inventors have previously developed a dominant negative peptide of BIG3, which specifically inhibits the BIG3-PHB2 interaction (PTL 1). This peptide has been confirmed to suppress breast cancer growth by reactivating the tumor suppressive activity of PHB2 to inhibit ERα-signaling pathways that bring about the growth of breast cancer (PTL 1).

[Citation List]

[Patent Literature]

[0004]

[PTL 1] WO 2013/018690

[Non-patent Literature]

[0005]

[NPL 1] Johnston, S. R., Clin. Cancer Res. 16, 1979-1987 (2010).
[NPL 2] Fisher, B. *et al.*, J. Natl. Cancer Inst. 97, 1652-1662 (2005).
[NPL 3] Jordan, V. C., Nature Rev. Drug Discov. 2, 205-213 (2003).
[NPL 4] Clarke, R. *et al.*, Pharmacol. Rev. 53, 25-71 (2001).
[NPL 5] Fisher, B. *et al.*, J. Natl. Cancer Inst. 93, 684-690 (2001).
[NPL 6] Chlebowski, R. *et al.*, Breast 2, S1-11 (2009).
[NPL 7] Chumsri, S. *et al.*, J. Steroid Biochem. Mol. Biol. 125, 13-22 (2011).
[NPL 8] Kim, J. W. *et al.*, Cancer Sci. 100, 1468-1478 (2009).
[NPL 9] Yoshimaru, T. *et al.*, Nat. Commun. 4, 2443 (2013).
[NPL 10] Yoshimaru, T. *et al.*, Sci Rep. 7(1), 1821 (2017)

[Summary]

[Technical Problem]

[0006]

As mentioned above, growth suppression actions on breast cancer cells by a dominant negative peptide of BIG 3 has been elucidated. However, the stability of the known dominant negative peptide cannot be said to be high, and the duration of inhibitory effects on the BIG3-PHB2 interaction is not that long. Then, the present inventors discovered that the duration of inhibitory effects on the BIG3-PHB2 interaction is improved by introducing a stapling structure (bridging structure) into the above-mentioned dominant negative peptide molecule (PCT/JP2017/001187, and Yoshimaru, T. *et al.*, Sci Rep. 7(1), 1821 (2017)). Peptides to which a stapling structure has been introduced (stapled peptides; stERAP No. 12 and such) were confirmed to show more stable effects of suppressing breast cancer growth.

The above-mentioned stapled peptides prolonged the duration of the inhibitory effects

on the BIG3-PHB2 interaction. However, inhibitory effects that last even longer are desired for clinical applications.

[0007]

Therefore, an objective of the present disclosure is to provide peptides having longer lasting inhibitory effects on the BIG3-PHB2 interaction.

[Solution to Problem]

[0007a]

In one aspect, the present disclosure provides a peptide comprising an amino acid sequence in which at least two pairs of amino acid residues are substituted with the same number of stapling structures in the amino acid sequence of SEQ ID NO: 4 or SEQ ID NO: 5, or a salt thereof, wherein the two pairs of amino acid residues are either (a) and (b), or (c) and (d) below: (a) the third and seventh amino acid residues from the N terminus of the amino acid sequence of SEQ ID NO: 4; and (b) the eighth and twelfth amino acid residues from the N terminus of the amino acid sequence of SEQ ID NO: 4, or (c) the third and seventh amino acid residues from the N terminus of the amino acid sequence of SEQ ID NO: 5; and (d) the tenth and fourteenth amino acid residues from the N terminus of the amino acid sequence of SEQ ID NO: 5.

[0007b]

In one aspect, the present disclosure provides a peptide which is a retro-inverso form of a peptide comprising an amino acid sequence in which at least two pairs of amino acid residues are substituted with the same number of stapling structures in the amino acid sequence of SEQ ID NO: 4 or SEQ ID NO: 5, or a salt thereof, wherein the two pairs of amino acid residues are (a) and (b) below: (a) the third and seventh amino acid residues from the N terminus of the amino acid sequence of SEQ ID NO: 4; and (b) the eighth and twelfth amino acid residues from the N terminus of the amino acid sequence of SEQ ID NO: 4, or wherein the two pairs of amino acid residues are (c) and (d) below: (c) the third and seventh amino acid residues from the N terminus of the amino acid sequence of SEQ ID NO: 5; and
(d) the tenth and fourteenth amino acid residues from the N terminus of the amino acid sequence of SEQ ID NO: 5.

[0007c]

In another aspect, the present disclosure provides a pharmaceutical composition comprising the peptide or the salt thereof as described herein and a pharmaceutically acceptable carrier.

[0007d]

In another aspect, the present disclosure provides use of the pharmaceutical composition as described herein in the manufacture of a medicament for treating cancer.

[0008]

The present inventors previously discovered that the duration of inhibitory effects on the BIG3-PHB2 interaction is improved by introducing a stapling structure into the above-mentioned dominant negative peptide molecule, and now the present inventors completed the present disclosure by discovering that stability is enhanced by further increasing the number of intramolecular crosslinks. More specifically, the present disclosure provides the following peptides and uses thereof:

[0009]

[1] a peptide comprising an amino acid sequence in which at least two pairs of amino acid residues are substituted with the same number of stapling structures in the amino acid sequence of SEQ ID NO: 4 or SEQ ID NO: 5, or a salt thereof;

[2] the peptide or the salt thereof of [1], wherein two pairs of amino acid residues are substituted with two stapling structures;

[3] the peptide or the salt thereof of [1] or [2], wherein the two pairs of amino acid residues are (a) and (b) below:

(a) the third and seventh amino acid residues from the N terminus of the amino acid sequence of SEQ ID NO: 4; and

(b) the eighth and twelfth amino acid residues from the N terminus of the amino acid sequence of SEQ ID NO: 4;

[4] the peptide or the salt thereof of [1] or [2], wherein the two pairs of amino acid residues are (c) and (d) below:

(c) the third and seventh amino acid residues from the N terminus of the amino acid sequence of SEQ ID NO: 5; and

(d) the tenth and fourteenth amino acid residues from the N terminus of the amino acid sequence of SEQ ID NO: 5;

[5] the peptide or the salt thereof of any one of [1] to [4], wherein the stapling structure is represented by Formula (I) below:

[Fig. 21]

Fig. 21 shows the effects of threonine phosphorylation of PHB2 caused by TTK and MK5, on the HER2 signal. (A, B) Immunoblots are shown which indicate that threonine phosphorylation of PHB2 caused by TTK and MK5 inhibits the HER2-HER3 binding and HER2-Shc binding. SK-BR-3 cells (A) and KPL-4 cells (B) in which TTK, MK5, and CHK1 expressions were suppressed by the siRNA method were treated for 24 hours with 1 μM stERAP, then the cells were lysed and subjected to immunoprecipitation using an anti-HER2 antibody, and immunoblot analyses were performed using the antibodies shown in the drawing. (C) Immunoblots are shown which indicate that inhibition of HER2-HER3 binding and HER2-Shc binding by PHB2 is avoided by a TTK inhibitor. SK-BR-3 cells were treated for 24 hours with 2 μM AZ3146 and 1 μM stERAP, and then cell lysates thereof were subjected to immunoprecipitation using an anti-HER2 antibody, and immunoblot analyses were performed using the antibodies shown in the drawing.

[Fig. 22]

Fig. 22 shows identification of the threonine phosphorylation sites in PHB2. (A) Immunoblots are shown which evaluate Thr42 and Thr169 as the threonine phosphorylation sites in PHB2. SK-BR-3 cells in which PHB2 expression was suppressed by the siRNA method were transfected with the HA-tagged PHB2 construct, alanine mutant at Thr42 (T42A), alanine mutant at Thr169 (T169A), and double alanine mutant at Thr42 and Thr169 (T42A+T169A). 48 hours later, these were treated for 24 hours with 1 μM stERAP. The cells were then lysed and subjected to immunoprecipitation using an anti-HA antibody, and immunoblot analyses were performed using the antibodies shown in the drawing. (B) Immunoblots are shown which indicate that phosphorylation of Thr42 and Thr169 in PHB2 inhibits HER2-HER3 binding and HER2-Shc binding. SK-BR-3 cells in which PHB2 expression was suppressed by the siRNA method were transfected with the HA-tagged PHB2 construct, alanine mutant at Thr42 (T42A), alanine mutant at Thr169 (T169A), and double alanine mutant at Thr42 and Thr169 (T42A+T169A). 48 hours later, these were treated for 24 hours with 1 μM stERAP. The cells were then lysed and subjected to immunoprecipitation using an anti-HER2 antibody and an anti-BIG3 antibody, and immunoblot analyses were performed using the antibodies shown in the drawing.

[Fig. 23]

Fig. 23 shows threonine phosphorylation of PHB2 caused by TTK and MK5. (A) Immunoblots are shown which indicate that PHB2 binds to TTK. SK-BR-3 cells were treated for 24 hours with 1 μM stERAP, then the cells were lysed and subjected to immunoprecipitation using an anti-PHB2 antibody, an anti-TTK antibody, and a rabbit IgG antibody, and immunoblot analyses were performed using the antibodies shown in the drawing. (B, C) Immunoblots are

solid-phase peptide synthesis using D-amino acids, with each amino acid residue of a pair at a position where one wants to form a stapling structure in the amino acid sequence of SEQ ID NO: 4 or SEQ ID NO: 5 being substituted with the D-glutamine derivative of Formula (III), and performing an olefin metathesis reaction according to Scheme (I). When synthesizing the retro-inverso form of a stapled ERAP or a sh stapled ERAP, solid-phase peptide synthesis can be performed based on the reversed amino acid sequence of the amino acid sequence of SEQ ID NO: 4 or 5 or a partial sequence thereof. Likewise the above, in this case too each amino acid residue of a pair, at a position where one wants to form a stapling structure, is substituted with the D-glutamine derivative of Formula (III) and then an olefin metathesis reaction is performed after synthesizing the peptide.

[0049]

When synthesizing a D-form stapled ERAP by Scheme (II) shown in Fig. 8, the D-form optical isomer of the glutamic acid derivative represented by Formula (IV) (hereinafter, "D-glutamic acid derivative of Formula (IV)") and the D-form optical isomer of the glutamine derivative represented by Formula (V) (hereinafter, "D-glutamine derivative of Formula (V)") can be used as the amino acid derivatives for stapling. Commercially available products can be used for the D-glutamic acid derivative of Formula (IV). The D-glutamine derivative of Formula (V) can be synthesized by using *N*-α-(*tert*-butoxycarbonyl)-D-glutamic acid α methyl ester instead of *N*-α-(*tert*-butoxycarbonyl)-L-glutamic acid α methyl ester (Compound 3) in Scheme (IV) shown in Fig. 6. Then, a D-form stapled ERAP can be obtained by synthesizing a D-form peptide through standard Fmoc solid-phase peptide synthesis using D-amino acids, with each one of the amino acid residues of a pair at a position where one wants to form a stapling structure in the amino acid sequence of SEQ ID NO: 4 or SEQ ID NO: 5 being substituted with the D-glutamic acid derivative of Formula (IV) and the D-glutamine derivative of Formula (V) respectively, and performing an intramolecular amidation reaction according to Scheme (II). When synthesizing the retro-inverso forms of stapled ERAPs, solid-phase peptide synthesis can be performed based on the reversed amino acid sequence of the amino acid sequence of SEQ ID NO: 4 or SEQ ID NO: 5. Likewise the above, in this case too each amino acid residue of a pair at a position where one wants to form a stapling structure is substituted with the D-glutamic acid derivative of Formula (IV) and the D-glutamine derivative of Formula (V), respectively, and then an intramolecular amidation reaction is performed after synthesizing the peptide.

[0050]

Peptides of the present invention may also be in the form of salts. The form of salts is not particularly limited, but pharmaceutically acceptable salts are preferred. Herein, the "pharmaceutically acceptable salt" refers to a salt that retains the pharmacological and

phosphorylation of HER2 (Y877) and the phosphorylation of Shc (Y239/Y240) (Fig. 25). In addition, although stERAP treatment induced the binding level of PHB2 to HER2 in a concentration-dependent manner, combined use with trastuzumab did not change the binding level of PHB2 (Fig. 25).

[0126]

Inhibitory Effects of stERAP on Trastuzumab-resistant HER2-positive Breast Cancer Cell Lines

Growth inhibitory effects of stERAP on trastuzumab-resistant SK-BR-3 cell were examined. As a result, administration of trastuzumab alone could not suppress the growth of trastuzumab-resistant SK-BR-3 cells at all whereas stERAP treatment showed dose-dependent suppressive effects on the growth of trastuzumab-resistant SK-BR-3 cells. Compared to the IC_{50} (0.054 μM, Fig. 24) for trastuzumab-sensitive SK-BR-3 cells, the IC_{50} was 10.64 μM which was higher (Fig. 26A), but regardless of whether the cells are resistant or sensitive, stERAP was found to have significant suppressive effects.

Next, effects of stERAP on the binding of the HER2-HER3 heterodimer and HER2-Shc in trastuzumab-resistant SK-BR-3 cells were examined. As a result, stERAP could nearly completely inhibit the binding of HER2-HER3 and HER2-Shc and the phosphorylation of Shc (Y239/Y240) in the sensitive and resistant cell lines (Fig. 26B). Furthermore, PHB2 which dissociated from BIG3 by stERAP treatment was observed to bind to HER2, and PHB2 was suggested to be involved in the inhibition of HER2-HER3 and HER2-Shc complexes. On the other hand, treatment with Herceptin alone hardly affected the binding of HER2-HER3 and HER2-Shc.

[0127]

Effects of stERAP on NF-κB Signaling in Trastuzumab-resistant HER2-positive Breast Cancer Cell Lines

It has been reported the possibility that activation of NF-κB signaling in HER2-positive breast cancer cells causes resistance to chemoradiotherapy (Anticancer Res., 26, 4235, 2006; Breast Cancer Res., 13, 221, 2011). Therefore, the effects of stERAP on NF-κB signaling in HER2-positive breast cancer cell lines were examined. As a result, compared to the parent SK-BR-3 cell line cells, a lot of NF-κB p65 was found to translocate into the nucleus in trastuzumab-resistant SK-BR-3 cells, and this signal was suggested to be involved in the resistance against trastuzumab treatment (Fig. 27). On the other hand, administration of stERAP remarkably inhibited the nuclear translocation of NF-κB p65 in trastuzumab-resistant SK-BR-3 cells (Fig. 27). Furthermore, stERAP was found to regulate the nuclear translocation of NF-κB p65 by nearly completely inhibiting the phosphorylation of IκBα which is activated only in trastuzumab-resistant SK-BR-3 cells (Fig. 27). The above result suggests that stERAP can avoid resistance to trastuzumab treatment by suppressing phosphorylation of IκBα and nuclear

Claims

1. A peptide comprising an amino acid sequence in which at least two pairs of amino acid residues are substituted with the same number of stapling structures in the amino acid sequence of SEQ ID NO: 4 or SEQ ID NO: 5, or a salt thereof,

wherein the two pairs of amino acid residues are either (a) and (b), or (c) and (d) below:

(a) the third and seventh amino acid residues from the N terminus of the amino acid sequence of SEQ ID NO: 4; and

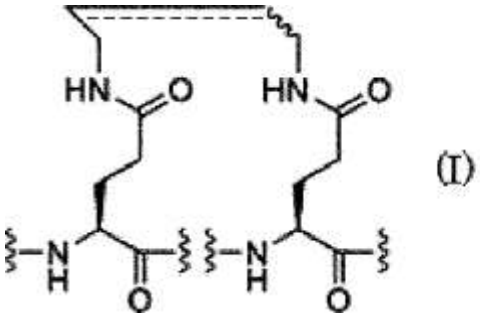
(b) the eighth and twelfth amino acid residues from the N terminus of the amino acid sequence of SEQ ID NO: 4, or

(c) the third and seventh amino acid residues from the N terminus of the amino acid sequence of SEQ ID NO: 5; and

(d) the tenth and fourteenth amino acid residues from the N terminus of the amino acid sequence of SEQ ID NO: 5.

2. The peptide or the salt thereof of claim 1, wherein two pairs of amino acid residues are substituted with two stapling structures.

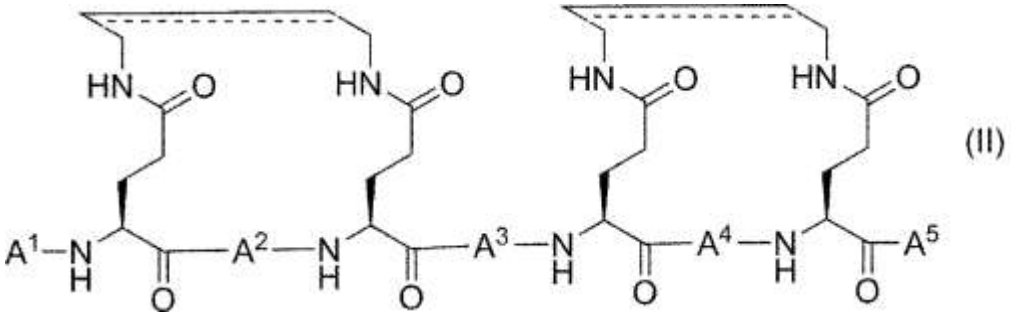
3. The peptide or the salt thereof of claim 1, wherein the stapling structure is represented by Formula (I) below:



(I)

(wherein, the double line drawn by a solid line and a dashed line indicates a single bond or a double bond).

4. The peptide or the salt thereof of claim 3, wherein the stapling structure is represented by Formula (II) below:



(II)

(wherein, the double line drawn by a solid line and a dashed line indicates a single bond or a double bond;

the combination of A^1, A^2, A^3, A^4, and A^5 is selected from the following:

A^1 = QM, A^2 = SDL, A^3 = –, A^4 = QLR, and A^5 = R; and

A^1 = QM, A^2 = SDL, A^3 = LQ, A^4 = RQR, and A^5 = OH;

wherein "–" indicates a peptide bond with no additional amino acid residue (that is, two stapling structures are connected); and "OH" indicates that one end of the above stapling structure constitutes the C terminus of the peptide derivative).

5. The peptide or the salt thereof of claim 1, wherein either one or both of N-terminal and C-terminal amino acid residues have been modified.

6. The peptide or the salt thereof of claim 5, wherein either one or both of N-terminal and C-terminal amino acid residues have been modified by any one or a combination of acetylation, amidation, and HA tagging.

7. The peptide or the salt thereof of claim 6, wherein the N-terminal amino acid residue is acetylated and the C-terminal amino acid residue is amidated.

8. The peptide or the salt thereof of claim 1, wherein all the amino acid residues have been substituted with D-form amino acid residues.

9. A peptide which is a retro-inverso form of a peptide comprising an amino acid sequence in which at least two pairs of amino acid residues are substituted with the same number of stapling structures in the amino acid sequence of SEQ ID NO: 4 or SEQ ID NO: 5, or a salt thereof, wherein the two pairs of amino acid residues are (a) and (b) below:

(a) the third and seventh amino acid residues from the N terminus of the amino acid sequence of SEQ ID NO: 4; and

(b) the eighth and twelfth amino acid residues from the N terminus of the amino acid sequence of SEQ ID NO: 4, or

wherein the two pairs of amino acid residues are (c) and (d) below:

(c) the third and seventh amino acid residues from the N terminus of the amino acid sequence of SEQ ID NO: 5; and

(d) the tenth and fourteenth amino acid residues from the N terminus of the amino acid sequence of SEQ ID NO: 5.

10. A pharmaceutical composition comprising the peptide or the salt thereof of claim 1 and a pharmaceutically acceptable carrier.

11. Use of the pharmaceutical composition of claim 10 in the manufacture of a medicament for treating cancer.

12. The use of claim 11, wherein the cancer is breast cancer or prostate cancer.

13. The use of claim 11, wherein the cancer is estrogen receptor-positive cancer.