of the peptides obtained by trimming of N-terminal amino acid by ERAP1 are shown in Table 55.

[0375]

Table 55

peptide used for trimming test			peptide obtained by trimming	
Example No.	amino acid sequence	sequence No.	amino acid sequence	sequence No.
2	CRMFPNAPYL	13	RMFPNAPYL	2
3	CALLPAVPSL	16	ALLPAVPSL	5
4	CRVPGVAPTL	18	RVPGVAPTL	7
5	CSLGEQQYSV	17	SLGEQQYSV	6

[0376]

Time-course changes in the formation rate of the peptides obtained by trimming are shown in Table 56 and Fig. 1.

[0377]

Table 56

Example No.	sequence No.	gradient	formation rate (%)			
			1 hr later	2 hr later	4 hr later	8 hr later
2	13	1	25.5	35.2	46.2	47.6
3	16	1	65.5	50.6	13.5	0
4	18	1	59.1	57.5	30.1	7.80
5	17	2	77.6	72.8	46.0	7.90

[0378]

The trimming results strongly suggest that, in any Cys-extended peptides (SEQ ID NOs: 13, 16, 17 and 18), Cys on the extended N-terminal is selectively cleaved by ERAP-1, namely, Cys-extended peptide undergoes appropriate trimming by ERAP-1 without marked dependence on the peptide sequence, and is finally converted to the object cancer antigen peptide (SEQ ID NO: 2, 5, 6 or 7).

[0379]

Experimental Example 2

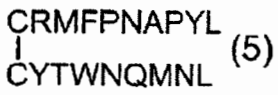
Evaluation of in vivo CTL induction ability using HLA-A0201 transgenic mouse and HLA-A2402 transgenic mouse

[0380]

The compound represented by the formula (5) synthesized in Example 1 was evaluated for the CTL induction ability by an in vivo CTL induction test using HLA-A0201 transgenic mouse and HLA-A2402 transgenic mouse. The compound represented by the

formula (5):

[0381]



[0382]

wherein the bond between C and C is a disulfide bond, is, in particular, a compound of the aforementioned formula (1), wherein cancer antigen peptide A is RMFPNAPYL (SEQ ID NO: 2) and cancer antigen peptide B is CYTWNQMNL (SEQ ID NO: 4). RMFPNAPYL (SEQ ID NO: 2) is a HLA-A0201-restricted WT1 peptide, and CYTWNQMNL (SEQ ID NO: 4) is a HLA-A24-restricted WT1 peptide.

[0383]

HLA-A0201 transgenic mouse (C57BL/6CrHLA-A2.1DR1) is a mouse which is defective in mouse MHC, and expresses chimera HLA of human MHC HLA-A0201 and mouse MHC H-2D^b, and HLA-DRB1*0101. Using this mouse, HLA-A02 positive peptide capable of inducing CTL in human can be selected (Eur J Immunol. 2004; 34: 3060-9). On the other hand, HLA-A2402 transgenic mouse (C57BL/6CrHLA-A2402/K^b) is a mouse that expresses chimera HLA of human MHC HLA-A2402 and mouse MHC H-2K^b. Using this mouse, HLA-A24 positive peptide capable of inducing CTL in human can be selected (Int J Cancer. 2002; 100: 565-70).

[0384]

Whether the administration of a compound represented by the formula (5) results in the induction of CTL specific to the object peptide (SEQ ID NO: 2, 4) was judged based on the measurement of IFNγ production by re-stimulation, with the peptide (SEQ ID NO: 2, 4), of the splenocyte derived from the above-mentioned mouse administered with a compound represented by the formula (5).

[0385]

Specifically, a compound represented by the formula (5) was dissolved in dimethyl sulfoxide (DMSO) at 40 mg/mL, further diluted with water for injection to 5 mg/mL, and emulsified by

mixing with an equal amount of incomplete Freund's adjuvant (IFA). The emulsified compound was intradermally administered to 4 sites at the base of tail of a mouse at 250 μg/site. One week later, the mouse was euthanized with CO_2 gas, the spleen was isolated, and splenocytes were prepared. IFNγ ELISPOT assay kit was used for the measurement of IFNγ production. On the previous day of splenocyte preparation, an ELISPOT plate was treated with an anti-mouse IFNγ antibody, and blocked with RPMI1640 medium containing 10% FBS the next day. The prepared HLA-A0201 transgenic mouse-derived splenocytes were plated at 0.15×10^6 cells/well, and HLA-A2402 transgenic mouse-derived splenocytes were plated at 1×10^6 cells/well, on the blocked ELISPOT plate. Peptide (SEQ ID NO: 2, 4) was dissolved in DMSO at 40 mg/mL, and further diluted with RPMI1640 medium containing 10% FBS to 40 μg/mL. The diluted peptide (SEQ ID NO: 2) was added to the HLA-A0201 transgenic mouse-derived splenocytes at a final concentration of 10 μg/mL. In addition, the diluted peptide (SEQ ID NO: 4) was added to the HLA-A2402 transgenic mouse-derived splenocytes at a final concentration of 10 μg/mL. The splenocytes added with the peptide were cultivated for 20 hr at 37°C, 5% CO_2, whereby peptide re-stimulation in vitro was performed. After culture, the supernatant was removed, and the ELISPOT plate was allowed to develop color according to the attached protocol. The number of spots that developed color was measured by ImmunoSpot Analyzer (manufactured by C.T.L.).

[0386]

The results of IFNγ ELISPOT assay using HLA-A0201 transgenic mouse are shown in Fig. 2, and the results of IFNγ ELISPOT assay using HLA-A2402 transgenic mouse are shown in Fig. 3.

In each Figure, the vertical axis shows the number of cells that reacted in the plated cells. In Fig. 2, the black bar and the white bar show the results of culture of HLA-A0201 transgenic mouse-derived splenocytes in the presence or absence

of the object peptide represented by SEQ ID NO: 2, and in Fig. 3, the black bar and the white bar show the results of culture of HLA-A2402 transgenic mouse-derived splenocytes in the presence or absence of the object peptide represented by SEQ ID NO: 4. That is, the difference in the values of the black bar and the white bar show the number of the object, each peptide-specific CTL induced in the mouse in vivo by the administration of a compound represented by the formula (5).

In each Figure, the value of the white bar is not detected. This means that the splenocytes of respective transgenic mice did not react at all in the absence of the object peptide. As a result of this test, IFNγ production specific to the object peptide shown by SEQ ID NO: 2 was detected in the HLA-A0201 transgenic mouse-derived splenocytes, and IFNγ production specific to the object peptide shown by SEQ ID NO: 4 was detected in the HLA-A2402 transgenic mouse-derived splenocytes.

[0387]

From the above, it was clarified that a compound represented by the formula (5) can induce CTL specific to the peptide shown by SEQ ID NO: 2 and CTL specific to the peptide shown by SEQ ID NO: 4. It was strongly suggested that the compound represented by the formula (5) undergoes cleavage of disulfide bond and appropriate trimming by ERAP-1 in mouse in vivo and is in fact processed into the peptides shown by SEQ ID NO: 2 and SEQ ID NO: 4.

That is, it was clarified that a compound represented by the formula (5), which is one embodiment of the compound of the present invention, is a conjugate wherein different two kinds of WT1 peptides form a composite via the disulfide bond shown in the formula (1), and is a WT1 cancer antigen peptide conjugate vaccine that in fact can induce different two kinds of CTLs in vivo.

[0388]

Reference Examples 1 - 7

By a method similar to that in Example 2, respective peptides consisting of the amino acid sequences of SEQ ID NOs: 22, 24, 23, 2, 4, 6 and 5 were synthesized. Table 57 shows the results of mass spectrometry. Since SEQ ID NOs: 22, 24, 23, 2, 4, 6 and 5 are not the compound of the present invention, they are described as Reference Examples .

[0389]

Table 57

Ref. Ex. No.	amino acid sequence	sequence No.	mass spectro-metry: LC-ESI/ MS m/z	mass spectro-metry: Calculated
1	CNKRYFKLSHLQMHSRK	22	1089.1 $[M+2H]^+$	1089.3
2	CNKRYFKLSHLQMHSRKHTG	24	825.1 $[M+3H]^+$	824.8
3	CNKRYFKLSHLQMHSRKH	23	772.4 $[M+3H]^+$	772.2
4	RMFPNAPYL	2	1109.0 $[M+H]^+$	1109.3
5	CYTWNQMNL	4	1172.9 $[M+H]^+$	1173.4
6	SLGEQQYSV	6	1010.9 $[M+H]^+$	1011.1
7	ALLPAVPSL	5	881.0 $[M+H]^+$	881.1

[0390]

Examples 6 - 9

By a method similar to that in Example 1, respective compounds (conjugates) represented by the formulas (3), (6), (7) and (8) were synthesized. Table 58 shows the results of mass spectrometry. (In each formula, the bond between C and C is a disulfide bond.)

[0391]

Table 58

Ex. No.	structural formula	formula No.	mass spectrometry: LC-ESI/ MS m/z	mass spectro-metry: Calculated
6	CRMFPNAPYL | CSLGEQQYSV	(3)	1162.3 $[M+2H]^+$	1162.0
7	CRMFPNAPYL | CNKRYFKLSHLQMHSRKHTG	(6)	1228.0 $[M+3H]^+$	1227.6
8	CRMFPNAPYL | CNKRYFKLSHLQMHSRKH	(7)	705.8 $[M+5H]^+$	705.3
9	CRMFPNAPYL | CNKRYFKLSHLQMHSRK	(8)	1129.8 $[M+3H]^+$	1129.2

[0392]

Experimental Example 3

Measurement of solubility

step 1. preparation of isotonic buffer

1. 75% aqueous solution of disodium hydrogen phosphate and 5.53% aqueous solution of citric acid were mixed, and respective buffers (pH 6.0 and 7.4) were prepared.

step 2. preparation of test solution

About 1 mg of a test product was measured, an isotonic buffer (0.5 mL) was added, and this was used as a test solution. The prepared test solution was shaken at room temperature for 90 min (shaking conditions: RECIPRO SHAKER SR-1N manufactured by TAITEC, Speed=8), centrifuged (15000 rpm, 5 min, room temperature), and the supernatant after centrifugation was used as a test solution.

step 3. preparation of standard solution

About 1 mg of the test product was accurately measured, dissolved in 0.1% TFA water/acetonitrile=1/1, made the total amount 10 mL, and this was used as a standard solution of the test product.

step 4. measurement of concentration of test product

The standard solution of the test product and the test solution were analyzed by HPLC (analysis conditions described in Table 59), and the solubility of the test product was calculated from the peak area ratio of the standard solution.

HPLC measurement conditions

column: ChemcoPack Quicksorb (4.6 mmφ×150 mm, 5 μm) manufactured by Chemco Scientific Co., Ltd.

mobile phase: SOLUTION A; 0.1% TFA water, SOLUTION B; 0.1% TFA acetonitrile solution

column temperature: room temperature

flow rate: 1 mL/min

detection wavelength: UV 254 nm, 230 nm (2 wavelength detection)

sample injection volume: 10 μL

[0393]

Table 59

gradient analysis conditions		
time (min)	SOLUTION A (%)	SOLUTION B (%)
0.00	80	20
10.00	0	100
15.00	0	100
15.01	80	20
25.00	80	20
25.01	STOP	

[0394]

The peptides synthesized in Reference Examples 1 - 2 and 4 - 7 and the compounds (conjugates) synthesized in Examples 1, 7 and 9 were subjected to the above-mentioned solubility measurement. Each solubility is shown in Table 60.

[0395]

Table 60

Reference Example No. or Example No.	amino acid sequence or structural formula	SEQ ID NO: or formula No.	pH 6.0 (mg/mL)	pH 7.4 (mg/mL)
Reference Example 6	SLGEQQYSV	SEQ ID NO: 6	>1.0	>1.0
Reference Example 7	ALLPAVPSL	SEQ ID NO: 5	>1.0	>1.0
Reference Example 2	CNKRYFKLSHLQMHSRKHTG	SEQ ID NO: 24	>1.0	0.556
Reference Example 1	CNKRYFKLSHLQMHSRK	SEQ ID NO: 22	>1.0	0.931
Example 7	CRMFPNAPYL | CNKRYFKLSHLQMHSRKHTG	formula (6)	>1.0	0.279
Example 9	CRMFPNAPYL | CNKRYFKLSHLQMHSRK	formula (8)	>1.0	0.789
Reference Example 4	RMFPNAPYL	SEQ ID NO: 2	>1.0	>1.0
Reference Example 5	CYTWNQMNL	SEQ ID NO: 4	0.106	0.167
Example 1	CRMFPNAPYL | CYTWNQMNL	formula (5)	0.511	0.200

[0396]

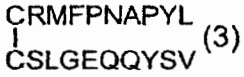
Experimental Example 4

Evaluation of in vivo CTL induction ability using HLA-A0201 transgenic mouse

[0397]

The compound represented by the formula (3) synthesized in Example 6 was evaluated for the CTL induction ability by an in vivo CTL induction test using HLA-A0201 transgenic mouse. The compound represented by the formula (3):

[0398]



(3)

[0399]

wherein the bond between C and C is a disulfide bond, is, in

particular, a compound of the aforementioned formula (1), wherein cancer antigen peptide A is RMFPNAPYL (SEQ ID NO: 2) and cancer antigen peptide B is SLGEQQYSV (SEQ ID NO: 6). RMFPNAPYL (SEQ ID NO: 2) and SLGEQQYSV(SEQ ID NO: 6) are a HLA-A0201-restricted WT1 peptides.

[0400]

The HLA-A0201 transgenic mouse is as described in Experimental Example 2.

[0401]

Whether the administration of a compound represented by the formula (3) results in the induction of CTL specific to the object peptide (SEQ ID NO: 2, 6) was judged based on the measurement of IFNγ production by re-stimulation, with the peptide (SEQ ID NO: 2, 6), of the splenocyte derived from the above-mentioned mouse administered with a compound represented by the formula (3).

[0402]

Specifically, a compound represented by the formula (3) was dissolved in water for injection at 10 mg/mL, and emulsified by mixing with an equal amount of incomplete Freund's adjuvant (IFA). The emulsified compound was intradermally administered to 2 sites at the base of tail of a mouse at 500 μg/site. One week later, the mouse was euthanized with CO_2 gas, the spleen was isolated, and splenocytes were prepared. IFNγ ELISPOT assay kit was used for the measurement of IFNγ production. On the previous day of splenocyte preparation, an ELISPOT plate was treated with an anti-mouse IFNγ antibody, and blocked with RPMI1640 medium containing 10% FBS the next day. The prepared HLA-A0201 transgenic mouse-derived splenocytes were plated at 0.75×10^6 cells/well on the blocked ELISPOT plate. Peptide (SEQ ID NO: 2, 6) was dissolved in DMSO at 40 mg/mL, and further diluted with RPMI1640 medium containing 10% FBS to 40 μg/mL. The diluted peptide (SEQ ID NO: 2, 6) was added to the HLA-A0201 transgenic mouse-derived splenocytes at a final concentration of

10 μg/mL. The splenocytes added with the peptide were cultured for 20 hr at 37°C, 5% CO_2, whereby peptide re-stimulation in vitro was performed. After culture, the supernatant was removed, and the ELISPOT plate was allowed to develop color according to the attached protocol. The number of spots that developed color was measured by ImmunoSpot Analyzer (manufactured by C.T.L.).

[0403]

The results of IFNγ ELISPOT assay using the HLA-A0201 transgenic mouse are shown in Fig. 4.

In Fig. 4, the vertical axis shows the number of cells that reacted in the plated cells. In Fig. 4, the black bar and the shaded bar show the results of culture of HLA-A0201 transgenic mouse-derived splenocytes while being pulsed with each peptide shown by SEQ ID NO: 2, 6, and the white bar show the results of culture without pulsing. That is, the difference in the values of the black or shaded bar and the white bar shows the number of peptide-specific CTL, and that the administration of a compound represented by the formula (3) resulted in the induction of CTL specific to each peptide shown by SEQ ID NOs: 2, 6 in vivo in the mouse. In Figure 4, the value of the white bar is not detected. This means that the splenocytes of HLA-A0201 transgenic mice did not react at all in the absence of pulsing with the object peptide. As a result of this test, IFNγ production specific to the peptide shown by SEQ ID NO: 2, 6 was detected in the HLA-A0201 transgenic mouse-derived splenocytes.

[0404]

From the above, it was clarified that a compound represented by the formula (3) can induce CTL specific to the peptide shown by SEQ ID NO: 2, 6. It was strongly suggested that the compound represented by the formula (3) undergoes cleavage of disulfide bond and appropriate trimming by ERAP-1 in mouse in vivo and is in fact processed into the peptides shown by SEQ ID NO: 2 and 6. That is, it was clarified that a compound represented by the formula (3), which is one embodiment

of the compound of the present invention, is a conjugate wherein different two kinds of peptides form a composite via the disulfide bond shown in the formula (1), and is a WT1 cancer antigen peptide conjugate vaccine that in fact can induce different two kinds of CTLs in vivo.

[0405]

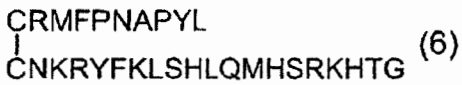
Experimental Example 5

Evaluation of in vivo CTL induction ability using HLA-A0201 transgenic mouse

[0406]

The compound represented by the formula (6) synthesized in Example 7 was evaluated for the CTL induction ability by an in vivo CTL induction test using HLA-A0201 transgenic mouse. The compound represented by the formula (6):

[0407]



(6)

[0408]

wherein the bond between C and C is a disulfide bond, is, in particular, a compound of the aforementioned formula (1), wherein cancer antigen peptide A is RMFPNAPYL (SEQ ID NO: 2) and cancer antigen peptide C is CNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 24). RMFPNAPYL (SEQ ID NO: 2) is a HLA-A0201-restricted WT1 peptide, and CNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 24) is an MHC class II-restricted WT1 peptide (namely, helper peptide).

[0409]

The HLA-A0201 transgenic mouse is as described in Experimental Example 2. Using this mouse, HLA-A02 positive peptide capable of inducing CTL in human can be selected, as well as the CTL induction enhancing activity of helper peptide capable of inducing helper T cell by binding to human HLA-DRB1*0101 can be evaluated.

[0410]

Whether the administration of a compound represented by

the formula (6) results in the induction of CTL specific to the object peptide (SEQ ID NO: 2) and cells reactive with helper peptide(SEQ ID NO: 24) was judged based on the measurement of IFNγ production by re-stimulation, with the peptide (SEQ ID NO: 2, 24), of the splenocyte derived from the above-mentioned mouse administered with a compound represented by the formula (6). In addition, the splenocytes derived from the above-mentioned mouse administered with a compound represented by the formula (6) and the splenocytes derived from the above-mentioned mouse administered with a compound represented by SEQ ID NO: 2 were re-stimulated with the peptide (SEQ ID NO: 2), and the IFNγ-producing cell numbers were compared.

[0411]

Specifically, a peptide represented by SEQ ID NO: 2 was dissolved in water for injection at 6 mg/mL, and emulsified by mixing with an equal amount of incomplete Freund's adjuvant (IFA). The emulsified peptide was intradermally administered to 2 sites at the base of tail of a mouse at 150 μg/site. A compound represented by the formula (6) was dissolved in water for injection (19.8 mg/mL), and emulsified by mixing with an equal amount of incomplete Freund's adjuvant (IFA). The emulsified compound was intradermally administered to 2 sites at the base of tail of a mouse at 495 μg/site. The mole number of the peptide of SEQ ID NO: 2 contained in the dose of the compound represented by the formula (6) per one mouse was adjusted to be equal to that of the peptide of SEQ ID NO: 2 contained in the dose per mouse. One week later, the mouse was euthanized with CO_2 gas, the spleen was isolated, and splenocytes were prepared. IFNγ ELISPOT assay kit was used for the measurement of IFNγ production. On the previous day of splenocyte preparation, an ELISPOT plate was treated with an anti-mouse IFNγ antibody, and blocked with RPMI1640 medium containing 10% FBS the next day. The prepared HLA-A0201 transgenic mouse-derived splenocytes were plated at 0.25×10^6

cells/well or 0.5×10^6 cells/well on the blocked ELISPOT plate. Peptide (SEQ ID NO: 2, 24) was dissolved in DMSO at 40 mg/mL, and further diluted with RPMI1640 medium containing 10% FBS to 40 μg/mL. The diluted peptide (SEQ ID NO: 2, 24) was added to the HLA-A0201 transgenic mouse-derived splenocytes at a final concentration of 10 μg/mL. The splenocytes added with the peptide were cultured for 20 hr at 37°C, 5% CO_2, whereby peptide re-stimulation in vitro was performed. After culture, the supernatant was removed, and the ELISPOT plate was allowed to develop color according to the attached protocol. The number of spots that developed color was measured by ImmunoSpot Analyzer (manufactured by C.T.L.).

[0412]

The results of IFNγ ELISPOT assay using the HLA-A0201 transgenic mouse are shown in Figs. 5 and 6.

In Fig. 5 and 6, the vertical axis shows the number of cells that reacted in the plated cells, and the horizontal axis shows compound or peptide administered to the mouse. In Fig. 5, the black bar shows the results of culture of HLA-A0201 transgenic mouse-derived splenocytes while being pulsed with the peptide shown by SEQ ID NO: 2, and the white bar show the results of culture without pulsing. That is, the difference in the values of the black bar and the white bar shows the number of peptide-specific CTL, and that the administration of the peptide shown by SEQ ID NO: 2 or a compound represented by the formula (6) resulted in the induction of CTL specific to the peptide shown by SEQ ID NO: 2 in vivo in the mouse. In Figure 5, the value of the white bar is not detected. This means that the splenocytes of HLA-A0201 transgenic mice did not react at all in the absence of pulsing with the object peptide. As a result of this test, IFNγ production specific to the peptide shown by SEQ ID NO: 2 was detected in the HLA-A0201 transgenic mouse-derived splenocytes. Moreover, in Fig. 5, the number of IFNγ-producing cells specific to the peptide shown by SEQ ID NO: 2, which were

induced by the administration of a compound represented by the formula (6), was higher than that of the peptide-specific IFNγ-producing cells induced by the administration of the peptide shown by SEQ ID NO: 2.

In Fig. 6, furthermore, the black bar shows the results of culture of HLA-A0201 transgenic mouse-derived splenocytes while being pulsed with peptide shown by SEQ ID NO: 24, and the white bar show the results of culture without pulsing. That is, the difference in the values of the black bar and the white bar shows the number of peptide-reactive cells, and that the administration of a compound represented by the formula (6) resulted in the induction of cells reactive with the helper peptide shown by SEQ ID NO: 24 in vivo in the mouse, and administration of a compound represented by SEQ ID NO: 2 did not induce cells reactive with the peptide shown by SEQ ID NO: 24 in vivo in the mouse. In Figure 6, the value of the white bar is not detected. This means that the splenocytes of HLA-A0201 transgenic mice did not react at all in the absence of pulsing with the object peptide.

[0413]

From the above, it was clarified that a compound represented by the formula (6) can induce CTL specific to the peptide shown by SEQ ID NO: 2 and cells reactive with the helper peptide shown by SEQ ID NO: 24. It was strongly suggested that the compound represented by the formula (6) undergoes cleavage of disulfide bond and appropriate trimming by ERAP-1 in mouse in vivo and is in fact processed into the peptides shown by SEQ ID NO: 2 and 24. It was assumed that the induction of the cell reactive with the helper peptide shown by SEQ ID NO: 24 produced from a compound represented by the formula (6) enhanced induction of CTL specific to the peptide shown by SEQ ID NO: 2, and many IFNγ-producing cells specific to the peptide shown by SEQ ID NO: 2 were found, as compared to the administration of the peptide shown by SEQ ID NO: 2.

That is, it was clarified that a compound represented by the formula (6), which is one embodiment of the compound of the present invention, is a conjugate wherein two different kinds of peptides form a composite via the disulfide bond shown in the formula (1), and is a WT1 cancer antigen peptide conjugate vaccine that in fact can induce CTLs and helper peptide reactive cells in vivo.

[0414]

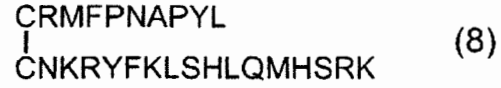
Experimental Example 6

Evaluation of in vivo CTL induction ability using HLA-A0201 transgenic mouse

[0415]

The compound represented by the formula (8) synthesized in Example 9 was evaluated for the CTL induction ability by an in vivo CTL induction test using HLA-A0201 transgenic mouse. The compound represented by the formula (8):

[0416]



(8)

[0417]

wherein the bond between C and C is a disulfide bond, is, in particular, a compound of the aforementioned formula (1), wherein cancer antigen peptide A is RMFPNAPYL (SEQ ID NO: 2) and cancer antigen peptide C is CNKRYFKLSHLQMHSRK(SEQ ID NO: 22). RMFPNAPYL (SEQ ID NO: 2) is a HLA-A0201-restricted WT1 peptide, and CNKRYFKLSHLQMHSRK (SEQ ID NO: 22) is an MHC class II-restricted WT1 peptide (namely, helper peptide).

[0418]

The HLA-A0201 transgenic mouse is as described in Experimental Examples 2 and 5.

[0419]

Whether the administration of a compound represented by the formula (8) results in the induction of CTL specific to the object peptide (SEQ ID NO: 2) and cells reactive with helper

peptide (SEQ ID NO: 22) reactive cell was judged based on the measurement of IFNγ production by re-stimulation, with the peptide (SEQ ID NO: 2, 22), of the splenocyte derived from the above-mentioned mouse administered with a compound represented by the formula (8). In addition, the splenocytes derived from the above-mentioned mouse administered with a compound represented by the formula (8) and the splenocytes derived from the above-mentioned mouse administered with a compound represented by SEQ ID NO: 2 were re-stimulated with the peptide (SEQ ID NO: 2), and the IFNγ-producing cell numbers were compared.

[0420]

Specifically, a peptide represented by SEQ ID NO: 2 was dissolved in water for injection at 6 mg/mL, and emulsified by mixing with an equal amount of incomplete Freund's adjuvant (IFA). The emulsified peptide was intradermally administered to 2 sites at the base of tail of a mouse at 150 μg/site. A compound represented by the formula (8) was dissolved in water for injection (18 mg/mL), and emulsified by mixing with an equal amount of incomplete Freund's adjuvant (IFA). The emulsified compound was intradermally administered to 2 sites at the base of tail of a mouse at 450 μg/site. The mole number of the peptide of SEQ ID NO: 2 contained in the dose of the compound represented by the formula (8) per one mouse was adjusted to be equal to that of the peptide of SEQ ID NO: 2 contained in the dose per mouse. One week later, the mouse was euthanized with CO_2 gas, the spleen was isolated, and splenocytes were prepared. IFNγ ELISPOT assay kit was used for the measurement of IFNγ production. On the previous day of splenocyte preparation, an ELISPOT plate was treated with an anti-mouse IFNγ antibody, and blocked with RPMI1640 medium containing 10% FBS the next day. The prepared HLA-A0201 transgenic mouse-derived splenocytes were plated at 0.25×10^6 cells/well or 0.5×10^6 cells/well on the blocked ELISPOT plate. Peptide (SEQ ID NO: 2, 22) was dissolved

in DMSO at 40 mg/mL, and further diluted with RPMI1640 medium containing 10% FBS to 40 μg/mL. The diluted peptide (SEQ ID NO: 2, 22) was added to the HLA-A0201 transgenic mouse-derived splenocytes at a final concentration of 10 μg/mL. The splenocytes added with the peptide were cultured for 20 hr at 37°C, 5% CO_2, whereby peptide re-stimulation in vitro was performed. After culture, the supernatant was removed, and the ELISPOT plate was allowed to develop color according to the attached protocol. The number of spots that developed color was measured by ImmunoSpot Analyzer (manufactured by C.T.L.).

[0421]

The results of IFNγ ELISPOT assay using the HLA-A0201 transgenic mouse are shown in Figs. 7 and 8.

In Figs. 7 and 8, the vertical axis shows the number of cells that reacted in the plated cells, and the horizontal axis shows compound or peptide administered to the mouse. In Fig. 7, the black bar shows the results of culture of HLA-A0201 transgenic mouse-derived splenocytes while being pulsed with the peptide shown by SEQ ID NO: 2, and the white bar shows the results of culture without pulsing. That is, the difference in the values of the black bar and the white bar shows the number of peptide-specific CTL, and that the administration of the peptide shown by SEQ ID NO: 2 or a compound represented by the formula (8) resulted in the induction of CTL specific to the peptide shown by SEQ ID NO: 2 in vivo in the mouse. In Figure 7, the value of the white bar is not detected. This means that the splenocytes of HLA-A0201 transgenic mice did not react at all in the absence of pulsing with the object peptide. As a result of this test, IFNγ production specific to the peptide shown by SEQ ID NO: 2 was detected in the HLA-A0201 transgenic mouse-derived splenocytes. Moreover, in Fig. 7, the number of IFNγ-producing cells specific to the peptide shown by SEQ ID NO: 2, which were induced by the administration of a compound represented by the formula (8), was higher than that of the peptide-specific IFNγ-

producing cells induced by the administration of the peptide shown by SEQ ID NO: 2.

In Fig. 8, furthermore, the black bar shows the results of culture of HLA-A0201 transgenic mouse-derived splenocytes while being pulsed with peptide shown by SEQ ID NO: 22, and the white bar shows the results of culture without pulsing. That is, the difference in the values of the black bar and the white bar shows the number of peptide-reactive cells, and that the administration of a compound represented by the formula (8) resulted in the induction of cells reactive with the helper peptide shown by SEQ ID NO: 22 in vivo in the mouse, and administration of a peptide represented by SEQ ID NO: 2 did not induce cells reactive with the peptide shown by SEQ ID NO: 22 in vivo in the mouse. In Figure 8, the value of the white bar is not detected. This means that the splenocytes of HLA-A0201 transgenic mice did not react at all in the absence of pulsing with the object peptide.

[0422]

From the above, it was clarified that a compound represented by the formula (8) can induce CTL specific to the peptide shown by SEQ ID NO: 2 and cells reactive with the helper peptide shown by SEQ ID NO: 22. It was strongly suggested that the compound represented by the formula (8) undergoes cleavage of disulfide bond and appropriate trimming by ERAP-1 in mouse in vivo and is in fact processed into the peptides shown by SEQ ID NO: 2 and 22. It was assumed that the induction of the cell reactive with the helper peptide shown by SEQ ID NO: 22 produced from a compound represented by the formula (8) enhanced induction of CTL specific to the peptide shown by SEQ ID NO: 2, and many IFNγ-producing cells specific to the peptide shown by SEQ ID NO: 2 were found, as compared to the administration of the compound shown by SEQ ID NO: 2.

That is, it was clarified that a compound represented by the formula (8), which is one embodiment of the compound of the

present invention, is a conjugate wherein two different kinds of peptides form a composite via the disulfide bond shown in the formula (1), and is a WT1 cancer antigen peptide conjugate vaccine that in fact can induce CTLs and helper peptide reactive cells in vivo.

[0423]

Example 10

By a method similar to that in Example 1, respective compounds (conjugates) represented by the formula (9) were synthesized. Table 61 shows the results of mass spectrometry. (In each formula, the bond between C and C is a disulfide bond.)

[0424]

[Table 61]

Ex. No.	structural formula	formula No.	mass spectro-metry:LC-ESI/MS m/Z	mass spectro-metry: Calculated
10	CALLPAVPSL | CNKRYFKLSHLQMHSRKHTG	9	1151.9 $[M+3H]^{3+}$	1152.0

[0425]

Reference Example 8 - 9

By a method similar to that in Example 2, peptides consisting of the amino acid sequences shown by SEQ ID NOs: 238 - 239 were synthesized. The results of mass spectrometry are shown in Table 62. Since the peptides described in the Table are not the compound of the present invention, they are indicated as Reference Examples.

[0426]

[Table 62]

Ref. Ex. No.	amino acid sequence	SEQ ID NO:	mass spectro-metry:LC-ESI/MS m/Z	mass spectro-metry: Calculated
8	RMFPNAPYLCYTWNQMNL	238	1132.2 $[M+2H]^{2+}$	1132.3
9	CYTWNQMNLRMFPNAPYL	239	1133.0 $[M+2H]^{2+}$	1132.3

[0427]

Reference Examples 10 - 11

By a method similar to that in Example 2, peptides consisting of the amino acid sequences shown by SEQ ID NOs: 240 - 241 were synthesized. The results of mass spectrometry are shown in Table 63. Since the peptides described in the Table are not the compound of the present invention, they are indicated as Reference Examples.

[0428]

[Table 63]

Ref. Ex. No.	amino acid sequence	SEQ ID NO:	mass spectro-metry:LC-ESI/MS m/	mass spectro-metry: Calculated
10	RMFPNAPYLGGGGGGCYTWNQMNL	240	1303.7 $[M+2H]^{2+}$	1303.5
11	CYTWNQMNGGGGGGRMFPNAPYL	241	1303.0 $[M+2H]^{2+}$	1303.5

[0429]

The peptides shown in Table 63 were synthesized by referring to the non-patent document, Cancer Science January 2012, Vol. 103, no. 1, 150-153.

[0430]

Experimental Example 7

Stability test of conjugate and cocktail vaccine

[0431]

step 1

Conjugate (formula No.: (6)) (2.4 mg) was dissolved in 120 μL of water for injection and preserved under shading at room temperature.

step 2

As a cocktail vaccine, the peptide shown by SEQ ID NO: 2 (1.1 mg) was dissolved in 180 μL of water for injection, 123 μL thereof was used to dissolve the peptide shown by SEQ ID NO: 24 (1.3 mg), and the solution was preserved under shading at room temperature.

step 3

The solutions (2.5 μL) obtained in step 1 and step 2 were diluted with water for injection (50 μL) and subjected to HPLC analysis (analysis conditions are shown below), and the content percentage of the conjugate and peptide in the aqueous solution were measured with the area value immediately after the start of the preservation as 100%. The content percentage of the conjugate is shown in Table 64, and that of each peptide in the cocktail vaccine is shown in Table 65.

analysis conditions

pump: UFLC manufactured by Shimadzu

column: Kinetex 2.6u C18 100A 3.0 mm i.d. x 75 mm

mobile phase: SOLUTION A; 0.1% TFA water, SOLUTION B; 0.1% TFA acetonitrile solution

column temperature: 40°C

flow rate: 1 mL/min

detection wavelength: UV 220, 254 nm (2 wavelengths detection)

sample injection volume: 10 μL

[0432]

[Table 64]

elapsed time	formula No. (6) content percentage (%)
1 day	107
2 weeks	96

[0433]

[Table 65]

elapsed time	SEQ ID NO: 2 content percentage (%)	SEQ ID NO: 24 content percentage (%)
1 day	97	65
1 week	99	9
2 weeks	94	6

[0434]

In Experimental Example 7, the conjugate represented by formula No. (6) contained 96% of the compound represented by the formula (6) at the time point of 2 weeks from the solution preparation. In contrast, in a mixed solution of SEQ ID NO: 2 and SEQ ID NO: 24, which is a cocktail vaccine, the content percentage of SEQ ID NO: 24 decreased to 65% at the time point of 1 day elapse, and to 6% 2 weeks later. These results show that the conjugate preserved in the form of an aqueous solution was stabler than cocktail vaccine preserved under the same conditions.

[0435]

Experimental Example 8

Evaluation of in vivo CTL induction ability using HLA-A0201 transgenic mouse

[0436]

The compound represented by the formula (7) synthesized in Example 8 was evaluated for the CTL induction ability by an in vivo CTL induction test using HLA-A0201 transgenic mouse. The compound represented by the formula (7):

[0437]

CRMFPNAPYL
|
CNKRYFKLSHLQMHSRKH (7)

[0438]

wherein the bond between C and C is a disulfide bond, is, in particular, a compound of the aforementioned formula (1), wherein cancer antigen peptide A is RMFPNAPYL (SEQ ID NO: 2) and cancer antigen peptide C is CNKRYFKLSHLQMHSRKH (SEQ ID NO: 23). RMFPNAPYL (SEQ ID NO: 2) is a HLA-A0201-restricted WT1 peptide, and CNKRYFKLSHLQMHSRKH (SEQ ID NO: 23) is an MHC class II-restricted WT1 peptide (namely, helper peptide).

[0439]

The HLA-A0201 transgenic mouse is as described in Experimental Examples 2 and 5.

[0440]

Whether the administration of a compound represented by the formula (7) results in the induction of CTL specific to the object peptide (SEQ ID NO: 2) and cells reactive with helper peptide (SEQ ID NO: 23) was judged based on the measurement of IFNγ production by re-stimulation, with the peptide (SEQ ID NO: 2, 23), of the splenocyte derived from the above-mentioned mouse administered with a compound represented by the formula (7). In addition, the splenocytes derived from the above-mentioned mouse administered with a compound represented by the formula (7) and the splenocytes derived from the above-mentioned mouse administered with a compound represented by SEQ ID NO: 2 were re-stimulated with the peptide (SEQ ID NO: 2), and the IFNγ-producing cell numbers were compared.

[0441]

Specifically, a peptide represented by SEQ ID NO: 2 was dissolved in water for injection at 6 mg/mL, and emulsified by mixing with an equal amount of incomplete Freund's adjuvant (IFA). The emulsified peptide was intradermally administered to 2 sites at the base of tail of a mouse at 150 μg/site. A

compound represented by the formula (7) was dissolved in water for injection (19 mg/mL), and emulsified by mixing with an equal amount of incomplete Freund's adjuvant (IFA). The emulsified compound was intradermally administered to 2 sites at the base of tail of a mouse at 475 μg/site. The mole number of the peptide of SEQ ID NO: 2 contained in the dose of the compound represented by the formula (7) per one mouse was adjusted to be equal to that of the peptide of SEQ ID NO: 2 contained in the dose per mouse. One week later, the mouse was euthanized with CO_2 gas, the spleen was isolated, and splenocytes were prepared. IFNγ ELISPOT assay kit was used for the measurement of IFNγ production. On the previous day of splenocyte preparation, an ELISPOT plate was treated with an anti-mouse IFNγ antibody, and blocked with RPMI1640 medium containing 10% FBS the next day. The prepared HLA-A0201 transgenic mouse-derived splenocytes were plated at 0.25×10^6 cells/well or 0.5×10^6 cells/well on the blocked ELISPOT plate. Peptide (SEQ ID NO: 2, 23) was dissolved in DMSO at 40 mg/mL, and further diluted with RPMI1640 medium containing 10% FBS to 40 μg/mL. The diluted peptide (SEQ ID NO: 2, 23) was added to the HLA-A0201 transgenic mouse-derived splenocytes at a final concentration of 10 μg/mL. The splenocytes added with the peptide were cultured for 17 hr at 37°C, 5% CO_2, whereby peptide re-stimulation in vitro was performed. After culture, the supernatant was removed, and the ELISPOT plate was allowed to develop color according to the attached protocol. The number of spots that developed color was measured by ImmunoSpot Analyzer (manufactured by C.T.L.).

[0442]

The results of IFNγ ELISPOT assay using the HLA-A0201 transgenic mouse are shown in Figs. 9 and 10. In Figs. 9 and 10, the vertical axis shows the number of cells that reacted in the plated cells, and the horizontal axis shows compound or peptide administered to the mouse. In Fig. 9, the black bar shows the results of culture of HLA-A0201 transgenic mouse-derived

splenocytes while being pulsed with the peptide shown by SEQ ID NO: 2, and the white bar shows the results of culture without pulsing. That is, the difference in the values of the black bar and the white bar shows the number of peptide-specific CTL, and that the administration of the peptide shown by SEQ ID NO: 2 or a compound represented by the formula (7) resulted in the induction of CTL specific to the peptide shown by SEQ ID NO: 2 in vivo in the mouse. In Figure 9, the value of the white bar is not detected. This means that the splenocytes of HLA-A0201 transgenic mice did not react at all in the absence of pulsing with the object peptide. As a result of this test, IFNγ production specific to the peptide shown by SEQ ID NO: 2 was detected in the HLA-A0201 transgenic mouse-derived splenocytes. Moreover, in Fig. 9, the number of IFNγ-producing cells specific to the peptide shown by SEQ ID NO: 2, which were induced by the administration of a compound represented by the formula (7), was higher than that of the peptide-specific IFNγ-producing cells induced by the administration of the peptide shown by SEQ ID NO: 2.

In Fig. 10, furthermore, the black bar shows the results of culture of HLA-A0201 transgenic mouse-derived splenocytes while being pulsed with peptide shown by SEQ ID NO: 23, and the white bar shows the results of culture without pulsing. That is, the difference in the values of the black bar and the white bar shows the number of peptide-reactive cells, and that the administration of a compound represented by the formula (7) resulted in the induction of cells reactive with the helper peptide shown by SEQ ID NO: 23 in vivo in the mouse, and administration of a peptide represented by SEQ ID NO: 2 did not induce cells reactive with the peptide shown by SEQ ID NO: 23 in vivo in the mouse. In Fig. 10, the value of the white bar is not detected. This means that the splenocytes of HLA-A0201 transgenic mice did not react at all in the absence of pulsing with the object peptide.

[0443]

From the above, it was clarified that a compound represented by the formula (7) can induce CTL specific to the peptide shown by SEQ ID NO: 2 and cells reactive with the helper peptide shown by SEQ ID NO: 23. It was strongly suggested that the compound represented by the formula (7) undergoes cleavage of disulfide bond and appropriate trimming by ERAP-1 in mouse in vivo and is in fact processed into the peptides shown by SEQ ID NO: 2 and 23. It was assumed that the induction of the cell reactive with the helper peptide shown by SEQ ID NO: 23 produced from a compound represented by the formula (7) enhanced induction of CTL specific to the peptide shown by SEQ ID NO: 2, and many IFNγ-producing cells specific to the peptide shown by SEQ ID NO: 2 were found, as compared to the administration of the compound shown by SEQ ID NO: 2.

That is, it was clarified that a compound represented by the formula (7), which is one embodiment of the compound of the present invention, is a conjugate wherein two different kinds of peptides form a composite via the disulfide bond shown in the formula (1), and is a WT1 cancer antigen peptide conjugate vaccine that in fact can induce CTLs and helper peptide reactive cells in vivo.

[0444]

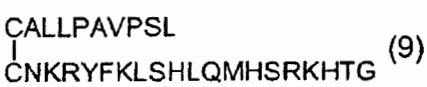
Experimental Example 9

Evaluation of in vivo CTL induction ability using HLA-A0201 transgenic mouse

[0445]

The compound represented by the formula (9) synthesized in Example 10 was evaluated for the CTL induction ability by an in vivo CTL induction test using HLA-A0201 transgenic mouse. The compound represented by the formula (9):

[0446]



(9)

[0447]

wherein the bond between C and C is a disulfide bond, is, in particular, a compound of the aforementioned formula (1), wherein cancer antigen peptide A is ALLPAVPSL (SEQ ID NO: 5) and cancer antigen peptide C is CNKRYFKLSHLQMHSRKHG (SEQ ID NO: 24). ALLPAVPSL (SEQ ID NO: 5) is a HLA-A0201 and HLA-A2402-restricted WT1 peptide, and CNKRYFKLSHLQMHSRKHG (SEQ ID NO: 24) is an MHC class II-restricted WT1 peptide (namely, helper peptide).

[0448]

The HLA-A0201 transgenic mouse is as described in Experimental Examples 2 and 5.

[0449]

Whether the administration of a compound represented by the formula (9) results in the induction of CTL specific to the object peptide (SEQ ID NO: 5) and cells reactive with helper peptide (SEQ ID NO: 24) was judged based on the measurement of IFNγ production by re-stimulation, with the peptide (SEQ ID NO: 5, 24), of the splenocyte derived from the above-mentioned mouse administered with a compound represented by the formula (9). In addition, the splenocytes derived from the above-mentioned mouse administered with a compound represented by the formula (9) and the splenocytes derived from the above-mentioned mouse administered with a compound represented by SEQ ID NO: 5 were re-stimulated with the peptide (SEQ ID NO: 5), and the IFNγ-producing cell numbers were compared.

[0450]

Specifically, a peptide represented by SEQ ID NO: 5 was dissolved in water for injection at 6 mg/mL, and emulsified by mixing with an equal amount of incomplete Freund's adjuvant (IFA). The emulsified peptide was intradermally administered to 2 sites at the base of tail of a mouse at 150 μg/site. A compound represented by the formula (9) was dissolved in water for injection (23.6 mg/mL), and emulsified by mixing with an equal amount of incomplete Freund's adjuvant (IFA). The

emulsified compound was intradermally administered to 2 sites at the base of tail of a mouse at 590 μg/site. The mole number of the peptide of SEQ ID NO: 5 contained in the dose of the compound represented by the formula (9) per one mouse was adjusted to be equal to that of the peptide of SEQ ID NO: 5 contained in the dose per mouse. One week later, the mouse was euthanized with CO_2 gas, the spleen was isolated, and splenocytes were prepared. IFNγ ELISPOT assay kit was used for the measurement of IFNγ production. On the previous day of splenocyte preparation, an ELISPOT plate was treated with an anti-mouse IFNγ antibody, and blocked with RPMI1640 medium containing 10% FBS the next day. The prepared HLA-A0201 transgenic mouse-derived splenocytes were plated at 0.25×10^6 cells/well or 0.75×10^6 cells/well on the blocked ELISPOT plate. Peptide (SEQ ID NO: 5, 24) was dissolved in DMSO at 40 mg/mL, and further diluted with RPMI1640 medium containing 10% FBS to 40 μg/mL. The diluted peptide (SEQ ID NO: 5, 24) was added to the HLA-A0201 transgenic mouse-derived splenocytes at a final concentration of 10 μg/mL. The splenocytes added with the peptide were cultured for 17 hr at 37°C, 5% CO_2, whereby peptide re-stimulation in vitro was performed. After culture, the supernatant was removed, and the ELISPOT plate was allowed to develop color according to the attached protocol. The number of spots that developed color was measured by ImmunoSpot Analyzer (manufactured by C.T.L.).

[0451]

The results of IFNγ ELISPOT assay using the HLA-A0201 transgenic mouse are shown in Figs. 11 and 12. In Figs. 11 and 12, the vertical axis shows the number of cells that reacted in the plated cells, and the horizontal axis shows the compound or peptide administered to the mouse. In Fig. 11, the black bar shows the results of culture of HLA-A0201 transgenic mouse-derived splenocytes while being pulsed with the peptide shown by SEQ ID NO: 5, and the white bar shows the results of culture

without pulsing. That is, the difference in the values of the black and the white bar shows the number of peptide-specific CTL, and that the administration of the peptide shown by SEQ ID NO: 5 or a compound represented by the formula (9) resulted in the induction of CTL specific to the peptide shown by SEQ ID NO: 5 in vivo in the mouse. In Fig. 11, the value of the white bar is not detected. This means that the splenocytes of HLA-A0201 transgenic mice did not react in the absence of pulsing with the object peptide. As a result of this test, IFNγ production specific to the peptide shown by SEQ ID NO: 5 was detected in the HLA-A0201 transgenic mouse-derived splenocytes. In Fig. 11, the number of IFNγ-producing cells specific to the peptide shown by SEQ ID NO: 5, which were induced by the administration of a compound represented by the formula (9), was higher than that of the peptide-specific IFNγ-producing cells induced by the administration of the peptide shown by SEQ ID NO: 5.

In Fig. 12, furthermore, the black bar shows the results of culture of HLA-A0201 transgenic mouse-derived splenocytes while being pulsed with peptide shown by SEQ ID NO: 24, and the white bar show the results of culture without pulsing. That is, the difference in the values of the black bar and the white bar shows the number of peptide-reactive cells, and that the administration of a compound represented by the formula (9) resulted in the induction of cells reactive with the helper peptide shown by SEQ ID NO: 24 in vivo in the mouse, and administration of the peptide represented by SEQ ID NO: 5 did not induce cells reactive with the peptide shown by SEQ ID NO: 24 in vivo in the mouse. In Figure 12, the value of the white bar is scarcely detected. This means that the splenocytes of HLA-A0201 transgenic mice did not react in the absence of pulsing with the object peptide.

[0452]

From the above, it was clarified that a compound represented by the formula (9) can induce CTL specific to the

peptide shown by SEQ ID NO: 5 and CTL reactive with the helper peptide shown by SEQ ID NO: 24. It was strongly suggested that the compound represented by the formula (9) undergoes cleavage of disulfide bond and appropriate trimming by ERAP-1 in mouse in vivo and is in fact processed into the peptides shown by SEQ ID NOs: 5 and 24. It was assumed that the induction of the cell reactive with the helper peptide shown by SEQ ID NO: 24 produced from a compound represented by the formula (9) enhanced induction of CTL specific to the peptide shown by SEQ ID NO: 5, and many IFNγ-producing cells specific to the peptide shown by SEQ ID NO: 5 were found, as compared to the administration of the compound shown by SEQ ID NO: 5.

That is, it was clarified that a compound represented by the formula (9), which is one embodiment of the compound of the present invention, is a conjugate wherein two different kinds of peptides form a composite via the disulfide bond shown in the formula (1), and is a WT1 cancer antigen peptide conjugate vaccine that in fact can induce CTLs and helper peptide reactive cells in vivo.

[0453]

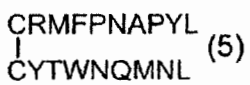
Comparative Example 1

Evaluation of in vivo CTL induction ability using HLA-A0201 transgenic mouse and HLA-A2402 transgenic mouse

[0454]

The compound represented by the formula (5) synthesized in Example 1 and the peptide shown by SEQ ID NO: 238 and 239 synthesized in Reference Example 8 and 9 were evaluated for the CTL induction ability by an in vivo CTL induction test using HLA-A0201 transgenic mouse and HLA-A2402 transgenic mouse. The compound represented by the formula (5):

[0455]



(5)

[0456]

wherein the bond between C and C is a disulfide bond, is as described in Experimental Example 2. The peptide shown by SEQ ID NOs: 238 and 239 is a long chain peptide wherein RMFPNAPYL (SEQ ID NO: 2), which is an HLA-A0201-restricted WT1 peptide, and CYTWNQMNL (SEQ ID NO: 4), which is HLA-A2402-restricted WT1 peptide, are linked by an amide bond.

[0457]

The HLA-A0201 transgenic mouse and HLA-A2402 transgenic mouse are as described in Experimental Example 2.

[0458]

Whether the administration of a compound represented by the formula (5) and the peptide shown by SEQ ID NO: 238, 239 results in the induction of CTL specific to the object peptide (SEQ ID NO: 2, 4) was judged based on the measurement of IFNγ production by re-stimulation, with the peptide (SEQ ID NO: 2, 4), of the splenocyte derived from the above-mentioned mouse administered with a compound represented by the formula (5) and the peptide shown by SEQ ID NO: 238, 239.

[0459]

Specifically, a compound represented by the formula (5) and the peptide shown by SEQ ID NOs: 238, 239 were each dissolved in dimethyl sulfoxide (DMSO) at 40 mg/mL, further diluted with water for injection to 5 mg/mL, and emulsified by mixing with an equal amount of incomplete Freund's adjuvant (IFA). The emulsified compound was intradermally administered to 4 sites at the base of tail of a mouse at 250 μg/site. One week later, the mouse was euthanized with CO_2 gas, the spleen was isolated, and splenocytes were prepared. IFNγ ELISPOT assay kit was used for the measurement of IFNγ production. On the previous day of splenocyte preparation, an ELISPOT plate was treated with an anti-mouse IFNγ antibody, and blocked with RPMI1640 medium containing 10% FBS the next day. The prepared HLA-A0201 transgenic mouse-derived splenocytes were plated at 0.25×10^6 cells/well, and HLA-A2402 transgenic mouse-derived

splenocytes were plated at 1×10^6 cells/well, on the blocked ELISPOT plate. Peptide (SEQ ID NO: 2, 4) was dissolved in DMSO at 40 mg/mL, and further diluted with RPMI1640 medium containing 10% FBS to 40 μg/mL. The diluted peptide (SEQ ID NO: 2) was added to the HLA-A0201 transgenic mouse-derived splenocytes at a final concentration of 10 μg/mL. In addition, the diluted peptide (SEQ ID NO: 4) was added to the HLA-A2402 transgenic mouse-derived splenocytes at a final concentration of 10 μg/mL. The splenocytes added with the peptide were cultivated for 18 hr at 37°C, 5% CO_2, whereby peptide re-stimulation in vitro was performed. After culture, the supernatant was removed, and the ELISPOT plate was allowed to develop color according to the attached protocol. The number of spots that developed color was measured by ImmunoSpot Analyzer (manufactured by C.T.L.).

[0460]

The results of IFNγ ELISPOT assay using HLA-A0201 transgenic mouse are shown in Fig. 13, and the results of IFNγ ELISPOT assay using HLA-A2402 transgenic mouse are shown in Fig. 14.

In each Figure, the vertical axis shows the number of cells that reacted in the plated cells. In Fig. 13, the black bar and the white bar show the results of culture of HLA-A0201 transgenic mouse-derived splenocytes in the presence or absence of the object peptide represented by SEQ ID NO: 2, and in Fig. 14, the black bar and the white bar show the results of culture of HLA-A2402 transgenic mouse-derived splenocytes in the presence or absence of the object peptide represented by SEQ ID NO: 4. That is, the difference in the values of the black bar and the white bar show the number of the object, each peptide-specific CTL induced in the mouse in vivo by the administration of a compound represented by the formula (5) and the peptide shown by SEQ ID NO: 238, 239.

In each Figure, the value of the white bar is not detected. This means that the splenocytes of respective transgenic mice

did not react at all in the absence of the object peptide. As a result of this test, IFNγ production specific to the object peptide shown by SEQ ID NO: 2 was detected in the splenocytes derived from HLA-A0201 transgenic mouse administered with a compound represented by the formula (5), and IFNγ production specific to the object peptide shown by SEQ ID NO: 4 was detected in the splenocytes derived from HLA-A02402 transgenic mouse administered with a compound represented by the formula (5). On the other hand, while IFNγ production specific to the object peptide shown by SEQ ID NO: 2 was detected in the splenocytes derived from HLA-A0201 transgenic mouse administered with the peptide shown by SEQ ID NO: 238; however, when compared to the splenocytes derived from HLA-A2402 transgenic mouse administered with a compound represented by the formula (5), the number thereof was very small. IFNγ production specific to the object peptide shown by SEQ ID NO: 4 was detected in the splenocytes derived from HLA-A2402 transgenic mouse administered with the peptide shown by SEQ ID NO: 238. While IFNγ production specific to the object peptide shown by SEQ ID NO: 2 was detected in the splenocytes derived from HLA-A0201 transgenic mouse administered with the peptide shown by SEQ ID NO: 239; however, when compared to the splenocytes derived from HLA-A0201 transgenic mouse administered with a compound represented by the formula (5), the number thereof was small. IFNγ production specific to the object peptide shown by SEQ ID NO: 4 was detected in the splenocytes derived from HLA-A2402 transgenic mouse administered with peptide SEQ ID NO: 239.

[0461]

Therefrom, the compound represented by the formula (5) of the present invention has been clarified to be able to efficiently induce CTL specific to the peptide shown by SEQ ID NO: 2 and CTL specific to the peptide shown by SEQ ID NO: 4. On the other hand, the long chain peptide shown by SEQ ID NOs: 238, 239 could not efficiently induce both the CTL specific to the

peptide shown by SEQ ID NO: 2 and the CTL specific to the peptide shown by SEQ ID NO: 4.

[0462]

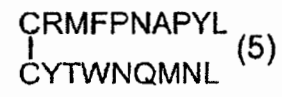
Comparative Example 2

Evaluation of in vivo CTL induction ability using HLA-A0201 transgenic mouse and HLA-A2402 transgenic mouse

[0463]

The compound represented by the formula (5) synthesized in Example 1 and peptides shown by SEQ ID NOs: 240 and 241 synthesized in Reference Examples 10 and 11 were evaluated for the CTL induction ability by an in vivo CTL induction test using HLA-A0201 transgenic mouse and HLA-A2402 transgenic mouse. The compound represented by the formula (5):

[0464]



(5)

[0465]

wherein the bond between C and C is a disulfide bond, is as described in Experimental Example 2. The peptide shown by SEQ ID NOs: 240 and 241 is a long chain peptide wherein RMFPNAPYL (SEQ ID NO: 2), which is an HLA-A0201-restricted WT1 peptide, and CYTWNQMNL (SEQ ID NO: 4), which is an HLA-A2402-restricted WT1 peptide, are linked by an amide bond via 6 glycines as a peptide spacer.

[0466]

HLA-A0201 transgenic mouse and HLA-A2402 transgenic mouse are as indicated in Experimental Example 2.

[0467]

Whether the administration of a compound represented by the formula (5) and SEQ ID NO: 240, 241 peptide shown by results in the induction of CTL specific to the object peptide (SEQ ID NO: 2, 4) was judged based on the measurement of IFNγ production by re-stimulation, with the peptide (SEQ ID NO: 2, 4), of the splenocyte derived from the above-mentioned mouse

administered with a compound represented by the formula (5) and the peptide shown by SEQ ID NO: 240, 241.

[0468]

Specifically, a compound represented by the formula (5) was dissolved in dimethyl sulfoxide (DMSO) at 80 mg/mL, further diluted with water for injection to 10 mg/mL, and emulsified by mixing with an equal amount of incomplete Freund's adjuvant (IFA). The emulsified compound was intradermally administered to 2 sites at the base of tail of a mouse at 500 μg/site. In addition, the peptides shown by SEQ ID NOs: 240, 241 were dissolved in dimethyl sulfoxide (DMSO) at 80 mg/mL, further diluted with water for injection to 11 mg/mL, and emulsified by mixing with an equal amount of incomplete Freund's adjuvant (IFA). The emulsified compound was intradermally administered to 2 sites at the base of tail of a mouse at 550 μg/site. One week later, the mouse was euthanized with CO_2 gas, the spleen was isolated, and splenocytes were prepared. IFNγ ELISPOT assay kit was used for the measurement of IFNγ production. On the previous day of splenocyte preparation, an ELISPOT plate was treated with an anti-mouse IFNγ antibody, and blocked with RPMI1640 medium containing 10% FBS the next day. The prepared HLA-A0201 transgenic mouse-derived splenocytes at 0.25×10^6 cells/well and HLA-A2402 transgenic mouse-derived splenocytes at 1.5×10^6 cells/well were plated on the blocked ELISPOT plate. Peptide (SEQ ID NO: 2, 4) was dissolved in DMSO at 40 mg/mL, and further diluted with RPMI1640 medium containing 10% FBS to 40 μg/mL. The diluted peptide (SEQ ID NO: 2) was added to the HLA-A0201 transgenic mouse-derived splenocytes at a final concentration of 10 μg/mL. In addition, the diluted peptide (SEQ ID NO: 4) was added to HLA-A2402 transgenic mouse-derived splenocytes at a final concentration of 10 μg/mL. The splenocytes added with the peptide were cultured for 17 hr at 37°C, 5% CO_2, whereby peptide re-stimulation in vitro was performed. After culture, the supernatant was removed, and the

ELISPOT plate was allowed to develop color according to the attached protocol. The number of spots that developed color was measured by ImmunoSpot Analyzer (manufactured by C.T.L.).

[0469]

The results of IFNγ ELISPOT assay using HLA-A0201 transgenic mouse are shown in Fig. 15, and the results of IFNγ ELISPOT assay using HLA-A2402 transgenic mouse are shown in Fig. 16.

In each Figure, the vertical axis shows the number of cells that reacted in the plated cells. In Fig. 15, the black bar and the white bar show the results of culture of HLA-A0201 transgenic mouse-derived splenocytes in the presence or absence of the object peptide represented by SEQ ID NO: 2, and in Fig. 16, the black bar and the white bar show the results of culture of HLA-A2402 transgenic mouse-derived splenocytes in the presence or absence of the object peptide represented by SEQ ID NO: 4. That is, the difference in the values of the black bar and the white bar show the number of the object, each peptide-specific CTL induced in the mouse in vivo by the administration of a compound represented by the formula (5) and the peptides shown by SEQ ID NOs: 240, 241.

In each Figure, the value of the white bar is not detected. This means that the splenocytes of respective transgenic mice did not react in the absence of the object peptide. As a result of this test, IFNγ production specific to the object peptide shown by SEQ ID NO: 2 was detected in the splenocytes derived from HLA-A0201 transgenic mouse administered with a compound represented by the formula (5), and IFNγ production specific to the object peptide shown by SEQ ID NO: 4 was detected in the splenocytes derived from HLA-A2402 transgenic mouse administered with a compound represented by the formula (5). On the other hand, IFNγ production specific to the object peptide shown by SEQ ID NO: 2 was extremely less in the splenocytes derived from HLA-A0201 transgenic mouse administered with the peptide shown

by SEQ ID NO: 240; however, IFNγ production specific to the object peptide shown by SEQ ID NO: 4 was detected in the splenocytes derived from HLA-A02402 transgenic mouse administered with a compound represented by SEQ ID NO: 240. In addition, IFNγ production specific to the object peptide shown by SEQ ID NO: 2 was extremely less in the splenocytes derived from HLA-A0201 transgenic mouse administered with the peptide shown by SEQ ID NO: 241. While IFNγ production specific to the object peptide shown by SEQ ID NO: 4 was detected in the splenocytes derived from HLA-A0201 transgenic mouse administered with the peptide shown by SEQ ID NO: 241; however, when compared to the splenocytes derived from HLA-A2402 transgenic mouse administered with a compound represented by the formula (5), the number thereof was very small.

[0470]

Therefrom, the compound represented by the formula (5) of the present invention has been clarified to be able to efficiently induce CTL specific to the peptide shown by SEQ ID NO: 2 and CTL specific to the peptide shown by SEQ ID NO: 4. On the other hand, the long chain peptide containing the peptide spacer shown by SEQ ID NO: 240, 241 could not efficiently induce both the CTL specific to the peptide shown by SEQ ID NO: 2 and the CTL specific to the peptide shown by SEQ ID NO: 4.

[0471]

Experimental Example 10

The peptide synthesized in Reference Example 3 and the compounds (conjugates) synthesized in Examples 6 and 9 were subjected to the solubility measurement by a method similar to that in Experimental Example 3. Each solubility is shown in Table 66.

[0472]

[Table 66]

Ex. No. and Ref. Ex.	amino acid sequence or structural formula	SEQ ID NO: or formula No.	pH 6.0 (mg/mL)	pH 7.4 (mg/mL)
Ref. Ex. 3	CNKRYFKLSHLQMHSRKH	SEQ ID NO: 23	>1.0	0.712
Ex. 6	CRMFPNAPYL | CSLGEQQYSV	formula (3)	>1.0	>1.0
Ex. 10	CALLPAVPSL | CNKRYFKLSHLQMHSRKHTG	formula (9)	>1.0	0.565

[0473]

Examples 11 - 12

By a method similar to that in Example 2, peptides consisting of the amino acid sequences of SEQ ID NOs: 242 - 243 were synthesized. The results of mass spectrometry are shown in Table 67. The peptides described in Table 67 are the compounds of the present invention.

[0474]

[Table 67]

Ex. No.	amino acid sequence	SEQ ID NO:	mass spectro-metry: LC-ESI/MS m/z	mass spectro-metry: Calculated
11	CWAPVLDFAPPGASAYGSL	242	1923.5 $[M+H]^{1+}$	1923.2
12	WAPVLDFAPPGASAYGSLC	243	1923.6 $[M+H]^{1+}$	1923.2

[0475]

Example 13

By a method similar to that in Example 1, each compound (conjugate) represented by the formula 10 was synthesized. The

results of mass spectrometry are shown in Table 68, wherein the bond between C and C is a disulfide bond.

[0476]

[Table 68]

Ex. No.	structural formula	formula No.	mass spectrometry:LC-ESI/MS m/Z	mass spectrometry: Calculated
13	CRMFPNAPYL | CWAPVLDFAPPGASAYGSL	10	1566.6 $[M+2H]^{2+}$	1566.8

[0477]

Reference Example 12

By a method similar to that in Example 1, each compound (conjugate) represented by the formula 11 was synthesized. The results of mass spectrometry are shown in Table 69, wherein the bond between C and C is a disulfide bond. The peptide described in the Table is not the compound of the present invention, and therefore, it is described as Reference Example.

[0478]

[Table 69]

Ref. Ex. No.	structural formula	formula No.	mass spectrometry: LC-ESI/MS m/z	mass spectrometry: Calculated
12	WAPVLDFAPPGASAYGSLC | CRMFPNAPYL	11	1044.8 $[M+3H]^{3+}$	1044.9

[0479]

Example 14

Synthesis of the compound represented by the formula (12):

[0480]

CRMFPNAPYL
|
CACYTWNQMNL (12)
|
CNKRYFKLSHLQMHSRK

[0481]

wherein the bond between C and C is a disulfide bond

[0482]

step 1. Synthesis of Fmoc-Cys(Mmt)-Ala-SBn (Mmt is 4-Methoxytrityl)

(Synthesis of Fmoc-C(Mmt)A-SBn)

A solution of Fmoc-Cys(Mmt)-OH (4.80 g), N,N-diisopropylethylamine (2.56 mL), hexafluorophosphoric acid (benzotriazol-1-yloxy)tripyrrolidinophosphonium (4.50 g) and H-Ala-SBn synthesized by a known method (for example, Journal of Organic Chemistry, Vol. 64, No. 24 8761-8769) in chloroform (20 ml) was stirred at room temperature for 1 hr. The reaction mixture was purified by column chromatography (elution solvent, hexane/ethyl acetate) to give the object compound, Fmoc-C(Mmt)A-SBn (2.80 g).

NMR: ^{1}H NMR ($CDCl_3$) δ 7.72 (t, J = 7.6 Hz, 2H), 7.54 (d, J = 7.2 Hz, 1H), 7.38-7.34 (m, 7H), 7.29-7.25 (m, 6H), 7.23-7.15 (m, 7H), 6.76 (d, J = 8.8 Hz, 2H), 6.15 (d, J = 8.0 Hz, 1H), 4.95 (d, J = 7.2 Hz, 1H), 4.57 (quin, J = 7.6 Hz, 1H), 4.35 (d, J = 6.8 Hz, 2H) 4.19-4.17 (m, 1H), 4.04 (s, 2H), 3.73 (s, 3H), 2.72 (dd, J = 13.2, 8.4 Hz, 1H), 2.61 (d, J = 9.6 Hz, 1H), 1.31 (d, J = 7.2 Hz, 3H).

[0483]

step 2. Synthesis of H-Cys(Mmt)-Ala-Cys-Tyr-Thr-Trp-Asn-Gln-Met-Asn-Leu-OH

(Synthesis of C(Mmt)ACYTWNQMNL)

A solution of Fmoc-Cys(Mmt)-Ala-SBn(11mg) obtained in step 1, H-Cys-Tyr-Thr-Trp-Asn-Gln-Met-Asn-Leu-OH (21 mg) synthesized by a known method (for example, WO07/063903), N,N-diisopropylethylamine (200 µL), 3,3',3''-Phosphanetriyl tripropanoic acid hydrochloride (1 mg), 4-mercaptophenylacetic

acid (1 mg) and 0.1M sodium phosphate buffer (pH 7.5, 200 µL) in DMF (400 µL) was stirred at room temperature for 4 hr. To the reaction mixture was added diethylamine (200 µL) and the mixture was further stirred for 15 min. The reaction mixture was purified by reversed-phase HPLC to give the object compound, C(Mmt)ACYTWNQMNL (7 mg).

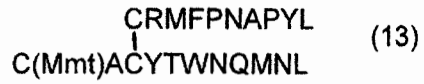
mass spectrometry: LC-ESI/MS m/z=810.2 $[M+2H]^{2+}$ (Calculated=810.5)

[0484]

step 3. Synthesis of (H-Cys(Mmt)-Ala-Cys-Tyr-Thr-Trp-Asn-Gln-Met-Asn-Leu-OH) (H-Cys-Arg-Met-Phe-Pro-Asn-Ala-Pro-Tyr-Leu-OH) disulfide bond

[i.e., synthesis of a compound represented by the formula (13):

[0485]



(13)

[0486]

wherein the bond between C and C is a disulfide bond.

A solution of H-Cys(Mmt)-Ala-Cys-Tyr-Thr-Trp-Asn-Gln-Met-Asn-Leu-OH (51 mg) obtained in step 2 and (H-Cys(Npys)-Arg-Met-Phe-Pro-Asn-Ala-Pro-Tyr-Leu-OH (43 mg) obtained in Example 1, step 1 in DMF (4 mL) was stirred at room temperature for 2 hr. The reaction mixture was purified by reversed-phase HPLC to give 39 mg of the object compound, (H-Cys(Mmt)-Ala-Cys-Tyr-Thr-Trp-Asn-Gln-Met-Asn-Leu-OH) (H-Cys-Arg-Met-Phe-Pro-Asn-Ala-Pro-Tyr-Leu-OH) disulfide bond [i.e., a compound represented by the formula (13)].

mass spectrometry: LC-ESI/MS m/z=1414.4 $[M+2H]^{2+}$ (Calculated=1415.2)

[0487]

step 4. Synthesis of H-Cys(SPy)-Asn-Lys-Arg-Tyr-Phe-Lys-Leu-Ser-His-Leu-Gln-Met-His-Ser-Arg-Lys-OH

(Synthesis of C(SPy)NKRYFKLSHLQMHSRK)

A 20% w/w solution of H-Cys-Asn-Lys-Arg-Tyr-Phe-Lys-Leu-

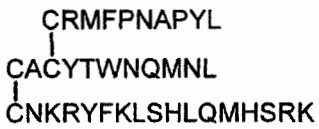
Ser-His-Leu-Gln-Met-His-Ser-Arg-Lys-OH (182 mg) obtained in Reference Example 1 and 2,2'-dipyridylbisulfide (0.2M isopropanol solution, 544 µL) in acetic acid water (4 mL) was stirred at room temperature for 17 hr. The reaction mixture was purified by reversed-phase HPLC to give the object compound, H-Cys(SPy)-Asn-Lys-Arg-Tyr-Phe-Lys-Leu-Ser-His-Leu-Gln-Met-His-Ser-Arg-Lys-OH (177 mg).

mass spectrometry: LC-ESI/MS m/z=1143.5 $[M+2H]^{2+}$ (Calculated=1142.9)

[0488]

step 5. Synthesis of a compound represented by the formula (12):

[0489]



(12)

[0490]

wherein the bond between C and C is a disulfide bond

A solution of (H-Cys(Mmt)-Ala-Cys-Tyr-Thr-Trp-Asn-Gln-Met-Asn-Leu-OH)(H-Cys-Arg-Met-Phe-Pro-Asn-Ala-Pro-Tyr-Leu-OH) disulfide bond obtained in step 3 [i.e., a compound represented by the formula (13)] (9 mg), H-Cys(SPy)-Asn-Lys-Arg-Tyr-Phe-Lys-Leu-Ser-His-Leu-Gln-Met-His-Ser-Arg-Lys-OH (24 mg) obtained in step 4 and triisopropylsilane (10 µL) in trifluoroacetic acid (190 µL) was stirred at room temperature for 1 hr. The reaction mixture was purified by reversed-phase HPLC to give the object compound, a compound represented by the formula 12 (5 mg).

mass spectrometry: LC-ESI/MS m/z=1577.2 $[M+3H]^{3+}$ (Calculated=1577.9)

[0491]

Examples 15 - 16

By a method similar to that in Example 14, each compound (conjugate) represented by the formula 14 or 15 was synthesized. The results of mass spectrometry are shown in Table 70, wherein the bond between C and C is a disulfide bond.

[0492]

[Table 70]

Ex. No.	structural formula	formula No.	mass spectro-metry: LC-ESI/MS m/Z	mass spectro-metry: Calculated
15	CRMFPNAPYL | CACYTWNQMNL | CWAPVLDFAPPGASAYGSL	14	1492.5 $[M+3H]^{3+}$	1493.1
16	CWAPVLDFAPPGASAYGSL | CACYTWNQMNL | CRMFPNAPYL	15	1492.5 $[M+3H]^{3+}$	1493.1

[0493]

Reference Example 13

By a method similar to that in Example 2, peptides consisting of the amino acid sequence of SEQ ID NO: 244 were synthesized. Table 71 shows the results of mass spectrometry. The peptide described in the Table is not the compound of the present invention, and therefore, it is described as Reference Example.

[0494]

[Table 71]

Ref. Ex. No.	amino acid sequence	SEQ ID NO:	mass spectro-metry: LC-ESI/MS m/Z	mass spectro-metry: Calculated
13	WAPVLDFAPPGASAYGSL	244	1819.8 $[M+H]^{+}$	1819.1

[0495]

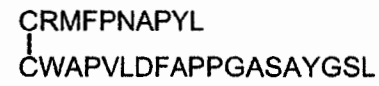
Experimental Example 11

Evaluation of in vivo CTL induction ability using HLA-A0201 transgenic mouse

[0496]

The compound represented by the formula (10) synthesized in Example 13 was evaluated for the CTL induction ability by an in vivo CTL induction test using HLA-A0201 transgenic mouse. The compound represented by the formula (10):

[0497]



(10)

[0498]

wherein the bond between C and C is a disulfide bond, is, in particular, a compound of the aforementioned formula (1), wherein cancer antigen peptide A is RMFPNAPYL (SEQ ID NO: 2) and cancer antigen peptide B is WAPVLDFAPPGASAYGSL (SEQ ID NO: 244). RMFPNAPYL (SEQ ID NO: 2) is an HLA-A0201-restricted WT1 peptide, and WAPVLDFAPPGASAYGSL (SEQ ID NO: 244) is MHC class II-restricted WT1 peptide (namely, helper peptide).

[0499]

The HLA-A0201 transgenic mouse is as described in Experimental Examples 2 and 5.

[0500]

Whether the administration of a compound represented by the formula (10) results in the induction of CTL specific to the object peptide (SEQ ID NO: 2) was judged based on the measurement of IFNγ production by re-stimulation, with the peptide (SEQ ID NO: 2), of the splenocyte derived from the above-mentioned mouse administered with a compound represented by the formula (10). Whether or not the helper peptide (SEQ ID NO: 244) works in the living body was judged by comparison of the number of IFNγ-producing cells when the splenocytes derived from the above-mentioned mouse administered with a compound represented by the formula (10) and the splenocytes derived from the above-mentioned mouse administered with the peptide shown by SEQ ID NO: 2 were re-stimulated with the peptide (SEQ ID NO: 2).

[0501]

Specifically, a compound shown by SEQ ID NO: 2 was

dissolved in dimethyl sulfoxide (DMSO) at 80 mg/mL, further diluted with water for injection to 3 mg/mL, and emulsified by mixing with an equal amount of incomplete Freund's adjuvant (IFA). The emulsified compound was intradermally administered to 2 sites at the base of tail of a mouse at 150 μg/site. In addition, a compound represented by the formula (10) was dissolved in dimethyl sulfoxide (DMSO) at 80 mg/mL, further diluted with water for injection to 8.5 mg/mL, and emulsified by mixing with an equal amount of incomplete Freund's adjuvant (IFA). The emulsified compound was intradermally administered to 2 sites at the base of tail of a mouse at 425 μg/site. The mole number of the peptide of the SEQ ID NO: 2 contained in the dose of a compound represented by the formula (10) per mouse was controlled to be equal to the mole number contained in the dose of the peptide shown by SEQ ID NO: 2 per mouse. In addition, the concentration of DMSO contained in each emulsion was also set to the same level. One week later, the mouse was euthanized with CO_2 gas, the spleen was isolated, and splenocytes were prepared. IFNγ ELISPOT assay kit was used for the measurement of IFNγ production. On the previous day of splenocyte preparation, an ELISPOT plate was treated with an anti-mouse IFNγ antibody, and blocked with RPMI1640 medium containing 10% FBS the next day. The prepared HLA-A0201 transgenic mouse-derived splenocytes were plated at 0.125×10^6 cells/well on the blocked ELISPOT plate. Peptide (SEQ ID NO: 2, 4) was dissolved in DMSO at 40 mg/mL, and further diluted with RPMI1640 medium containing 10% FBS to 40 μg/mL. The diluted peptide (SEQ ID NO: 2) was added to the HLA-A0201 transgenic mouse-derived splenocytes at a final concentration of 10 μg/mL. The splenocytes added with the peptide were cultivated for 19 hr at 37°C, 5% CO_2, whereby peptide re-stimulation in vitro was performed. After culture, the supernatant was removed, and the ELISPOT plate was allowed to develop color according to the attached protocol. The number of spots that developed color was

measured by ImmunoSpot Analyzer (manufactured by C.T.L.).

[0502]

The results of IFNγ ELISPOT assay using the HLA-A0201 transgenic mouse are shown in Fig. 17. In Fig. 17, the vertical axis shows the number of cells that reacted in the plated cells, and the horizontal axis shows compound or peptide administered to the mouse. In Fig. 17, the black bar shows the results of culture of HLA-A0201 transgenic mouse-derived splenocytes while being pulsed with the peptide shown by SEQ ID NO: 2, and the white bar shows the results of culture without pulsing. That is, the difference in the values of the black bar and the white bar shows the number of peptide-specific CTL, and that the administration of the peptide shown by SEQ ID NO: 2 or a compound represented by the formula (10) resulted in the induction of CTL specific to the peptide shown by SEQ ID NO: 2 in vivo in the mouse. In Fig. 17, the value of the white bar is not detected. This means that the splenocytes of HLA-A0201 transgenic mice did not react at all in the absence of pulsing with the object peptide. As a result of this test, IFNγ production specific to the peptide shown by SEQ ID NO: 2 was detected in the HLA-A0201 transgenic mouse-derived splenocytes. Moreover, in Fig. 17, the number of IFNγ-producing cells specific to the peptide shown by SEQ ID NO: 2, which were induced by the administration of a compound represented by the formula (10), was higher than that of the peptide-specific IFNγ-producing cells induced by the administration of the peptide shown by SEQ ID NO: 2.

[0503]

From the above, it was clarified that a compound represented by the formula (10) can induce CTL specific to the peptide shown by SEQ ID NO: 2. When a compound represented by the formula (10) was administered, many IFNγ producing cells specific to the peptide shown by SEQ ID NO: 2 were observed as compared to administration of the peptide shown by SEQ ID NO: 2.

It was assumed that the induction of the cell reactive with the helper peptide shown by SEQ ID NO: 244 produced from a compound represented by the formula (10) enhanced induction of CTL specific to the peptide shown by SEQ ID NO: 2. Therefore, it was strongly suggested that the compound represented by the formula (10) undergoes cleavage of disulfide bond and appropriate trimming by ERAP-1 in mouse in vivo and is in fact processed into the peptides shown by SEQ ID NOs: 2 and 244.

That is, it was clarified that a compound represented by the formula (10), which is one embodiment of the compound of the present invention, is a conjugate wherein two different kinds of peptides form a composite via the disulfide bond shown in the formula (1), and is a WT1 cancer antigen peptide conjugate vaccine that in fact can induce CTLs and helper peptide reactive cells in vivo.

[0504]

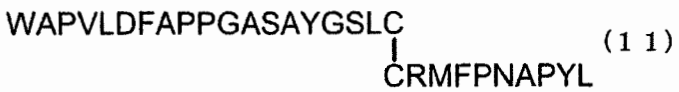
Comparative Example 3

Evaluation of in vivo CTL induction ability using HLA-A0201 transgenic mouse

[0505]

The compound represented by the formula (11) synthesized in Reference Example 12 was evaluated for the CTL induction ability by an in vivo CTL induction test using HLA-A0201 transgenic mouse. The compound represented by the formula (11):

[0506]



(11)

[0507]

wherein the bond between C and C is a disulfide bond, is, in particular, a compound of the aforementioned formula (1), wherein cancer antigen peptide A is RMFPNAPYL (SEQ ID NO: 2) and cancer antigen peptide C is WAPVLDFAPPGASAYGSLC (SEQ ID NO: 243). RMFPNAPYL (SEQ ID NO: 2) is a HLA-A0201-restricted WT1 peptide, and WAPVLDFAPPGASAYGSL (SEQ ID NO: 244) is an MHC class II-

restricted WT1 peptide (namely, helper peptide).

[0508]

The HLA-A0201 transgenic mouse is as described in Experimental Examples 2 and 5.

[0509]

Whether the administration of a compound represented by the formula (11) results in the induction of CTL specific to the object peptide (SEQ ID NO: 2) was judged based on the measurement of IFNγ production by re-stimulation, with the peptide (SEQ ID NO: 2), of the splenocytes derived from the above-mentioned mouse administered with a compound represented by the formula (11). Whether or not the helper peptide (SEQ ID NO: 244) works in the living body was judged by comparison of the number of IFNγ-producing cells when the splenocytes derived from the above-mentioned mouse administered with a compound represented by the formula (11) and the splenocytes derived from the above-mentioned mouse administered with the peptide shown by SEQ ID NO: 2 were re-stimulated with the peptide (SEQ ID NO: 2).

[0510]

By a method similar to that in Experimental Example 11, CTL induction test was performed.

[0511]

The results of IFNγ ELISPOT assay using the HLA-A0201 transgenic mouse are shown in Fig. 18. In Fig. 18, the vertical axis shows the number of cells that reacted in the plated cells, and the horizontal axis shows compound or peptide administered to the mouse. In Fig. 18, the black bar shows the results of culture of HLA-A0201 transgenic mouse-derived splenocytes while being pulsed with the peptide shown by SEQ ID NO: 2, and the white bar shows the results of culture without pulsing. That is, the difference in the values of the black bar and the white bar shows the number of peptide-specific CTL, and that the administration of the peptide shown by SEQ ID NO: 2 or a compound represented by the formula (11) resulted in the

induction of CTL specific to the peptide shown by SEQ ID NO: 2 in vivo in the mouse. In Fig. 18, the value of the white bar is not detected. This means that the splenocytes of HLA-A0201 transgenic mice did not react at all in the absence of pulsing with the object peptide. As a result of this test, IFNγ production specific to the peptide shown by SEQ ID NO: 2 was detected in the HLA-A0201 transgenic mouse-derived splenocytes. On the other hand, in Fig. 18, an increase in the IFNγ producing cells specific to the peptide shown by SEQ ID NO: 2, which was induced by the administration of the peptide shown by SEQ ID NO: 2, could not be detected by the administration of a compound represented by the formula (11).

[0512]

The results of Experimental Example 11 and Comparative Example 3 suggest that, when WAPVLDFAPPGASAYGSL (SEQ ID NO: 244) is used as an MHC class II-restricted WT1 peptide, WAPVLDFAPPGASAYGSL (SEQ ID NO: 244) as the cancer antigen peptide B in the aforementioned formula (1) is a more preferable embodiment of the invention than WAPVLDFAPPGASAYGSLC (SEQ ID NO: 243) as the cancer antigen peptide C.

[0513]

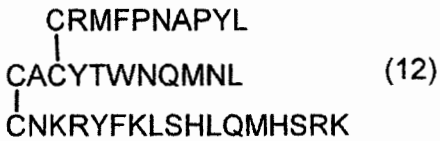
Experimental Example 12

Evaluation of in vivo CTL induction ability using HLA-A0201 transgenic mouse and HLA-A2402 transgenic mouse

[0514]

The CTL induction ability of the compound represented by the formula 12 synthesized in Example 14 was evaluated by an in vivo CTL induction test using an HLA-A0201 transgenic mouse and an HLA-A2402 transgenic mouse. RMFPNAPYL (SEQ ID NO: 2) contained in the compound represented by the formula (12):

[0515]



(12)

[0516]

wherein the bond between C and C is a disulfide bond, is an HLA-A0201-restricted WT1 peptide, CYTWNQMNL (SEQ ID NO: 4) is HLA-A2402-restricted WT1 peptide, and CNKRYFKLSHLQMHSRK (SEQ ID NO: 22) is MHC class II-restricted WT1 peptide (namely, helper peptide).

[0517]

The HLA-A0201 transgenic mouse and HLA-A2402 transgenic mouse are as described in Experimental Examples 2 and 5.

[0518]

Whether the administration of a compound represented by the formula (12) results in the induction of CTL specific to the object peptide (SEQ ID NO: 2, 4) was judged based on the measurement of IFNγ production by re-stimulation, with the peptide (SEQ ID NO: 2, 4), of the splenocyte derived from the above-mentioned mouse administered with a compound represented by the formula (12). Whether or not the helper peptide (SEQ ID NO: 22) works in the living body was judged by comparison of the number of IFNγ-producing cells when the splenocytes derived from the above-mentioned mouse administered with a compound represented by the formula (12) and the splenocytes derived from the above-mentioned mouse administered with a compound represented by the formula (5) were re-stimulated with the peptide (SEQ ID NOs: 2, 4).

[0519]

Specifically, a compound represented by the formula (5) was dissolved in dimethyl sulfoxide (DMSO) at 80 mg/mL, further diluted with water for injection to 3 mg/mL, and emulsified by mixing with an equal amount of Montanide ISA51VG. The emulsified compound was intradermally administered to 2 sites at the base of tail of a mouse at 150 μg/site. In addition, a compound represented by the formula (12) was dissolved in dimethyl sulfoxide (DMSO) at 80 mg/mL, further diluted with water for injection to 6 mg/mL, and emulsified by mixing with an

equal amount of Montanide ISA51VG. The emulsified compound was intradermally administered to 2 sites at the base of tail of a mouse at 300 μg/site. The mole number of the compound represented by the formula (5) contained in the dose of the compound represented by the formula (12) per mouse was controlled to be equal to the mole number contained in the dose of the compound represented by the formula (5) per mouse. In addition, the concentration of DMSO contained in each emulsion was also set to the same level. One week later, the mouse was euthanized with CO_2 gas, the spleen was isolated, and splenocytes were prepared. IFNγ ELISPOT assay kit was used for the measurement of IFNγ production. On the previous day of splenocyte preparation, an ELISPOT plate was treated with an anti-mouse IFNγ antibody, and blocked with RPMI1640 medium containing 10% FBS the next day. The prepared HLA-A0201 transgenic mouse-derived splenocytes were plated, and HLA-A2402 transgenic mouse-derived splenocytes were each plated at 0.25×10^6 cells/well, on the blocked ELISPOT plate. Peptide (SEQ ID NO: 2, 4) was dissolved in DMSO at 40 mg/mL, and further diluted with RPMI1640 medium containing 10% FBS to 40 μg/mL. The diluted peptide (SEQ ID NO: 2) was added to the HLA-A0201 transgenic mouse-derived splenocytes at a final concentration of 10 μg/mL. In addition, the diluted peptide (SEQ ID NO: 4) was added to the HLA-A2402 transgenic mouse-derived splenocytes at a final concentration of 10 μg/mL. The splenocytes added with the peptide were cultivated for 17 hr at 37°C, 5% CO_2, whereby peptide re-stimulation in vitro was performed. After culture, the supernatant was removed, and the ELISPOT plate was allowed to develop color according to the attached protocol. The number of spots that developed color was measured by ImmunoSpot Analyzer (manufactured by C.T.L.).

[0520]

The results of IFNγ ELISPOT assay using HLA-A0201 transgenic mouse are shown in Fig. 19, and the results of IFNγ

ELISPOT assay using HLA-A2402 transgenic mouse are shown in Fig. 20.

In each Figure, the vertical axis shows the number of cells that reacted in the plated cells. In Fig. 19, the black bar and the white bar show the results of culture of HLA-A0201 transgenic mouse-derived splenocytes in the presence or absence of the object peptide represented by SEQ ID NO: 2, and in Fig. 20, the black bar and the white bar show the results of culture of HLA-A2402 transgenic mouse-derived splenocytes in the presence or absence of the object peptide represented by SEQ ID NO: 4. That is, the difference in the values of the black bar and the white bar show the number of the object, each peptide-specific CTL induced in the mouse in vivo by the administration of compounds represented by the formula (5) and formula (12).

In each Figure, the value of the white bar is not detected. This means that the splenocytes of respective transgenic mice did not react in the absence of the object peptide. As a result of this test, IFNγ production specific to the object peptide shown by SEQ ID NO: 2 was detected in the HLA-A0201 transgenic mouse-derived splenocytes administered compounds represented by the formula (5) and formula (12), and IFNγ production specific to the object peptide shown by SEQ ID NO: 4 was detected in the HLA-A2402 transgenic mouse-derived splenocytes administered compounds represented by the formula (5) and formula (12). In Fig. 19, the number of the IFNγ-producing cells specific to the peptide shown by SEQ ID NO: 2, which was induced by the administration of a compound represented by the formula (12), was higher than the number of the IFNγ producing cells specific to peptide, which was induced by the administration of a compound represented by the formula (5). On the other hand, in Fig. 20, the number of the IFNγ-producing cells specific to the peptide shown by SEQ ID NO: 4, which was induced by the administration of a compound represented by the formula (12), did not differ much from the number of the IFNγ producing cells

specific to peptide, which was induced by the administration of a compound represented by the formula (5).

[0521]

From the above, it was clarified that a compound represented by the formula (12) can induce CTL specific to the peptide shown by SEQ ID NOs: 2, 4. When a compound represented by the formula (12) was administered, many IFNγ producing cells specific to the peptide shown by SEQ ID NO: 2 were observed as compared to administration of the compound represented by the formula (5). It was assumed that the induction of the cell reactive with the helper peptide shown by SEQ ID NO: 22 produced from a compound represented by the formula (12) enhanced induction of CTL specific to the peptide shown by SEQ ID NO: 2. The absence of much difference in the number of IFNγ-producing cells specific to the peptide shown by SEQ ID NO: 4 between the administration of a compound represented by the formula 12 and the administration of a compound represented by the formula (5) was assumed to be attributable to the absence of induction of the cells reactive with the helper peptide shown by SEQ ID NO: 22, since the HLA-A2402 transgenic mouse does not express human MHC class II. Accordingly, it was strongly suggested that the compound represented by the formula (12) undergoes cleavage of disulfide bond and appropriate trimming by ERAP-1 in mouse in vivo and is in fact processed into the peptides shown by SEQ ID NOs: 2, 4 and 22.

That is, it was clarified that a compound represented by the formula (12), which is one embodiment of the compound of the present invention, is a conjugate wherein three different kinds of peptides form a composite via the disulfide bond, and is a WT1 cancer antigen peptide conjugate vaccine that in fact can induce CTLs and helper peptide reactive cells in vivo.

[0522]

Experimental Example 13

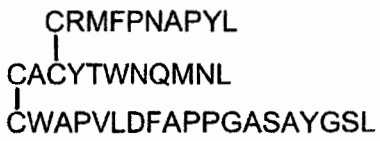
Evaluation of in vivo CTL induction ability using HLA-

A0201 transgenic mouse and HLA-A2402 transgenic mouse

[0523]

The CTL induction ability of the compound represented by the formula (14) synthesized in Example 15 was evaluated by an in vivo CTL induction test using an HLA-A0201 transgenic mouse and an HLA-A2402 transgenic mouse. RMFPNAPYL (SEQ ID NO: 2) contained in a compound represented by the formula (14):

[0524]



(14)

[0525]

wherein the bond between C and C is a disulfide bond, is an HLA-A0201-restricted WT1 peptide, CYTWNQMNL (SEQ ID NO: 4) is HLA-A2402-restricted WT1 peptide, WAPVLDFAPPGASAYGSL (SEQ ID NO: 244) is MHC class II-restricted WT1 peptide (namely, helper peptide).

[0526]

The HLA-A0201 transgenic mouse and HLA-A2402 transgenic mouse are as described in Experimental Examples 2 and 5.

[0527]

Whether the administration of a compound represented by the formula (14) results in the induction of CTL specific to the object peptide (SEQ ID NO: 2, 4) was judged based on the measurement of IFNγ production by re-stimulation, with the peptide (SEQ ID NO: 2, 4), of the splenocyte derived from the above-mentioned mouse administered with a compound represented by the formula (14). Whether or not helper peptide (SEQ ID NO: 244) works in the living body was judged by comparison of the number of IFNγ-producing cells when the splenocytes derived from the above-mentioned mouse administered with a compound represented by the formula (14) and the splenocytes derived from the above-mentioned mouse administered with a compound represented by the formula (5) were re-stimulated with the

peptide (SEQ ID NO: 2).

[0528]

By a method similar to that in Experimental Example 12, a CTL induction test was performed. The compound represented by the formula (14) was dissolved in dimethyl sulfoxide (DMSO) at 80 mg/mL, diluted with water for injection at 5.6 mg/mL, and mixed with an equal amount of Montanide ISA51VG to give an emulsion. The emulsified compound was intradermally administered to 2 sites at the base of tail of a mouse at 280 μg/site.

[0529]

The results of IFNγ ELISPOT assay using HLA-A0201 transgenic mouse are shown in Fig. 21, and the results of IFNγ ELISPOT assay using HLA-A2402 transgenic mouse are shown in Fig. 22.

In each Figure, the vertical axis shows the number of cells that reacted in the plated cells. In Fig. 21, the black bar and the white bar show the results of culture of HLA-A0201 transgenic mouse-derived splenocytes in the presence or absence of the object peptide represented by SEQ ID NO: 2, and in Fig. 22, the black bar and the white bar show the results of culture of HLA-A2402 transgenic mouse-derived splenocytes in the presence or absence of the object peptide represented by SEQ ID NO: 4. That is, the difference in the values of the black bar and the white bar show the number of the object, each peptide-specific CTL induced in the mouse in vivo by the administration of compounds represented by the formulas (5) and (14).

In each Figure, the value of the white bar is not detected. This means that the splenocytes of respective transgenic mice did not react in the absence of the object peptide. As a result of this test, IFNγ production specific to the object peptide shown by SEQ ID NO: 2 was detected in the splenocytes derived from HLA-A0201 transgenic mouse administered with compounds represented by the formulas (5) and (14), and IFNγ production

specific to the object peptide shown by SEQ ID NO: 4 was detected in the splenocytes derived from HLA-A2402 transgenic mouse administered with compounds represented by the formulas (5) and (14). In Figs. 21 and 22, the number of the IFNγ producing cells specific to the peptide shown by SEQ ID NO: 2, 4, which was induced by the administration of a compound represented by the formula (14), was higher than the number of the peptide-specific IFNγ producing cells, which was induced by the administration of a compound represented by the formula (5).

[0530]

From the above, it was clarified that a compound represented by the formula (14) can induce CTL specific to the peptides shown by SEQ ID NOs: 2 and 4. It was assumed that the induction of the cell reactive with the helper peptide shown by SEQ ID NO: 244 produced from a compound represented by the formula (14) enhanced induction of CTL specific to the peptide shown by SEQ ID NO: 2, and many IFNγ-producing cells specific to the peptide shown by SEQ ID NO: 2 were found when a compound represented by the formula (14) was administered as compared to the administration of a compound represented by the formula (5). On the other hand, many IFNγ-producing cells specific to the peptide shown by SEQ ID NO: 4 were found when a compound represented by the formula (14) was administered as compared to the administration of a compound represented by the formula (5). It was assumed that the peptide shown by SEQ ID NO: 244 was bound to mouse MHC class II expressed in HLA-A2402 transgenic mouse to induce the cell reactive with the helper peptide, which in turn enhanced induction of CTL specific to the peptide shown by SEQ ID NO: 4. Therefore, it was strongly suggested that the compound represented by the formula (14) undergoes cleavage of disulfide bond and appropriate trimming by ERAP-1 in mouse in vivo and is in fact processed into the peptides shown by SEQ ID NOs: 2, 4 and 244.

That is, it was clarified that a compound represented by

the formula (14), which is one embodiment of the compound of the present invention, is a conjugate wherein three different kinds of peptides form a composite via the disulfide bond, and is a WT1 cancer antigen peptide conjugate vaccine that in fact can induce CTLs and helper peptide reactive cells in vivo.

[0531]

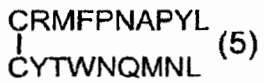
Experimental Example 14

Evaluation of in vivo CTL induction ability using HLA-A0201 transgenic mouse

[0532]

A cocktail vaccine which is a mixture of the compound represented by the formula (5) synthesized in Example 1 and the peptide shown by SEQ ID NO: 22 synthesized in Reference Example 1 was evaluated for the CTL induction ability by an in vivo CTL induction test using HLA-A0201 transgenic mouse. RMFPNAPYL (SEQ ID NO: 2) contained in the compound represented by the formula (5):

[0533]



[0534]

wherein the bond between C and C is a disulfide bond, is an HLA-A0201-restricted WT1 peptide, CYTWNQMNL (SEQ ID NO: 4) is HLA-A2402-restricted WT1 peptide, and CNKRYFKLSHLQMHSRK (SEQ ID NO: 22) is MHC class II-restricted WT1 peptide (namely, helper peptide).

[0535]

The HLA-A0201 transgenic mouse is as described in Experimental Examples 2 and 5.

[0536]

Whether the administration of a compound represented by the formula (5) results in the induction of CTL specific to the object peptide (SEQ ID NO: 2) was judged based on the measurement of IFNγ production by re-stimulation, with the

peptide (SEQ ID NO: 2), of the splenocyte derived from the above-mentioned mouse administered with a compound represented by the formula (5). Whether or not the helper peptide (SEQ ID NO: 22) mixed with the formula (5) works in the living body was judged by comparison of the number of IFNγ-producing cells when the splenocytes derived from the above-mentioned mouse administered with a compound represented by the formula (5) alone and the splenocytes derived from the above-mentioned mouse administered with a cocktail vaccine of a compound represented by the formula (5) and the peptide shown by SEQ ID NO: 22 were re-stimulated with the peptide (SEQ ID NO: 2).

[0537]

Specifically, a compound represented by the formula (5) was dissolved in dimethyl sulfoxide (DMSO) at 80 mg/mL, further diluted with water for injection to 3 mg/mL, and emulsified by mixing with an equal amount of Montanide ISA51VG. The emulsified compound was intradermally administered to 2 sites at the base of tail of a mouse at 150 μg/site. In addition, a compound represented by the formula (5) and the peptide shown by SEQ ID NO: 22 were dissolved in dimethyl sulfoxide (DMSO) at 80 mg/mL, diluted with water for injection and mixed such that the concentration after dilution was 3 mg/mL for the compound represented by the formula (5), and 2.7 mg/mL for the peptide shown by SEQ ID NO: 22. The diluted solution was mixed with an equal amount of Montanide ISA51VG to give an emulsion. The cocktail vaccine containing a compound represented by the formula (5) at 150 μg/site, and the peptide shown by SEQ ID NO: 22 at 137 μg/site was intradermally administered to 2 sites at the base of tail of a mouse. The DMSO concentration of each emulsion was set to the same level. One week later, the mouse was euthanized with CO_2 gas, the spleen was isolated, and splenocytes were prepared. IFNγ ELISPOT assay kit was used for the measurement of IFNγ production. On the previous day of splenocyte preparation, an ELISPOT plate was treated with an

anti-mouse IFNγ antibody, and blocked with RPMI1640 medium containing 10% FBS the next day. The prepared HLA-A0201 transgenic mouse-derived splenocytes were plated at 0.25×10^6 cells/well on the blocked ELISPOT plate. Peptide (SEQ ID NO: 2) was dissolved in DMSO at 40 mg/mL, and further diluted with RPMI1640 medium containing 10% FBS to 40 μg/mL. The diluted peptide (SEQ ID NO: 2) was added to the HLA-A0201 transgenic mouse-derived splenocytes at a final concentration of 10 μg/mL. The splenocytes added with the peptide were cultured for 17 hr at 37°C, 5% CO_2, whereby peptide re-stimulation in vitro was performed. After culture, the supernatant was removed, and the ELISPOT plate was allowed to develop color according to the attached protocol. The number of spots that developed color was measured by ImmunoSpot Analyzer (manufactured by C.T.L.).

[0538]

The results of IFNγ ELISPOT assay using the HLA-A0201 transgenic mouse are shown in Fig. 23.

In Fig. 23, the vertical axis shows the number of cells that reacted in the plated cells. In Fig. 23, the black bar and the white bar show the results of culture of HLA-A0201 transgenic mouse-derived splenocytes in the presence or absence of the object peptide represented by SEQ ID NO: 2. That is, the difference in the values of the black bar and the white bar shows the number of the object, each peptide-specific CTL induced in the mouse in vivo by the administration of a cocktail vaccine containing a compound represented by the formula (5) and a helper peptide (SEQ ID NO: 22).

In Fig. 23, the value of the white bar is not detected. This means that the splenocytes of respective transgenic mice did not react in the absence of the object peptide. As a result of this test, IFNγ production specific to the object peptide shown by SEQ ID NO: 2 was detected in the splenocytes derived from HLA-A0201 transgenic mouse administered with a compound represented by the formula (5) alone, and a cocktail vaccine

containing a helper peptide (SEQ ID NO: 22). In Fig. 23, the number of the IFNγ producing cells specific to the peptide shown by SEQ ID NO: 2, which was induced by the administration of a cocktail vaccine containing a helper peptide (SEQ ID NO: 22), was higher than the number of the peptide-specific IFNγ producing cells, which was induced by the administration of a compound represented by the formula (5) alone.

[0539]

From the above, it was clarified that a cocktail vaccine containing a compound represented by the formula (5) and the peptide shown by SEQ ID NO: 22 can induce CTL specific to the peptides shown by SEQ ID NO: 2. In addition, many IFNγ-producing cells specific to the peptide shown by SEQ ID NO: 2 were found when a cocktail vaccine was administered as compared to the single administration of a compound represented by the formula (5). It was assumed that the induction of the cell reactive with the helper peptide shown by SEQ ID NO: 22 contained in the cocktail vaccine enhanced induction of CTL specific to the peptide shown by SEQ ID NO: 2. Therefore, it was clarified that a cocktail vaccine containing a compound represented by the formula (5) and the helper peptide can strongly induce CTL in the body of mouse as compared to the single administration of a compound represented by the formula (5).

[0540]

Experimental Example 15

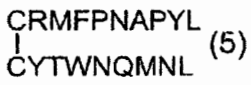
Evaluation of in vivo CTL induction ability using HLA-A0201 transgenic mouse

[0541]

The CTL induction ability of a cocktail vaccine of a compound represented by the formula (5) synthesized in Example 1 and the peptide shown by SEQ ID NO: 244 synthesized in Reference Example 13 was evaluated by an in vivo CTL induction test using an HLA-A0201 transgenic mouse. RMFPNAPYL (SEQ ID NO: 2)

contained in a compound represented by the formula (5):

[0542]



[0543]

wherein the bond between C and C is a disulfide bond, is an HLA-A0201-restricted WT1 peptide, CYTWNQMNL (SEQ ID NO: 4) is an HLA-A2402-restricted WT1 peptide, and WAPVLDFAPPGASAYGSL (SEQ ID NO: 244) is an MHC class II-restricted WT1 peptide (namely, helper peptide).

[0544]

The HLA-A0201 transgenic mouse is as described in Experimental Examples 2 and 5.

[0545]

Whether the administration of a compound represented by the formula (5) results in the induction of CTL specific to the object peptide (SEQ ID NO: 2) was judged based on the measurement of IFNγ production by re-stimulation, with the peptide (SEQ ID NO: 2), of the splenocyte derived from the above-mentioned mouse administered with a compound represented by the formula (5). Whether or not the helper peptide (SEQ ID NO: 244) mixed with the formula (5) works in the living body was judged by comparison of the number of IFNγ-producing cells when the splenocytes derived from the above-mentioned mouse administered with a compound represented by the formula (5) alone and the splenocytes derived from the above-mentioned mouse administered with a cocktail vaccine of a compound represented by the formula (5) and the peptide shown by SEQ ID NO: 244 were re-stimulated with the peptide (SEQ ID NO: 2).

[0546]

By a method similar to that in Experimental Example 14, a CTL induction test was performed. To give a cocktail vaccine, a compound represented by the formula (5) and the peptide shown by SEQ ID NO: 244 were dissolved in dimethyl sulfoxide (DMSO) at 80

mg/mL, diluted with water for injection and mixed such that the concentration after dilution was 3 mg/mL for the compound represented by the formula (5), and 2.3 mg/mL for the peptide shown by SEQ ID NO: 244. The diluted solution was mixed with an equal amount of Montanide ISA51VG to give an emulsion. A cocktail vaccine containing the compound represented by the formula (5) at 150 μg/site, and the peptide shown by SEQ ID NO: 244 at 115 μg/site was intradermally administered to 2 sites at the base of tail of a mouse.

[0547]

The results of IFNγ ELISPOT assay using the HLA-A0201 transgenic mouse are shown in Fig. 24.

In Fig. 24, the vertical axis shows the number of cells that reacted in the plated cells. In Fig. 24, the black bar and the white bar show the results of culture of HLA-A0201 transgenic mouse-derived splenocytes in the presence or absence of the object peptide represented by SEQ ID NO: 2. That is, the difference in the values of the black bar and the white bar shows the number of the object, each peptide-specific CTL induced in the mouse in vivo by the administration of a cocktail vaccine containing a compound represented by the formula (5) and a helper peptide (SEQ ID NO: 244).

In Fig. 24, the value of the white bar is not detected. This means that the splenocytes of respective transgenic mice did not react in the absence of the object peptide. As a result of this test, IFNγ production specific to the object peptide shown by SEQ ID NO: 2 was detected in the splenocytes derived from HLA-A0201 transgenic mouse administered with a compound represented by the formula (5) alone, and a cocktail vaccine containing a helper peptide (SEQ ID NO: 244). In Fig. 24, the number of the IFNγ producing cells specific to the peptide shown by SEQ ID NO: 2, which was induced by the administration of a cocktail vaccine containing a helper peptide (SEQ ID NO: 244), was higher than the number of the peptide-specific IFNγ

producing cells, which was induced by the administration of a compound represented by the formula (5) alone.

[0548]

From the above, it was clarified that a cocktail vaccine containing a compound represented by the formula (5) and the peptide shown by SEQ ID NO: 244 can induce CTL specific to the peptides shown by SEQ ID NO: 2. In addition, many IFNγ-producing cells specific to the peptide shown by SEQ ID NO: 2 were found when a cocktail vaccine was administered as compared to the single administration of a compound represented by the formula (5). It was assumed that the induction of the cell reactive with the helper peptide shown by SEQ ID NO: 244 contained in the cocktail vaccine enhanced induction of CTL specific to the peptide shown by SEQ ID NO: 2. Therefore, it was clarified that a cocktail vaccine containing a compound represented by the formula (5) and the helper peptide can strongly induce CTL in the body of mouse as compared to the single administration of a compound represented by the formula (5).

[0549]

As one embodiment of producing a vaccine containing two WT1 antigen peptides, a cocktail vaccine containing two different peptides as a single preparation can be mentioned. When producing a cocktail vaccine, the properties of the cancer antigen peptides to be mixed poses one problem. As shown in Table 60 and Table 66, production of a cocktail of two WT1 antigen peptides means processing of two peptides having different solubility, namely, property, into one preparation. In contrast, the conjugate of the present invention is a compound wherein two WT1 antigen peptides are bonded via a disulfide bond, and shows a single solubility, namely, property. This means that the conjugate of the present invention has single property and also has the property corresponding to the two WT1 antigen peptides, as shown in Experimental Example 2.

In this aspect, it was shown that the conjugate of the present invention is a compound capable of inducing a response to the two WT1 antigen peptides without the need to consider an interaction between the two WT1 antigen peptides and the like, unlike cocktail vaccines.

[0550]

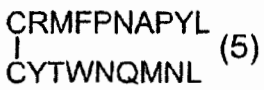
Experimental Example 16

Evaluation of in vivo CTL induction ability using HLA-A0201 transgenic mouse

[0551]

The CTL induction ability of a cocktail vaccine of a compound represented by the formula (5) synthesized in Example 1 and the peptide shown by SEQ ID NO: 24 synthesized in Reference Example 2 was evaluated by an in vivo CTL induction test using an HLA-A0201 transgenic mouse. RMFPNAPYL (SEQ ID NO: 2) contained in a compound represented by the formula (5):

[0552]



[0553]

wherein the bond between C and C is a disulfide bond, is an HLA-A0201-restricted WT1 peptide, CYTWNQMNL (SEQ ID NO: 4) is an HLA-A2402-restricted WT1 peptide, and CNKRYFKLSHLQMHSRKTG (SEQ ID NO: 24) is an MHC class II-restricted WT1 peptide (namely, helper peptide).

[0554]

The HLA-A0201 transgenic mouse is as described in Experimental Examples 2 and 5.

[0555]

Whether the administration of a compound represented by the formula (5) results in the induction of CTL specific to the object peptide (SEQ ID NO: 2) was judged based on the measurement of IFNγ production by re-stimulation, with the peptide (SEQ ID NO: 2), of the splenocyte derived from the

above-mentioned mouse administered with a compound represented by the formula (5). Whether or not the helper peptide (SEQ ID NO: 24) mixed with the formula (5) works in the living body was judged by comparison of the number of IFNγ-producing cells when the splenocytes derived from the above-mentioned mouse administered with a compound represented by the formula (5) alone and the splenocytes derived from the above-mentioned mouse administered with a cocktail vaccine of a compound represented by the formula (5) and the peptide shown by SEQ ID NO: 24 were re-stimulated with the peptide (SEQ ID NO: 2).

[0556]

Specifically, a compound represented by the formula (5) was dissolved in dimethyl sulfoxide (DMSO) at 80 mg/mL, further diluted with water for injection to 3 mg/mL, and emulsified by mixing with an equal amount of Montanide ISA51VG. The emulsified compound was intradermally administered to 2 sites at the base of tail of a mouse at 150 μg/site. In addition, a compound represented by the formula (5) and the peptide shown by SEQ ID NO: 24 were dissolved in dimethyl sulfoxide (DMSO) at 80 mg/mL, and diluted with water for injection. They were mixed such that the concentration after dilution is 3 mg/mL for the compound represented by the formula (5), and 3.11 mg/mL for the peptide shown by SEQ ID NO: 24. The diluted solution was mixed with an equal amount of Montanide ISA51VG to give an emulsion. The cocktail vaccine containing a compound represented by the formula (5) at 150 μg/site, and the peptide shown by SEQ ID NO: 24 at 156 μg/site was intradermally administered to 2 sites at the base of tail of a mouse. The DMSO concentration of each emulsion was set to the same level. One week later, the mouse was euthanized with CO_2 gas, the spleen was isolated, and splenocytes were prepared. IFNγ ELISPOT assay kit was used for the measurement of IFNγ production. On the previous day of splenocyte preparation, an ELISPOT plate was treated with an anti-mouse IFNγ antibody, and blocked with RPMI1640 medium

containing 10% FBS the next day. The prepared HLA-A0201 transgenic mouse-derived splenocytes were plated at 0.25×10^6 cells/well on the blocked ELISPOT plate. Peptide (SEQ ID NO: 2) was dissolved in DMSO at 40 mg/mL, and further diluted with RPMI1640 medium containing 10% FBS to 40 μg/mL. The diluted peptide (SEQ ID NO: 2) was added to the HLA-A0201 transgenic mouse-derived splenocytes at a final concentration of 10μg/mL. The splenocytes added with the peptide were cultured for 19 hr at 37°C, 5% CO_2, whereby peptide re-stimulation in vitro was performed. After culture, the supernatant was removed, and the ELISPOT plate was allowed to develop color according to the attached protocol. The number of spots that developed color was measured by ImmunoSpot Analyzer (manufactured by C.T.L.).

[0557]

The results of IFNγ ELISPOT assay using the HLA-A0201 transgenic mouse are shown in Fig. 25.

In Fig. 25, the vertical axis shows the number of cells that reacted in the plated cells. In Fig. 25, the black bar and the white bar show the results of culture of HLA-A0201 transgenic mouse-derived splenocytes in the presence or absence of the object peptide represented by SEQ ID NO: 2. That is, the difference in the values of the black bar and the white bar shows the number of the object, each peptide-specific CTL induced in the mouse in vivo by the administration of a cocktail vaccine containing a compound represented by the formula (5) and a helper peptide (SEQ ID NO: 24).

In Fig. 25, the value of the white bar is not detected. This means that the splenocytes of respective transgenic mice did not react in the absence of the object peptide. As a result of this test, IFNγ production specific to the object peptide shown by SEQ ID NO: 2 was detected in the splenocytes derived from HLA-A0201 transgenic mouse administered with a compound represented by the formula (5) alone, and a cocktail vaccine containing a helper peptide (SEQ ID NO: 24). In Fig. 25, the

number of the IFNγ producing cells specific to the peptide shown by SEQ ID NO: 2, which was induced by the administration of a cocktail vaccine containing a helper peptide (SEQ ID NO: 24), was higher than the number of the peptide-specific IFNγ producing cells, which was induced by the single administration of a compound represented by the formula (5) alone.

[0558]

From the above, it was clarified that a cocktail vaccine containing a compound represented by the formula (5) and the peptide shown by SEQ ID NO: 24 can induce CTL specific to the peptides shown by SEQ ID NO: 2. In addition, many IFNγ-producing cells specific to the peptide shown by SEQ ID NO: 2 were found when a cocktail vaccine was administered as compared to the single administration of a compound represented by the formula (5). It was assumed that the induction of the cell reactive with the helper peptide shown by SEQ ID NO: 24 contained in the cocktail vaccine enhanced induction of CTL specific to the peptide shown by SEQ ID NO: 2. Therefore, it was clarified that a cocktail vaccine containing a compound represented by the formula (5) and the helper peptide can strongly induce CTL in the body of mouse as compared to the single administration of a compound represented by the formula (5).

[0559]

Experimental Example 17

Evaluation of in vivo CTL induction ability using HLA-A0201 transgenic mouse

[0560]

SEQ ID NO: 242 synthesized in Example 11 is the peptide shown by SEQ ID NO: 244 having an extended cysteine at the N-terminal. SEQ ID NO: 244 in the cocktail vaccine in Experimental Example 15 shows a CTL induction enhancing activity. In this test, therefore, the CTL induction ability of a cocktail vaccine of a compound represented by the formula (5) synthesized

in Example 1 and the peptide shown by SEQ ID NO: 242 was evaluated by an in vivo CTL induction test using an HLA-A0201 transgenic mouse. RMFPNAPYL (SEQ ID NO: 2) contained in a compound represented by the formula (5):

[0561]



(5)

[0562]

wherein the bond between C and C is a disulfide bond, is an HLA-A0201-restricted WT1 peptide, CYTWNQMNL (SEQ ID NO: 4) is an HLA-A2402-restricted WT1 peptide, and WAPVLDFAPPGASAYGSL(SEQ ID NO: 244) contained in CWAPVLDFAPPGASAYGSL (SEQ ID NO: 242) is an MHC class II-restricted WT1 peptide (namely, helper peptide).

[0563]

The HLA-A0201 transgenic mouse is as described in Experimental Examples 2 and 5.

[0564]

Whether the administration of a compound represented by the formula (5) results in the induction of CTL specific to the object peptide (SEQ ID NO: 2) was judged based on the measurement of IFNγ production by re-stimulation, with the peptide (SEQ ID NO: 2), of the splenocyte derived from the above-mentioned mouse administered with a compound represented by the formula (5). Whether or not the helper peptide (SEQ ID NO: 242) mixed with the formula (5) works in the living body was judged by comparison of the number of IFNγ-producing cells when the splenocytes derived from the above-mentioned mouse administered with a compound represented by the formula (5) alone and the splenocytes derived from the above-mentioned mouse administered with a cocktail vaccine of a compound represented by the formula (5) and the peptide shown by SEQ ID NO: 242 were re-stimulated with the peptide (SEQ ID NO: 2).

[0565]

By a method similar to that in Experimental Example 16, a

CTL induction test was performed. To give a cocktail vaccine, a compound represented by the formula (5) and the peptide shown by SEQ ID NO: 242 were dissolved in dimethyl sulfoxide (DMSO) at 80 mg/mL, diluted with water for injection and mixed such that the concentration after dilution was 3 mg/mL for the compound represented by the formula (5), and 2.42 mg/mL for the peptide shown by SEQ ID NO: 242. The diluted solution was mixed with an equal amount of Montanide ISA51VG to give an emulsion. A cocktail vaccine containing the compound represented by the formula (5) at 150 μg/site, and the peptide shown by SEQ ID NO: 242 at 121 μg/site was intradermally administered to 2 sites at the base of tail of a mouse.

[0566]

The results of IFNγ ELISPOT assay using the HLA-A0201 transgenic mouse are shown in Fig. 26.

In Fig. 26, the vertical axis shows the number of cells that reacted in the plated cells. In Fig. 26, the black bar and the white bar show the results of culture of HLA-A0201 transgenic mouse-derived splenocytes in the presence or absence of the object peptide represented by SEQ ID NO: 2. That is, the difference in the values of the black bar and the white bar shows the number of the object, each peptide-specific CTL induced in the mouse in vivo by the administration of a cocktail vaccine containing a compound represented by the formula (5) and a helper peptide (SEQ ID NO: 242).

In Fig. 26, the value of the white bar is not detected. This means that the splenocytes of respective transgenic mice did not react in the absence of the object peptide. As a result of this test, IFNγ production specific to the object peptide shown by SEQ ID NO: 2 was detected in the splenocytes derived from HLA-A0201 transgenic mouse administered with a compound represented by the formula (5) alone, and a cocktail vaccine containing a helper peptide (SEQ ID NO: 242). In Fig. 26, the number of the IFNγ producing cells specific to the peptide shown

by SEQ ID NO: 2, which was induced by the administration of a cocktail vaccine containing a helper peptide (SEQ ID NO: 242), was higher than the number of the peptide-specific IFNγ producing cells, which was induced by the administration of a compound represented by the formula (5) alone.

[0567]

From the above, it was clarified that a cocktail vaccine containing a compound represented by the formula (5) and the peptide shown by SEQ ID NO: 242 can induce CTL specific to the peptides shown by SEQ ID NO: 2. In addition, many IFNγ-producing cells specific to the peptide shown by SEQ ID NO: 2 were found when a cocktail vaccine was administered as compared to the single administration of a compound represented by the formula (5). It was assumed that the induction of the cell reactive with the helper peptide shown by SEQ ID NO: 244 contained in the peptide shown by SEQ ID NO: 242 contained in the cocktail vaccine enhanced induction of CTL specific to the peptide shown by SEQ ID NO: 2. Therefore, it was clarified that a cocktail vaccine containing a compound represented by the formula (5) and the helper peptide can strongly induce CTL in the body of mouse as compared to the single administration of a compound represented by the formula (5).

[0568]

Experimental Example 18

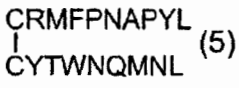
Evaluation of in vivo CTL induction ability using HLA-A0201 transgenic mouse

[0569]

SEQ ID NO: 243 synthesized in Example 12 is the peptide shown by SEQ ID NO: 244 having an extended cysteine at the N-terminal. SEQ ID NO: 244 in the cocktail vaccine in Experimental Example 15 shows a CTL induction enhancing activity. In this test, therefore, the CTL induction ability of a cocktail vaccine of a compound represented by the formula (5) synthesized in Example 1 and the peptide shown by SEQ ID NO: 243 was

evaluated by an in vivo CTL induction test using an HLA-A0201 transgenic mouse. RMFPNAPYL (SEQ ID NO: 2) contained in a compound represented by the formula (5):

[0570]



[0571]

wherein the bond between C and C is a disulfide bond, is an HLA-A0201-restricted WT1 peptide, CYTWNQMNL (SEQ ID NO: 4) is an HLA-A2402-restricted WT1 peptide, and WAPVLDFAPPGASAYGSL(SEQ ID NO: 244) contained in WAPVLDFAPPGASAYGSLC (SEQ ID NO: 243) is an MHC class II-restricted WT1 peptide (namely, helper peptide).

[0572]

The HLA-A0201 transgenic mouse is as described in Experimental Examples 2 and 5.

[0573]

Whether the administration of a compound represented by the formula (5) results in the induction of CTL specific to the object peptide (SEQ ID NO: 2) was judged based on the measurement of IFNγ production by re-stimulation, with the peptide (SEQ ID NO: 2), of the splenocyte derived from the above-mentioned mouse administered with a compound represented by the formula (5). Whether or not the helper peptide (SEQ ID NO: 243) mixed with the formula (5) works in the living body was judged by comparison of the number of IFNγ-producing cells when the splenocytes derived from the above-mentioned mouse administered with a compound represented by the formula (5) alone and the splenocytes derived from the above-mentioned mouse administered with a cocktail vaccine of a compound represented by the formula (5) and the peptide shown by SEQ ID NO: 243 were re-stimulated with the peptide (SEQ ID NO: 2).

[0574]

By a method similar to that in Experimental Example 16, a CTL induction test was performed. To give a cocktail vaccine, a

compound represented by the formula (5) and the peptide shown by SEQ ID NO: 243 were dissolved in dimethyl sulfoxide (DMSO) at 80 mg/mL, diluted with water for injection and mixed such that the concentration after dilution was 3 mg/mL for the compound represented by the formula (5), and 2.42 mg/mL for the peptide shown by SEQ ID NO: 243. The diluted solution was mixed with an equal amount of Montanide ISA51VG to give an emulsion. A cocktail vaccine containing the compound represented by the formula (5) at 150 μg/site, and the peptide shown by SEQ ID NO: 243 at 121 μg/site was intradermally administered to 2 sites at the base of tail of a mouse.

[0575]

The results of IFNγ ELISPOT assay using the HLA-A0201 transgenic mouse are shown in Fig. 27.

In Fig. 27, the vertical axis shows the number of cells that reacted in the plated cells. In Fig. 27, the black bar and the white bar show the results of culture of HLA-A0201 transgenic mouse-derived splenocytes in the presence or absence of the object peptide represented by SEQ ID NO: 2. That is, the difference in the values of the black bar and the white bar shows the number of the object, each peptide-specific CTL induced in the mouse in vivo by the administration of a cocktail vaccine containing a compound represented by the formula (5) and a helper peptide (SEQ ID NO: 243).

In Fig. 27, the value of the white bar is not detected. This means that the splenocytes of respective transgenic mice did not react in the absence of the object peptide. As a result of this test, IFNγ production specific to the object peptide shown by SEQ ID NO: 2 was detected in the splenocytes derived from HLA-A0201 transgenic mouse administered with a compound represented by the formula (5) alone, and a cocktail vaccine containing a helper peptide (SEQ ID NO: 243). In Fig. 27, the number of the IFNγ producing cells specific to the peptide shown by SEQ ID NO: 2, which was induced by the administration of a

cocktail vaccine containing a helper peptide (SEQ ID NO: 243), was higher than the number of the peptide-specific IFNγ producing cells, which was induced by the administration of a compound represented by the formula (5) alone.

[0576]

From the above, it was clarified that a cocktail vaccine containing a compound represented by the formula (5) and the peptide shown by SEQ ID NO: 243 can induce CTL specific to the peptides shown by SEQ ID NO: 2. In addition, many IFNγ-producing cells specific to the peptide shown by SEQ ID NO: 2 were found when a cocktail vaccine was administered as compared to the single administration of a compound represented by the formula (5). It was assumed that the induction of the cell reactive with the helper peptide shown by SEQ ID NO: 244 contained in the peptide shown by SEQ ID NO: 243 contained in the cocktail vaccine enhanced induction of CTL specific to the peptide shown by SEQ ID NO: 2. Therefore, it was clarified that a cocktail vaccine containing a compound represented by the formula (5) and the helper peptide can strongly induce CTL in the body of mouse as compared to the single administration of a compound represented by the formula (5).

[0577]

As one embodiment of producing a vaccine containing two WT1 antigen peptides, a cocktail vaccine containing two different peptides as a single preparation can be mentioned. When producing a cocktail vaccine, the properties of the cancer antigen peptides to be mixed poses one problem. As shown in Table 60 and Table 66, production of a cocktail of two WT1 antigen peptides means processing of two peptides having different solubility, namely, property, into one preparation. In contrast, the conjugate of the present invention is a compound wherein two WT1 antigen peptides are bonded via a disulfide bond, and shows a single solubility, namely, property. This means that the conjugate of the present invention has

single property and also has the property corresponding to the two WT1 antigen peptides, as shown in Experimental Example 2. In this aspect, it was shown that the conjugate of the present invention is a compound capable of inducing a response to the two WT1 antigen peptides without the need to consider an interaction between the two WT1 antigen peptides and the like, unlike cocktail vaccines.

[0578]

Experimental Example 19

Evaluation of in vivo CTL induction ability using HLA-A2402 transgenic mouse after filter filtration

The homodimer shown by SEQ ID NO: 4 formed via a disulfide bond and a compound represented by the formula (5) are dissolved in water for injection at 3-10 mg/mL. The pharmacological activity of each compound is evaluated using an HLA-A2402 transgenic mouse (C57BL/6CrHLA-A2402/K^b) with the CTL induction activity as an index. For administration to the HLA-A2402 transgenic mouse, the compound is dissolved in water for injection, sterilized by filtration using a low protein-binding filter (membrane filter of the grade aiming at sterilization treatment of injection) and mixed with incomplete Freund's adjuvant to give an emulsion.

The emulsified compound is intradermally administered to the tail root of an HLA-A2402 transgenic mouse. One week later, the mouse is euthanized with CO_2 gas, the spleen or inguinal lymph node is isolated, and splenocytes or or lymph node cells are prepared. IFNγ ELISPOT assay kit is used for the measurement of IFNγ production. On the previous day of cell preparation, an ELISPOT plate is treated with an anti-mouse IFNγ antibody, and blocked with RPMI1640 medium containing 10% FBS the next day. The prepared mouse-derived cells are plated on the blocked ELISPOT plate. Peptide (SEQ ID NO: 4) is dissolved in DMSO at 40 mg/mL, and further diluted with RPMI1640 medium containing 10% FBS to 40 μg/mL. The diluted peptide (SEQ ID NO:

4) is added to the HLA-A2402 transgenic mouse-derived splenocytes or lymph node cells at a final concentration of 10 μg/mL. The cells added with the peptide are cultivated for 16-20 hr at 37°C, 5% CO_2, whereby peptide re-stimulation in vitro is performed. After culture, the supernatant is removed, and the ELISPOT plate is allowed to develop color according to the attached protocol. The number of spots that developed color is measured by ImmunoSpot Analyzer (manufactured by C.T.L.).

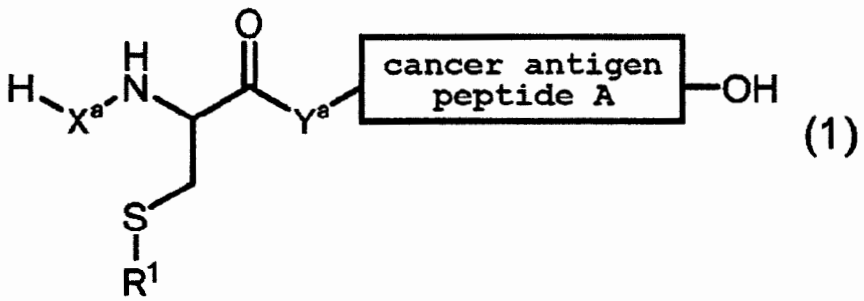
INDUSTRIAL APPLICABILITY

[0579]

The compound of the present invention is useful as an active ingredient of a cancer vaccine that efficiently induces CTL and is easy to produce. This application is based on a patent application Nos. 2013-072173 (filing date: March 29, 2013) and 2013-158383 filed in Japan (filing date: July 31, 2013), the whole contents of which are incorporated into this specification.

CLAIMS

1. A compound represented by the formula (1):

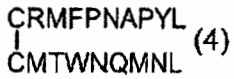


(1)

wherein X^a and Y^a are each a single bond,
cancer antigen peptide A is a peptide consisting of any amino acid sequence selected from the following amino acid sequences:
RMFPNAPYL (SEQ ID NO: 2),
ALLPAVPSL (SEQ ID NO: 5),
SLGEQQYSV (SEQ ID NO: 6) and
RVPGVAPTL (SEQ ID NO: 7),
an amino group of an N-terminal amino acid of the cancer antigen peptide A binds to Y^a in the formula (1), and a carbonyl group of a C-terminal amino acid of the cancer antigen peptide A binds to a hydroxyl group in the formula (1),
R^1 is a cancer antigen peptide C,
the cancer antigen peptide C has a sequence different from that of the cancer antigen peptide A, which is a peptide consisting of any amino acid sequence selected from the following amino acid sequences:
CMTWNQMNL (SEQ ID NO: 3) and
CYTWNQMNL (SEQ ID NO: 4),
and a thioether group of the cysteine residue of the cancer antigen peptide C is bonded to the thioether group in the formula (1),
or a pharmaceutically acceptable salt thereof.

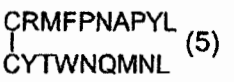
2. The compound according to claim 1, wherein the compound

represented by the formula (1) is a compound represented by the formula (4):



wherein the bond between C and C is a disulfide bond, or a pharmaceutically acceptable salt thereof.

3. The compound according to claim 1, wherein the compound represented by the formula (1) is a compound represented by the formula (5):

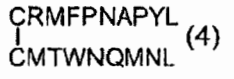


wherein the bond between C and C is a disulfide bond, or a pharmaceutically acceptable salt thereof.

4. A pharmaceutical composition comprising the compound according to any one of claims 1 to 3, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier.

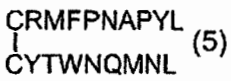
5. The pharmaceutical composition according to claim 4, comprising at least one peptide consisting of an amino acid sequence selected from the group consisting of the following amino acid sequences:
CNKRYFKLSHLQMHSRK (SEQ ID NO: 22),
CNKRYFKLSHLQMHSRKH (SEQ ID NO: 23),
CNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 24),
WAPVLDFAPPGASAYGSL (SEQ ID NO: 244),
CWAPVLDFAPPGASAYGSL (SEQ ID NO: 242) and
WAPVLDFAPPGASAYGSLC (SEQ ID NO: 243).

6. A composition comprising a compound selected from the group consisting of a compound represented by the formula (4):



wherein the bond between C and C is a disulfide bond,

and a compound represented by the formula (5):



(5)

wherein the bond between C and C is a disulfide bond, and at least one peptide consisting of an amino acid sequence selected from the group consisting of the following amino acid sequences:

CNKRYFKLSHLQMHSRK (SEQ ID NO: 22),

CNKRYFKLSHLQMHSRKH (SEQ ID NO: 23),

CNKRYFKLSHLQMHSRKHTG (SEQ ID NO: 24),

WAPVLDFAPPGASAYGSL (SEQ ID NO: 244),

CWAPVLDFAPPGASAYGSL (SEQ ID NO: 242) and

WAPVLDFAPPGASAYGSLC (SEQ ID NO: 243).