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CA 2599150 C 2014/03/11

(11)(21) **2 599 150**

(12) **BREVET CANADIEN**
CANADIAN PATENT

(13) **C**

(86) **Date de dépôt PCT/PCT Filing Date:** 2005/08/19
(87) **Date publication PCT/PCT Publication Date:** 2006/08/31
(45) **Date de délivrance/Issue Date:** 2014/03/11
(85) **Entrée phase nationale/National Entry:** 2007/08/22
(86) **N° demande PCT/PCT Application No.:** JP 2005/015135
(87) **N° publication PCT/PCT Publication No.:** 2006/090494
(30) **Priorité/Priority:** 2005/02/25 (JP2005-052165)

(51) **Cl.Int./Int.Cl.** ***A61K 38/16*** (2006.01),
A61K 31/337 (2006.01), ***A61K 31/475*** (2006.01),
A61K 31/555 (2006.01), ***A61P 35/00*** (2006.01)

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(54) **Titre : AGENT ANTICANCEREUX COMPRENANT DU PACLITAXEL ET LA TOXINE DIPHTERIQUE MUTANTE CRM 197**
(54) **Title: ANTICANCER AGENT COMPRISING PACLITAXEL AND A DIPTHERIA TOXIN MUTANT, CRM 197**

(57) **Abrégé/Abstract:**
The present invention is an antineoplastic agent characterized by including at least one of taxol and taxol derivatives and a protein which is a mutant of diphtheria toxin, having an activity to inhibit a binding between HB-EGF and EGFR and substantially not having a toxicity of diphtheria toxin as active ingredients.

ABSTRACT

The present invention is an antineoplastic agent characterized by including at least one of taxol and taxol derivatives and a protein which is a mutant of diphtheria toxin, having an activity to inhibit a binding between HB-EGF and EGFR and substantially not having a toxicity of diphtheria toxin as active ingredients.

ANTICANCER AGENT COMPRISING PACLITAXEL AND A DIPTHERIA TOXIN MUTANT, CRM 197

Technical Field

The present invention relates to an antineoplastic agent or an antineoplastic agent composition, a method for treating a cancer and a kit for treating the cancer, which are effective for treating various cancers such as ovarian cancers, breast cancers, prostate cancers, uterus cancers, lung cancers, pancreatic cancers, stomach cancers, cancers of the colon and rectum and glioblastoma.

Background Art

Various therapeutic methods and therapeutic drugs for malignant tumors have been developed, but sufficient therapeutic effects often have not been able to be obtained yet. Antineoplastic agents such as taxol, carboplatin and irinotecan are effective, but their side effects are strong, and thus, reduction of the side effect has been desired.

Meanwhile, HB-EGF is known to be a cell growth factor of the EGF family and as a molecule which is essential for formation and regeneration process of a body as well as is involved in occurrence of vascular stenosis and arteriosclerosis (see, e.g., Non-patent literature 1). This molecule is synthesized as a membrane bound precursor (proHB-EGF), and is cleaved on the cell surface with protease to produce the soluble form HB-EGF. A growth promoting action is observed in the soluble form whereas a growth inhibitory action is observed in the membrane-anchored form. Thus, HB-EGF

seems to serve for formation and maintenance of tissues by appropriately using the soluble form or the membrane-anchored form as the situation demands.

HB-EGF is bound to EGF receptor (EGFR) (Her1) and Her4 (ErbB-4) in the EGFR family and activates them. However, members (Her1, Her2, Her3 and Her4) in the EGFR family can form heterodimers in all combinations as well as form homodimers. Thus, consequently, HB-EGF can activate all molecules in the EGFR family. HB-EGF is expressed in various tissues, and appears to act in broad cells and tissues, and is reported to promote well the growth of fibroblasts, smooth muscle cells and keratinocytes (see e.g., Non-patent literature 2).

HB-EGF is synthesized as the membrane bound precursor (proHB-EGF) as described above, and proHB-EGF is composed of, from an N terminus, a signal sequence, a prosequence, a heparin binding domain, an EGF-like domain, a juxtamembrane domain, a transmembrane domain and a cytoplasmic domain (FIG. 1). This proHB-EGF becomes the soluble form by being cleaved with protease (ectodomain shedding) at a portion indicated by an arrow in the figure. It has been proposed that the ectodomain shedding of proHB-EGF is stimulated by a pathway in which lysophosphatidic acid (LPA) activates Ras-Raf-MEK pathway through a G protein-coupled receptor or a pathway in which phorbol ester activates PKC (see e.g., Non-patent literature 3).

A function that the soluble form HB-EGF is bound to EGFR

and facilitates phosphorylation of EGFR is present in the EGF-like domain (see e.g., Non-patent literature 1).

It has been known that diphtheria toxin is a protein having a molecular weight of about 59,000 produced by diphtheria bacillus and is bound to the membrane-anchored form precursor (proHB-EGF) of HB-EGF as the receptor (see e.g., Non-patent literature 4). Also, a mutant such as CRM197 of diphtheria toxin is known as an inhibitor of the soluble form HB-EGF (see e.g., Non-patent literature 5). Database information of diphtheria toxin is available for its gene in EMBL; K01722, its amino acid sequence in SWISS-PROT; P00588 and its three dimensional structure in PDB; 1MDT or 1XDT. A phage lysogenized in a diphtheria bacilli encodes the gene of diphtheria toxin.

Diphtheria toxin is a simple protein composed of 535 amino acid residues (the amino acid sequence [SEQ ID NO:1] of diphtheria toxin and a base sequence [SEQ ID NO:2] of the gene encoding it are shown in FIGs. 2 and 3, and italic letters represent the signal sequence). Diphtheria toxin can be separated into fragment A and fragment B by treating with a reducing agent (FIG. 4). According to conformational analyses, the fragment B is further divided into two domains. For the function of each domain, a catalytic domain corresponding to the fragment A (amino acid numbers 1 to 193 when the signal sequence is excluded) has an ADP ribosylation activity, a transmembrane domain (amino acid numbers 194 to 378 when the signal sequence is excluded) corresponding to an N terminal

half of the fragment B has a nature to form a channel in an endosome membrane, and a receptor-binding domain (amino acid numbers 386 to 535 when the signal sequence is excluded) corresponding to a C terminal half of the fragment B has an activity to bind to a diphtheria toxin receptor on the cell surface.

The fragment A (catalytic domain) of diphtheria toxin has the action to ADP-ribosylate EF-2 (elongation factor 2) in the presence of NAD, thereby inhibiting protein synthesis. Therefore, in order to exert the toxicity of diphtheria toxin, the fragment A must enter in cytoplasm.

In the mechanism in which the fragment A enters in the cytoplasm, the receptor-binding domain in the fragment B is bound to proHB-EGF which is the receptor on the cell surface to internalize diphtheria toxin by endocytosis, then the transmembrane domain is inserted in the endosome membrane in the endosome, and finally the fragment A is released in the cytoplasm by passing through the endosome to inactivate EF-2 there (see e.g., Non-patent literature 6).

To exert the toxicity of diphtheria toxin, both the fragments A and B are necessary. Therefore, if either the fragment has a mutation, a protein having no toxicity of diphtheria toxin can be generated.

In diphtheria toxin, the detoxified mutant such as CRM197 having the mutation in the catalytic domain has been isolated.

Meanwhile, the mutant of diphtheria toxin has the activity to inhibit the binding between HB-EGF and EGFR

because diphtheria toxin is bound to the EGF-like domain of the soluble form HB-EGF. The receptor-binding domain of diphtheria toxin is involved in this binding. It has been reported that Lys at position 516 and Phe at position 530 in diphtheria toxin are important for the binding to HB-EGF (see e.g., Non-patent literature 7). A crystal structure of a complex composed of diphtheria toxin and the EGF domain of HB-EGF has been analyzed, and the important amino acid residues for binding to HB-EGF have been reported to be between positions 381 and 535 (see e.g., Non-patent literature 8).

This way, it has been observed that diphtheria toxin mutant is bound to HB-EGF and inhibits the activity of HB-EGF. Recently, it has been attempted to use diphtheria toxin mutant as the therapeutic agent for the cancer by targeting HB-EGF for cancer therapy, but the attempt has not come into practical use yet (Patent document 1, Non-patent literature 9).

Patent document 1: JP 2004-155776-A;

Non-patent literature 1: Mekata E. et al, "Idenshi Igaku" Vol. 5, No. 2, P.131-134, 2001, Medical Do Co., Ltd.;

Non-patent literature 2: Higashiyama, S. et al., J. Cell Biol., 122: 933-940, 1993;

Non-patent literature 3: Prenzel, N. et al., Nature 402: 884-888, 1999;

Non-patent literature 4: J. G. Naglich et al., Cell 69: 1051-1061, 1992;

Non-patent literature 5: T. Mitamura et al., J. Biol. Chem., 270: 1015, 1995;

Non-patent literature 6: T. Umata et al., J. Biol. Chem., 273: 8351, 1998;

Non-patent literature 7: Shen, HS et al., J. Biol. Chem., 269: 29077-29084, 1994;

Non-patent literature 8: Gordon VL et al., Molecular Cell 1: 67-78, 1997;

Non-patent literature 9: Miyamoto, S. et al., Cancer Res., 64: 5720-5727, 2004.

DISCLOSURE OF THE INVENTION

PROBLEMS TO BE SOLVED BY THE INVENTION

The present invention makes it a task to solve the above conventional various problems and accomplish the following objects. That is, it is an object of the present invention to provide an antineoplastic agent and a method for treating cancers, effective for the treatment of malignant tumors with no side effect.

MEANS FOR SOLVING THE PROBLEMS

In one particular embodiment there is provided an antineoplastic composition for the treatment of breast and ovarian cancers comprising (a) paclitaxel in an amount of 0.875 to 1.375 mg per kilogram of body weight of a recipient patient with (b) a protein consisting of an amino acid sequence shown in SEQ ID No:3 in an amount of 1 μg to 30 mg per kilogram of body weight of the recipient patient, wherein the amounts both independently have no sufficient anti-cancer action.

In another particular embodiment there is provided use to treat breast and ovarian cancers of (a) paclitaxel in an

amount of 0.875 to 1.375 mg per kilogram of body weight of a recipient patient, and (b) 1 μg to 30 mg per kilogram of body weight of the recipient patient of a protein consisting of an amino acid sequence shown in SEQ ID NO:3, wherein the use is a simultaneous use.

In yet another particular embodiment there is provided a kit characterized by comprising (a) paclitaxel in an amount of 0.875 to 1.375 mg per kilogram of body weight of a recipient patient and (b) a protein consisting of an amino acid sequence shown in SEQ ID NO:3, wherein (a) and (b) both independently have no sufficient anti-cancer action but an anti-cancer action is elicited by combining (a) and (b) together in the recipient patient.

In accordance with one aspect of the present invention there is provided an antineoplastic agent characterized by combining (a) at least one of paclitaxel and derivatives thereof with (b) at least one of a protein consisting of an amino acid sequence shown in SEQ ID NO:3, a protein consisting of an amino acid sequence shown in SEQ ID NO:3 wherein a Glu at position 173 is substituted by a Lys, and a protein consisting of an amino acid sequence shown in FIG. 5 or FIG. 6.

In accordance with another aspect of the present invention there is provided use of (a) at least one of paclitaxel and derivatives thereof, in combination with (b) at least one of a protein consisting of an amino acid sequence shown in SEQ ID NO:3, a protein consisting of an amino acid sequence shown in SEQ ID NO:3 wherein a Glu at position 173 is substituted by a Lys, and a protein consisting of an amino acid sequence shown in FIG. 5 or FIG. 6, for the manufacture of an antineoplastic agent.

In accordance with yet another aspect of the present invention there is provided a kit for treating cancers, composed of (a) at least one of paclitaxel and derivatives thereof and (b) at least one of a protein consisting of an amino acid sequence shown in SEQ ID NO:3, a protein consisting of an amino acid sequence shown in SEQ ID NO:3 wherein a Glu at position 173 is substituted by a Lys, and a protein consisting of an amino acid sequence shown in FIG. 5 or FIG. 6.

In accordance with still yet another aspect of the present invention there is provided an antineoplastic agent comprising (a) at least one of paclitaxel and derivatives thereof and (b) at least one of a protein consisting of an amino acid sequence shown in SEQ ID NO:3, a protein consisting of an amino acid sequence shown in SEQ ID NO:3 wherein a Glu at position 173 is substituted by a Lys, and a protein consisting of an amino acid sequence shown in FIG. 5 or FIG. 6.

The present inventors have obtained a finding that a synergistic effect is obtained by using a diphtheria toxin mutant in combination with one or two or more selected from the group consisting of paclitaxel (taxol), carboplatin, irinotecan and derivatives thereof, and led to the present invention.

That is, procedures to solve the above problems of the present invention are as follows.

[1] An antineoplastic agent characterized by combining (a) at least one of paclitaxel, carboplatin, irinotecan and derivatives thereof with (b) a protein which is a diphtheria toxin mutant having an activity to inhibit a binding between HB-EGF and EGFR and substantially not having toxicity of diphtheria toxin.

[2] The antineoplastic agent according to [1] characterized in that the protein comprises at least a receptor-binding domain with no mutation in an amino acid sequence of diphtheria toxin.

[3] The antineoplastic agent according to [1] wherein the protein is a protein composed of an amino acid sequence having one or more amino acid deletions, substitutions or additions in the amino acid sequence of diphtheria toxin.

[4] The antineoplastic agent according to [1] wherein the protein is either CRM197 or DT52E148K.

[5] The antineoplastic agent according to [1] wherein (a) at least one of paclitaxel, carboplatin, irinotecan and the derivatives thereof is combined with (b) the protein which is diphtheria toxin mutant having the activity to inhibit the binding between HB-EGF and EGFR and substantially not having toxicity of diphtheria toxin in amounts in which both independently have no sufficient anti-cancer action.

[6] A method for treating cancers characterized by administering (a) at least one of paclitaxel, carboplatin, irinotecan and derivatives thereof in combination with (b) a protein which is a diphtheria toxin mutant having an activity

to inhibit a binding between HB-EGF and EGFR and substantially not having toxicity of diphtheria toxin.

[7] The method according to [6] wherein (a) at least one of paclitaxel, carboplatin, irinotecan and the derivatives thereof and (b) the protein which is diphtheria toxin mutant having the activity to inhibit the binding between HB-EGF and EGFR and substantially not having the toxicity of diphtheria toxin are administered in amounts in which both independently have no sufficient anticancer action but the anticancer action is elicited by combining the both.

[8] A kit for treating cancers, composed of (a) at least one of paclitaxel, carboplatin, irinotecan and derivatives thereof and (b) a protein which is a diphtheria toxin mutant having an activity to inhibit a binding between HB-EGF and EGFR and substantially not having toxicity of diphtheria toxin.

[9] The kit according to [8] characterized by comprising (a) at least one of paclitaxel, carboplatin, irinotecan and the derivatives thereof and (b) the protein which is diphtheria toxin mutant having the activity to inhibit the binding between HB-EGF and EGFR and substantially not having the toxicity of diphtheria toxin in amounts in which both independently have no sufficient anticancer action but the anticancer action is elicited by combining the both.

[10] An antineoplastic agent composition comprising (a) at least one of paclitaxel, carboplatin, irinotecan and derivatives thereof and (b) a protein which is a diphtheria toxin mutant having an activity to inhibit a binding between

HB-EGF and EGFR and substantially not having toxicity of diphtheria toxin.

EFFECTS OF THE INVENTION

According to the present invention, it is possible to provide the antineoplastic agent or the antineoplastic agent composition, the method for treating the cancer and the kit for treating the cancer, which are effective for the treatment of the malignant tumors such as ovarian cancers, breast cancers, prostate cancers, uterus cancers, lung cancers, pancreatic cancers, stomach cancers, cancers of the colon and rectum and glioblastoma.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic view showing a structure of proHB-EGF;

FIG. 2 is a view showing an amino acid sequence and a base sequence of diphtheria toxin;

FIG. 3 is a view showing the amino acid sequence and the base sequence of diphtheria toxin (sequel to FIG. 2);

FIG. 4 is a view showing a domain structure of diphtheria toxin;

FIG. 5 is a view showing an amino acid sequence and a base sequence of GST-DT;

FIG. 6 is a view showing the amino acid sequence and the base sequence of GST-DT (sequel to FIG. 5);

FIG. 7 is a view showing effects by CRM197 administration

on tumor growth in nude mice injected with SKOV-3 cells;

FIG. 8 is a view showing effects by CRM197 administration on tumor growth in nude mice injected with SK-HB1 cells;

FIG. 9 is a view showing effects by CRM197 and taxol administration on tumor growth in nude mice injected with SKOV-3 cells;

FIG. 10 is a view showing effects by CRM197 and taxol administration on tumor growth in nude mice injected with SK-HB1 cells; and

FIG. 11 is a view showing effects by CRM197 and taxol administration on tumor growth in nude mice injected with MDA-MB-231 (breast cancer) cells.

BEST MODES FOR CARRYING OUT THE INVENTION

A first aspect is characterized by combining (a) at least one of paclitaxel, carboplatin, irinotecan and derivatives thereof with (b) a protein which is a diphtheria toxin mutant having an activity to inhibit a binding between HB-EGF and EGFR and substantially not having toxicity of diphtheria toxin. By combining the anticancer agent (a) with the protein (b), an amount of the anticancer agent (a) to be used can be reduced to inhibit side effects and elicit sufficient anticancer actions.

The above diphtheria toxin mutant indicates the protein composed of the amino acid sequence having one or multiple amino acid deletions, substitutions or additions in the amino acid sequence of diphtheria toxin, and is, for example the

protein composed of the amino acid sequence having one or more amino acid deletions, substitutions or additions. A signal sequence composed of 25 amino acid residues of diphtheria toxin may or may not be included, and both sequences are included in the scope of the present invention.

A paclitaxel derivative includes docetaxel (taxotale), a carboplatin derivative includes cisplatin, and an irinotecan derivative includes topotecan.

The antineoplastic agent of a second aspect of the present invention uses any of the following proteins (a), (b) and (c) as the above protein:

(a) a protein composed of a portion of diphtheria toxin and comprising at least a receptor-binding domain of diphtheria toxin;

(b) a protein composed of the amino acid sequence having one or more amino acid deletions, substitutions or additions in the amino acid sequence of the protein (a); and

(c) a complex protein comprising either the protein (a) or (b).

The above protein refers to one which is the portion of diphtheria toxin or the mutant thereof or the complex protein comprising such a protein and holds the receptor-binding domain.

Here, the toxicity of diphtheria toxin means that diphtheria toxin is bound to the receptor on the cell surface, enters into a cell and a protein synthesis function of the cell is inhibited by an activity of the fragment A to ADP-

ribosylate EF-2 (peptide elongation factor 2), and can be easily determined by degrees of the protein synthesis inhibition. That is, a certain amount of diphtheria toxin is added to cultured cells, which are then cultured for about 2 to 8 hours. Subsequently, the cells are cultured in the presence of a radioactive amino acid for a short time, and then the radioactive amino acid incorporated in the protein is quantified.

Specifically, Vero cells (1×10^5 cells) are seeded in a 24-well plate, and cultured in a CO_2 incubator for 16 hours. After confirming that the cells sufficiently adhere to the plate, each well is washed once with cold PBS (150 mM NaCl, 2.7 mM KCl 10 mM phosphate buffer, pH 7.2). At that time, the liquid is carefully added and discarded so that the cells are not detached. Then, 0.5 mL of medium for assay containing serum is added. As the medium for the assay, one in which a concentration of leucine has been reduced to about 1/10 compared with the ordinary medium is used. This is because an uptake efficiency of [^{3}H]-leucine added later is increased. But, Ham's F12 medium contains a less content of leucine, and thus this can also be used as the medium for the assay. The serum is added at the concentration typically used.

Subsequently, various concentrations of diphtheria toxin are added, and the cells are cultured in the CO_2 incubator for 2 to 5 hours. Then, 10 μL of 3.7 MBq/mL [^{3}H]-leucine is added, and the culture is continued for an additional one hour.

The medium is discarded, the well is washed once with PBS,

the cells are lysed with 0.5 mL of 0.1 M NaOH, and a cell lysate is collected in a tube. The well is washed again with 0.5 mL of 0.1 M NaOH, and the solution is collected in the same tube.

To this, 0.5 mL of 20% trichloroacetic acid solution is added, which is then agitated on a Vortex mixer. A produced precipitate is trapped with a glass filter, and the filter is further washed with 5% trichloroacetic acid solution.

Finally, the filter is washed with 100% ethanol, and dried.

The filter is immersed in toluene PPO scintillator, and a radioactivity trapped in the filter is measured by a liquid scintillation counter. A value in a sample in which diphtheria toxin was not added is measured, this value is made 100%, and a value in a sample in which the toxin was added is calculated as %.

The protein substantially not having the toxicity of diphtheria toxin refers to the protein in which the toxicity of diphtheria toxin has been detoxified or attenuated to an extremely low level, and in the present invention, refers to those having no significant difference from the value in the sample in which diphtheria toxin has not been added or the sample in which diphtheria toxin mutant not having the catalytic domain has been added when the toxicity is measured in the above Vero cell system using diphtheria toxin at a concentration of 1 ng/mL. For the significant difference, it is preferable that there is no significant difference at

significant level 5% in t-test, it is more preferable that there is no significant difference at significant level 1%, and it is still more preferable that there is no significant difference at significant level 0.1%.

But, in the mutants such as CRM197 and DT52E148K described to have no toxicity of diphtheria toxin until now, it has been proved that the extremely faint toxicity (e.g., about 10^{-10} in CRM197 compared with wild type diphtheria toxin) remains (Patent document 1). The mutants having such a faint toxicity are not excluded from the present invention. The toxicity level of diphtheria toxin is preferably the same as or lower than that of CRM197 in terms of eliminating the side effect by the toxicity of diphtheria toxin and increasing the safety.

The toxicity of diphtheria toxin can be controlled by mutating the catalytic domain essential for ADP-ribosylating the peptide elongation factor-2 or deleting a part or all of the catalytic domain.

The function of the mutated catalytic domain can be exactly examined by directly measuring the ADP ribosylation activity. The ADP ribosylation activity can be directly measured by adding the fragment A or the protein (mutated catalytic domain) in which the ADP ribosylation activity is to be measured and NAD labeled with a radioisotope to isolated and purified EF-2, ADP-ribosylating EF-2 *in vitro* and measuring the radioactivity incorporated into EF-2.

Specifically, Tris buffer (pH 7.8) at a final

concentration of 20 mM, 1 mM MTT (dithiothreitol) 0.1 to 1 μg/mL of the fragment A or 0.1 to 100 μg/mL of the protein in which the ADP ribosylation activity is to be measured are added to a rabbit reticulocyte EF-2 fraction obtained by the method described in the following reference, Moynihan, M. R. and Pappenheimer, A. M. Jr. Infect. Immun., 32: 575-582, 1981, further [^{32}P] NAD at a final activity of 370 KBq/mL (about 740 GBq/mM) is added thereto and mixed, and the mixture is reacted at 37°C for 10 minutes.

The same volume of 10% trichloroacetic acid solution is added to the reaction solution to precipitate a protein, a resulting precipitate is trapped in a glass filter, and the filter is further washed with 5% trichloroacetic acid solution.

Finally, the filter is washed with 100% ethanol and dried.

The filter is immersed in toluene PPO scintillator, and the radioactivity trapped in the filter is measured by the liquid scintillation counter.

The measured radioactivity indicates the degree of the ADP ribosylation activity, and the relative activity of the ADP ribosylation in the mutated protein can be determined based on the radioactivity using the unmutated fragment A.

According to more detailed investigation of the present inventors based on domain information, it has been found that including the amino acid sequence from positions 378 to 535 corresponding to the portion comprising the receptor-binding domain is only necessary for the characteristic of having the activity to inhibit the binding between the soluble form HB-

EGF and EGFR. That is, a gene in which the sequence from the positions 378 to 535 of diphtheria toxin had been fused to GST (gluthathione-S-transferase) was made, and this was expressed in *Escherichia coli* to produce a fusion protein (GST-DT) having the above structure. GST-DT inhibited the binding of the ^{125}I-labeled diphtheria toxin to HB-EGF in a dose dependent manner. It was found by the degree of the inhibition that GST-DT was bound to HB-EGF with similar strength to that of diphtheria toxin. Therefore, it was found that the sequence required for the binding was the sequence from the positions 378 to 535, i.e., the portion comprising the receptor-binding domain.

Whether having the activity to inhibit the binding between HB-EGF and EGFR can be determined by the above inhibition experiment in the binding of the aforementioned ^{125}I-labeled diphtheria toxin to HB-EGF.

Thus, the protein having the activity to inhibit the binding between HB-EGF and EGFR and substantially not having the toxicity of diphtheria toxin can be obtained by making a diphtheria toxin mutant protein having the mutation in the catalytic domain with holding the receptor-binding domain, or a protein which is a portion of diphtheria toxin obtained by deleting a part or all of the catalytic domain with holding the receptor-binding domain of diphtheria toxin.

Examples of such a mutant include CRM197, DT52E148K and GST-DT. These do not have the toxicity of diphtheria toxin substantially, and inhibit the binding of HB-EGF to EGFR.

CRM197 is the mutant obtained by mutating Gly to Glu at position 52 when counted without including the signal sequence composed of 25 amino acid residues. DT52E148K is the mutant obtained by mutating Glu to Lys at position 148 in addition to the above mutation when counted without including the signal sequence. GST-DT is the protein comprising the positions 378 to 535 of diphtheria toxin when counted without including the signal sequence of diphtheria toxin. The amino acid sequence (first 25 amino acid residues represent the signal sequence) for CRM197 and the base sequence of the gene encoding it are shown in SEQ ID NOS:3 and 4, respectively. The amino acid sequence (SEQ ID NO:5) for GST-DT and the base sequence (SEQ ID NO:6) of the gene encoding it are shown in FIGs. 5 and 6, respectively.

CRM197 has been already reported to not have the toxicity of diphtheria toxin, i.e., not have the ADP ribosylation activity (T. Uchida and A. M. Pappenheimer Jr. Science 175: 901-903, 1972). A 148K mutant having the mutation at position 148E has been known to have only an extremely faint activity (J. T. Barbieri and R. J. Collier, Infect. Immun., 55: 1647-1651, 1987). DT52E148K which is a double mutant having the 148K mutation in addition to CRM197 which is the 52E mutant is preferable as the safer mutant. The toxicity of these mutants was identified to have no significant difference from the value of the sample in which diphtheria toxin had not been added in the aforementioned protein synthesis inhibition experiment. It is obvious from completely lacking the

catalytic domain that GST-DT has no toxicity of diphtheria toxin.

SEQ ID NO:3

MSRKLFASILIGALLGIGAPPSAHAGADDVVDSSKSFVMENFSSYHGTKPGYVDSIQKGIQK
PKSGTQGNYDDDWKEFYSTDNKYDAAGYSVDNENPLSGKAGGVVKVTYPGLTKVLALKVDNA
ETIKKELGLSLTEPLMEQVGTEEFIKRFGDGASRVVLSLPFAEGSSSVEYINNWEQAKALSV
ELEINFETRGKRGQDAMYEYMAQACAGNRVRRSVGSSLSCINLDWDVIRDKTKTKIESLKEH
GPIKNKMSESPNKTVSEEKAKQYLEEFHQTALEHPELSELKTVTGTNPVFAGANYAAWAVNV
AQVIDSETADNLEKTTAALSILPGIGSVMGIADGAVHHNTEEIVAQSIALSSLMVAQAIPLV
GELVDIGFAAYNFVESIINLFQVVHNSYNRPAYSPGHKTQPFLHDGYAVSWNTVEDSIIRTG
FQGESGHDIKITAENTPLPIAGVLLPTIPGKLDVNKSKTHISVNGRKIRMRCRAIDGDVTFC
RPKSPVYVGNGVHANLHVAFHRSSSEKIHSNEISSDSIGVLGYQKTVDHTKVNSKLSLFFEI
KS

A fragment containing the receptor-binding domain can be made by synthesizing a DNA sequence of the receptor-binding domain by PCR using a gene (Pβ197) encoding CRM197 incorporated in a plasmid as a template, inserting this in multicloning site of an expression vector (pGEX-3X, pQE-30) for synthesizing a GST fusion protein or a histidine tag, incorporating a resulting plasmid in *Escherichia coli* and synthesizing the gene encoded by the plasmid in *Escherichia coli*.

The mutant having the mutation in the catalytic domain can be made as follows. A CRM197 region is synthesized by PCR using the gene (Pβ197) encoding CRM197 incorporated in the plasmid as the template, and using a portion to be mutated as a primer. The primer is synthesized by introducing a point

mutation so as to have the mutation, and used. The synthesized DNA can be introduced into a gene expression vector (pET-22b) for *Escherichia coli*, and *Escherichia coli* is transfected with the vector to express the mutant in *Escherichia coli*.

The antineoplastic agent of the present invention can be used for the treatment of malignant tumors in broad range such as ovarian cancers, breast cancers, prostate cancers, cancers of the uterine cervix, cancers of the uterine body, thyroid cancers, lung cancers, pancreatic cancers, stomach cancers, cancers of the colon and rectum and glioblastoma, and preferably can be used for the malignant tumors expressing HB-EGF. The preferable cancers subjected to the treatment are breast cancers, prostate cancers, pancreatic cancers, stomach cancers, cancers of the colon and rectum, ovarian cancers, glioblastoma, cancers of the uterine body and cancers of the uterine cervix.

In the antineoplastic agent of the present invention, the above active ingredient can be directly formulated or can be formulated in combination with a pharmaceutically acceptable carrier for pharmaceuticals.

The antineoplastic agent can be administered orally or parenterally (e.g., intravenous, intramuscular, intraperitoneal, subcutaneous or intradermal injection, intrarectal administration, permucosal administration, administration via respiratory tract). When applied to the malignant tumor such as ovarian cancer intraperitoneally seeded, it is preferable in terms of being directly

transported by cancer cells to administer by intraperitoneal injection.

Pharmaceutical compositions suitable for the oral administration include, for example, tablets, granules, capsules, powders, solutions, suspensions and syrups. The pharmaceutical compositions suitable for the parenteral administration include, for example, injectable agents, drops, suppositories and percutaneous absorbing agents, but the formulation is not limited thereto.

Types of additives for the formulations used for producing the antineoplastic agent are not particularly limited and can be appropriately selected by those skilled in the art. For example, excipients, disintegrants and disintegrant aids, binders, lubricants, coating agents, bases, solubilizers and solubilizer aids, dispersants, suspending agents, emulsifiers, buffers, antioxidants, preservatives, tonicity agents, pH adjusters, solubilizers and stabilizers can be used. Individual specific ingredients used for these purposes are well known to those skilled in the art.

As the additives for the formulations usable for preparing the formulations for the oral administration, for example, the excipients such as glucose, lactose, D-mannitol, starch or crystalline cellulose; the disintegrants and disintegrant aids such as carboxymethylcellulose, starch and calcium carboxymethylcellulose; the binders such as hydroxypropylcellulose, hydroxypropylmethylcellulose, polyvinyl pyrrolidone or gelatin; the lubricants such as

magnesium stearate and talc; the coating agents such as hydroxypropylmethylcellulose, saccharose, polyethylene glycol or titanium oxide; and the bases such as Vaseline, liquid paraffin, polyethylene glycol, gelatin, kaolin, glycerine, purified water or hard fat, and the like can be used.

As the additives for the formulations usable for preparing the formulations for the injection or drip infusion, the solubilizers and solubilizer aids such as injectable distilled water, saline or propylene glycol, which are aqueous or can constitute a solubilized type injectable agent in use; the tonicity agents such as glucose, sodium chloride, D-mannitol and glycerine; and the pH adjusters such as inorganic acids, organic acids, inorganic bases or organic bases can be used.

An amount of the active ingredient contained in the antineoplastic agent of the present invention varies depending on a formulation form or an administration route of the antineoplastic agent, can not be defined categorically, but can be typically determined by appropriately selecting from the range of about 0.0001 to 70% in the final formulation.

The antineoplastic agent of the present invention can be administered to mammalian animals including human beings.

The amount of the antineoplastic agent of the present invention to be administered should be appropriately increased or decreased depending on conditions such as patient's age, gender, body weight and symptom, and the administration route. As the amount of the active ingredient per day per adult, the

amount of the mutant protein of diphtheria toxin which is one of the active ingredients is preferably in the range of about 1 μg to 30 mg per day per kg body weight.

Paclitaxel is typically administered in an amount of 3.5 to 5.5 mg/kg mainly with a platinum based drug. However, in this administration range, myelosuppression becomes remarkable, and continuation of the treatment becomes sometimes difficult. In particular, for peripheral nerve toxicity, the side effect sometimes appears at an initial administration, and the side effect is irreversible. Thus, the reduction of the amount to be administered is important for not only the reduction of the transient side effect but also the reduction of accumulative side effect. By combining with the mutant protein (in particular, CRM197) of diphtheria toxin, it is possible to reduce the amount of paclitaxel to be administered to one forth or less. Thus, the combination of paclitaxel with CRM197 is thought to contribute to not only the improvement of clinical effects but also the inhibition of side effect occurrence.

Carboplatin is typically administered in an amount of 10 to 20 mg/kg in combination with a taxane based drug. Carboplatin is the drug which induces the remarkable myelosuppression. In females and elderly people basically having lowered bone marrow functions, the severe myelosuppression is compelled, and the lethal cases with complication of severe infection are often experienced clinically. By combining with the mutant protein (in

particular, CRM197) of diphtheria toxin, the amount of carboplatin to be administered is reduced to one forth or less as is the case with paclitaxel, thereby being capable of obtaining the clinical effect equivalent to or more than that by carboplatin alone. It is sufficiently anticipated to avoid the remarkable myelosuppression by this reduction of the amount to be administered.

Irinotecan is typically administered in an amount of 2 to 3 mg/kg mainly in combination with the platinum based drug or as a single drug. In this administration range, gastrointestinal symptoms including diarrhea frequently appear in addition to the remarkable myelosuppression. In particular, when the diarrhea symptom is severe, even if the effect is clinically observed, discontinuation of the chemotherapy using irinotecan is compelled. Irinotecan is also administered over three weeks, and thus, the treatment with irinotecan is often discontinued due to the myelosuppression. By combining with the mutant protein (in particular, CRM197) of diphtheria toxin, the amount of irinotecan to be administered is reduced to one forth or less, thereby avoiding the discontinuation of the treatment due to the myelosuppression and the cessation of the treatment due to exacerbation of the diarrhea symptom. Thus, it is possible to obtain the sufficient clinical effect by combining irinotecan with CRM197.

The pharmaceutical in the above amount may be administered once a day, or by dividing into several times. It may also be administered once several days to several weeks,

or singly. It can also be administered with a component such as steroid to inhibit the side effect. The mutant protein of diphtheria toxin and at least one of paclitaxel, carboplatin, irinotecan and the derivatives thereof may be administered simultaneously or with a time difference.

EXAMPLES

Examples of the present invention will be described below, but the present invention is not limited to these Examples.

(Example 1)

<Production of CRM 197 protein>

A stock of lysogenic bacterium of C7(β197) [available from ATCC (American Type Culture Collection) as C7(beta197)M1 (No. 39255), diphtheria bacillus in which C7(β197) phage was lysogenized] is cultured, and a bacterial solution in a logarithmic growth phase late stage is added to C-Y medium to which 2% filtrated maltose was added so that an initial OD_{590} value was about 0.05. This OD value corresponds to about 5 x 10^7 microbial cells/mL. A flask is placed on a rotary shaker at 200 rpm, and the microbial cells are cultured at 35°C for 16 to 17 hours. The culture is terminated when the OD value becomes 10 to 15.

The above C-Y medium is prepared as follows. That is, 10 g of casamino acids, 20 g of yeast extract solution and 5 g of KH_2PO_4 are dissolved in 1 L of distilled water. After adding 2 mL of 50% $CaCl_2.2H_2O$, pH is adjusted to 7.4. The solution is boiled and then filtrated. Subsequently, 2 mL of Mueller and

Miller's solution II (22.5 g of $MgSO_4$, 0.115 g of β-alanine, 0.115 g of nicotinic acid, 7.5 mg of pimelic acid, 1 g of $CuSO_4.5H_2O$, 1g of $ZnSO_4.5H_2O$, 1g of $MnCl_2.4H_2O$/100ml H_2O) and 1 mL of Mueller and Miller's solution III (20 g of L-cystine, 20 mL of concentrated hydrochloric acid/100ml H_2O) are added. The C-Y medium was obtained by dispensing each 100 mL and autoclaving it.

The CRM protein is purified as follows.

A culture medium is centrifuged at 10,000 g for 15 minutes. Ammonium sulfate is added to a culture supernatant at a saturation degree of 65%. The mixture is left stand in an ice room for 24 to 48 hours. Precipitates are collected, dissolved in 0.02 M Tris hydrochloride buffer pH 7.2, and dialyzed against the same buffer.

A dialyzed solution is centrifuged to remove insoluble matters, the supernatant is applied on a DE52 column, and eluted with NaCl concentration gradient in 0.02 M Tris hydrochloride buffer pH 7.2. CRM197 is eluted at 0.08 M of NaCl. An eluted solution is saturated to 65% with ammonium sulfate. Precipitates are dissolved in 0.01 M Tris hydrochloride buffer, and equilibrated again. Column elution by applying onto the DE52 column and the precipitation with ammonium persulfate are repeated. Subsequently, the sample is applied to a Sephacryl™ S-200 and eluted with the solution of HEPES-NaOH, pH 7.2 and 0.15 M NaCl. The eluted CRM197 is applied onto a DeToxi gel to remove LPS-like substances contained in the CRM197 sample, and the resulting CRM197 is

used for experiments. For absorbance of CRM197 at 280 nm, 1OD corresponds to about 0.67 mg/mL.

(Preparation of cell lines)

An ovarian cancer cell line, SKOV-3 and a breast cancer cell line, MDA-MB-231 were obtained from ATCC (American Type Culture Collection).

SK-HB-1 cells were obtained by transfecting SKOV-3 cells with human HB-EGF cDNA incorporated in pRC/CMV vector (Invitrogen). The transfection was performed using LipofectAMINE reagent (Invitrogen) in accordance with a manual attached to the product. The transfected cells were cultured in the medium (RPMI-1640-10FCS) containing 400 μg/mL of G418. Surviving cells were seeded again in a petri dish at low density and a growing colony was picked up to yield SK-HB-1 cells. It was identified that this cell expressed HB-EGF at high level, by adding the ^{125}I-labeled diphtheria toxin to the cells and comparing the radioactivity of diphtheria toxin bound to the cells with that in SKOV-3 cells.

(Tumorigenicity experiments using nude mice)

The ovarian cancer cell line, SKOV-3, SK-HB1 and the breast cancer cell line, MDA-MB-231 cultured in RPMI + 10% FBS were washed with EDTA/PBS(-), and collected with 0.25% trypsin. The cells were washed twice with RPMI + 10% FBS and twice with RPMI (no serum), and 250 μL of cell suspension in RPMI (with serum) at 5 x 10^6 cells was inoculated in a dorsal portion of nude mice by subcutaneous injection.

In one group of the nude mice, 10 days after inoculating

SKOV-3 or SK-HB1 cells, the administration of CRM197 was initiated, and CRM197 was intraperitoneally administered in amounts shown in FIGs. 7 and 8 once a week over 4 weeks. The nude mice to which CRM197 had not been administered were used as controls. Relations between administration time periods and tumor volumes are shown in FIGs. 7 and 8. Here, the tumor volume was obtained by measuring a major axis and a minor axis of the tumor every 3 to 4 days and calculating by Major axis x Minor axis x Minor axis x 1/2.

Subsequently, for other groups of the nude mice, 7 days after inoculating SKOV-3 or SK-HB1 or MDA-MB-231 cells, the administration of CRM197 in combination with taxol (Bristol-Myers K.K.) was initiated. Both drugs were intraperitoneally administered in amounts shown in FIGs. 9 to 11 once a week over 4 weeks. The nude mice to which CRM197 and taxol had not been administered were used as the controls. The relations between administration time periods and tumor volumes are shown in FIGs. 9 to 11.

From these results, it was found that inhibitory effects on tumor growth was synergistically enhanced by combining taxol with CRM197.

INDUSTRIAL APPLICABILITY

The present invention can be utilized for the production of the antineoplastic agent effective for the treatment of various cancers including ovarian cancers, breast cancers, prostate cancers, cancers of the uterine cervix, cancers of

the uterine body, thyroid cancers, lung cancers, pancreatic cancers, stomach cancers, cancers of the colon and rectum and glioblastoma.

CLAIMS

1. An antineoplastic composition for the treatment of breast and ovarian cancers comprising (a) paclitaxel in an amount of 0.875 to 1.375 mg per kilogram of body weight of a recipient patient with (b) a protein consisting of an amino acid sequence shown in SEQ ID No:3 in an amount of 1 μg to 30 mg per kilogram of body weight of the recipient patient, wherein the amounts both independently have no sufficient anti-cancer action.

2. Use to treat breast and ovarian cancers of (a) paclitaxel in an amount of 0.875 to 1.375 mg per kilogram of body weight of a recipient patient, and (b) 1 μg to 30 mg per kilogram of body weight of the recipient patient of a protein consisting of an amino acid sequence shown in SEQ ID NO:3, wherein the use is a simultaneous use.

3. A kit characterized by comprising (a) paclitaxel in an amount of 0.875 to 1.375 mg per kilogram of body weight of a recipient patient and (b) a protein consisting of an amino acid sequence shown in SEQ ID NO:3, wherein (a) and (b) both independently have no sufficient anti-cancer action but an anti-cancer action is elicited by combining (a) and (b) together in the recipient patient.

FIG. 1

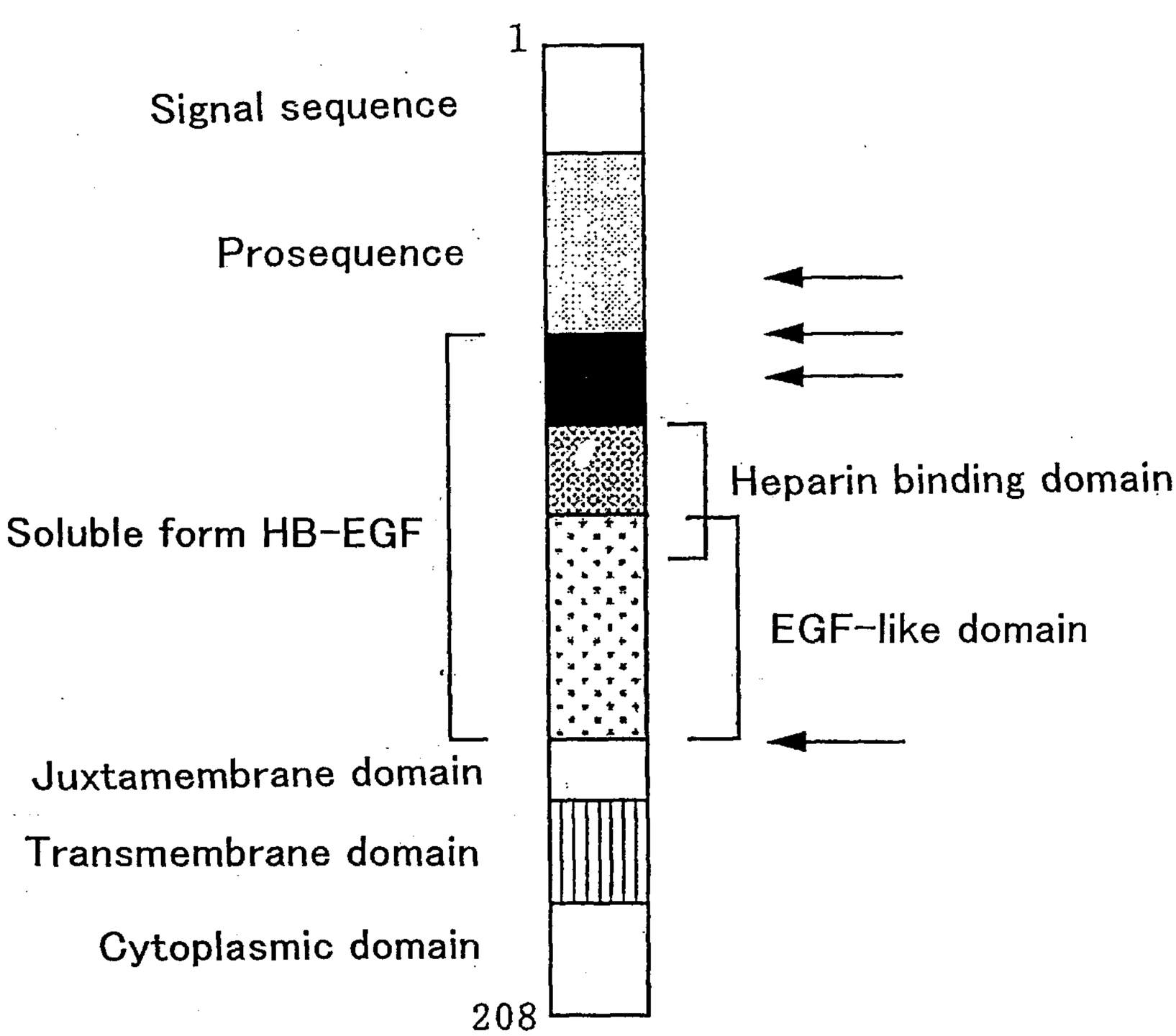

1
Signal sequence
Prosequence
Soluble form HB-EGF
Heparin binding domain
EGF-like domain
Juxtamembrane domain
Transmembrane domain
Cytoplasmic domain
208

FIG. 2

```
          10         20         30         40         50         60
GTGAGCAGAAAACTGTTTGCGTCAATCTTAATAGGGGCGCTACTGGGGATAGGGGCCCCA
M  S  R  K  L  F  A  S  I  L  I  G  A  L  L  G  I  G  A  P  -6

          70         80         90        100        110        120
CCTTCAGCCCATGCAGGCGCTGATGATGTTGTTGATTCTTCTAAATCTTTTGTGATGGAA
P  S  A  H  A  G  A  D  D  V  V  D  S  S  K  S  F  V  M  E  15

         130        140        150        160        170        180
AACTTTTCTTCGTACCACGGGACTAAACCTGGTTATGTAGATTCCATTCAAAAAGGTATA
N  F  S  S  Y  H  G  T  K  P  G  Y  V  D  S  I  Q  K  G  I  35

         190        200        210        220        230        240
CAAAAGCCAAAATCTGGTACACAAGGAAATTATGACGATGATTGGAAAGGGTTTTATAGT
Q  K  P  K  S  G  T  Q  G  N  Y  D  D  D  W  K  G  F  Y  S  55

         250        260        270        280        290        300
ACCGACAATAAATACGACGCTGCGGGATACTCTGTAGATAATGAAAACCCGCTCTCTGGA
T  D  N  K  Y  D  A  A  G  Y  S  V  D  N  E  N  P  L  S  G  75

         310        320        330        340        350        360
AAAGCTGGAGGCGTGGTCAAAGTGACGTATCCAGGACTGACGAAGGTTCTCGCACTAAAA
K  A  G  G  V  V  K  V  T  Y  P  G  L  T  K  V  L  A  L  K  95

         370        380        390        400        410        420
GTGGATAATGCCGAAACTATTAAGAAAGAGTTAGGTTTAAGTCTCACTGAACCGTTGATG
V  D  N  A  E  T  I  K  K  E  L  G  L  S  L  T  E  P  L  M  115

         430        440        450        460        470        480
GAGCAAGTCGGAACGGAAGAGTTTATCAAAAGGTTCGGTGATGGTGCTTCGCGTGTAGTG
E  Q  V  G  T  E  E  F  I  K  R  F  G  D  G  A  S  R  V  V  135

         490        500        510        520        530        540
CTCAGCCTTCCCTTCGCTGAGGGGAGTTCTAGCGTTGAATATATTAATAACTGGGAACAG
L  S  L  P  F  A  E  G  S  S  S  V  E  Y  I  N  N  W  E  Q  155

         550        560        570        580        590        600
GCGAAAGCGTTAAGCGTAGAACTTGAGATTAATTTTGAAACCCGTGGAAAACGTGGCCAA
A  K  A  L  S  V  E  L  E  I  N  F  E  T  R  G  K  R  G  Q  175

         610        620        630        640        650        660
GATGCGATGTATGAGTATATGGCTCAAGCCTGTGCAGGAAATCGTGTCAGGCGATCAGTA
D  A  M  Y  E  Y  M  A  Q  A  C  A  G  N  R  V  R  R  S  V  195

         670        680        690        700        710        720
GGTAGCTCATTGTCATGCATAAATCTTGATTGGGATGTCATAAGGGATAAAACTAAGACA
G  S  S  L  S  C  I  N  L  D  W  D  V  I  R  D  K  T  K  T  215

         730        740        750        760        770        780
AAGATAGAGTCTTTGAAAGAGCATGGCCCTATCAAAAATAAAATGAGCGAAAGTCCCAAT
K  I  E  S  L  K  E  H  G  P  I  K  N  K  M  S  E  S  P  N  235

         790        800        810        820        830        840
AAAACAGTATCTGAGGAAAAAGCTAAACAATACCTAGAAGAATTTCATCAAACGGCATTA
K  T  V  S  E  E  K  A  K  Q  Y  L  E  E  F  H  Q  T  A  L  255

         850        860        870        880        890        900
GAGCATCCTGAATTGTCAGAACTTAAAACCGTTACTGGGACCAATCCTGTATTCGCTGGG
E  H  P  E  L  S  E  L  K  T  V  T  G  T  N  P  V  F  A  G  275
```

FIG. 3

```
       910        920        930        940        950        960
GCTAACTATGCGGCGTGGGCAGTAAACGTTGCGCAAGTTATCGATAGCGAAACAGCTGAT
A  N  Y  A  A  W  A  V  N  V  A  Q  V  I  D  S  E  T  A  D  295

       970        980        990       1000       1010       1020
AATTTGGAAAAGACAACTGCTGCTCTTTCGATACTTCCTGGTATCGGTAGCGTAATGGGC
N  L  E  K  T  T  A  A  L  S  I  L  P  G  I  G  S  V  M  G  315

      1030       1040       1050       1060       1070       1080
ATTGCAGACGGTGCCGTTCACCACAATACAGAAGAGATAGTGGCACAATCAATAGCTTTA
I  A  D  G  A  V  H  H  N  T  E  E  I  V  A  Q  S  I  A  L  335

      1090       1100       1110       1120       1130       1140
TCGTCTTTAATGGTTGCTCAAGCTATTCCATTGGTAGGAGAGCTAGTTGATATTGGTTTC
S  S  L  M  V  A  Q  A  I  P  L  V  G  E  L  V  D  I  G  F  355

      1150       1160       1170       1180       1190       1200
GCTGCATATAATTTTGTAGAGAGTATTATCAATTTATTTCAAGTAGTTCATAATTCGTAT
A  A  Y  N  F  V  E  S  I  I  N  L  F  Q  V  V  H  N  S  Y  375

      1210       1220       1230       1240       1250       1260
AATCGTCCCGCGTATTCTCCGGGGCATAAAACGCAACCATTTCTTCATGACGGGTATGCT
N  R  P  A  Y  S  P  G  H  K  T  Q  P  F  L  H  D  G  Y  A  395

      1270       1280       1290       1300       1310       1320
GTCAGTTGGAACACTGTTGAAGATTCGATAATCCGAACTGGTTTTCAAGGGGAGAGTGGG
V  S  W  N  T  V  E  D  S  I  I  R  T  G  F  Q  G  E  S  G  415

      1330       1340       1350       1360       1370       1380
CACGACATAAAAATTACTGCTGAAAATACCCCGCTTCCAATCGCGGGTGTCCTACTACCG
H  D  I  K  I  T  A  E  N  T  P  L  P  I  A  G  V  L  L  P  435

      1390       1400       1410       1420       1430       1440
ACTATTCCTGGAAAGCTGGACGTTAATAAGTCCAAGACTCATATTTCCGTAAATGGTCGG
T  I  P  G  K  L  D  V  N  K  S  K  T  H  I  S  V  N  G  R  455

      1450       1460       1470       1480       1490       1500
AAAATAAGGATGCGTTGCAGAGCTATAGACGGTGATGTAACTTTTTGTCGCCCTAAATCT
K  I  R  M  R  C  R  A  I  D  G  D  V  T  F  C  R  P  K  S  475

      1510       1520       1530       1540       1550       1560
CCTGTTTATGTTGGTAATGGTGTGCATGCGAATCTTCACGTGGCATTTCACAGAAGCAGC
P  V  Y  V  G  N  G  V  H  A  N  L  H  V  A  F  H  R  S  S  495

      1570       1580       1590       1600       1610       1620
TCGGAGAAAATTCATTCTAATGAAATTTCGTCGGATTCCATAGGCGTTCTTGGGTACCAG
S  E  K  I  H  S  N  E  I  S  S  D  S  I  G  V  L  G  Y  Q  515

      1630       1640       1650       1660       1670       1680
AAAACAGTAGATCACACCAAGGTTAATTCTAAGCTATCGCTATTTTTTGAAATCAAAAGC
K  T  V  D  H  T  K  V  N  S  K  L  S  L  F  F  E  I  K  S  535

      1690
TGA
*
```

FIG. 4

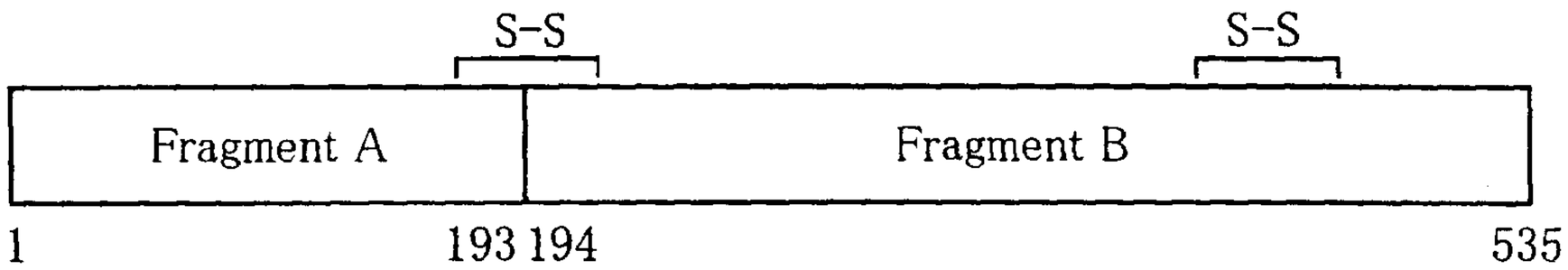


FIG. 5

```
          10         20         30         40         50         60
ATGTCCCCTATACTAGGTTATTGGAAAATTAAGGGCCTTGTGCAACCCACTCGACTTCTT
M  S  P  I  L  G  Y  W  K  I  K  G  L  V  Q  P  T  R  L  L

          70         80         90        100        110        120
TTGGAATATCTTGAAGAAAAATATGAAGAGCATTTGTATGAGCGCGATGAAGGTGATAAA
L  E  Y  L  E  E  K  Y  E  E  H  L  Y  E  R  D  E  G  D  K

         130        140        150        160        170        180
TGGCGAAACAAAAAGTTTGAATTGGGTTTGGAGTTTCCCAATCTTCCTTATTATATTGAT
W  R  N  K  K  F  E  L  G  L  E  F  P  N  L  P  Y  Y  I  D

         190        200        210        220        230        240
GGTGATGTTAAATTAACACAGTCTATGGCCATCATACGTTATATAGCTGACAAGCACAAC
G  D  V  K  L  T  Q  S  M  A  I  I  R  Y  I  A  D  K  H  N

         250        260        270        280        290        300
ATGTTGGGTGGTTGTCCAAAAGAGCGTGCAGAGATTTCAATGCTTGAAGGAGCGGTTTTG
M  L  G  G  C  P  K  E  R  A  E  I  S  M  L  E  G  A  V  L

         310        320        330        340        350        360
GATATTAGATACGGTGTTTCGAGAATTGCATATAGTAAAGACTTTGAAACTCTCAAAGTT
D  I  R  Y  G  V  S  R  I  A  Y  S  K  D  F  E  T  L  K  V

         370        380        390        400        410        420
GATTTTCTTAGCAAGCTACCTGAAATGCTGAAAATGTTCGAAGATCGTTTATGTCATAAA
D  F  L  S  K  L  P  E  M  L  K  M  F  E  D  R  L  C  H  K

         430        440        450        460        470        480
ACATATTTAAATGGTGATCATGTAACCCATCCTGACTTCATGTTGTATGACGCTCTTGAT
T  Y  L  N  G  D  H  V  T  H  P  D  F  M  L  Y  D  A  L  D

         490        500        510        520        530        540
GTTGTTTTATACATGGACCCAATGTGCCTGGATGCGTTCCCAAAATTAGTTTGTTTTAAA
V  V  L  Y  M  D  P  M  C  L  D  A  F  P  K  L  V  C  F  K

         550        560        570        580        590        600
AAACGTATTGAAGCTATCCCACAAATTGATAAGTACTTGAAATCCAGCAAGTATATAGCA
K  R  I  E  A  I  P  Q  I  D  K  Y  L  K  S  S  K  Y  I  A
```

FIG. 6

```
          610         620         630         640         650         660
TGGCCTTTGCAGGGCTGGCAAGCCACGTTTGGTGGTGGCGACCATCCTCCAAAATCGGAT
W  P  L  Q  G  W  Q  A  T  F  G  G  G  D  H  P  P  K  S  D

          670         680         690         700         710         720
CTGATCGAAGGTCGTGGGATCCCCGCGTATTCTCCGGGGCATAAAACGCAACCATTTCTT
L  I  E  G  R  G  I  P  A  Y  S  P  G  H  K  T  Q  P  F  L

          730         740         750         760         770         780
CATGACGGGTATGCTGTCAGTTGGAACACTGTTGAAGATTCGATAATCCGAACTGGTTTT
H  D  G  Y  A  V  S  W  N  T  V  E  D  S  I  I  R  T  G  F

          790         800         810         820         830         840
CAAGGGGAGAGTGGGCACGACATAAAAATTACTGCTGAAAATACCCCGCTTCCAATCGCG
Q  G  E  S  G  H  D  I  K  I  T  A  E  N  T  P  L  P  I  A

          850         860         870         880         890         900
GGTGTCCTACTACCGACTATTCCTGGAAAGCTGGACGTTAATAAGTCCAAGACTCATATT
G  V  L  L  P  T  I  P  G  K  L  D  V  N  K  S  K  T  H  I

          910         920         930         940         950         960
TCCGTAAATGGTCGGAAAATAAGGATGCGTTGCAGAGCTATAGACGGTGATGTAACTTTT
S  V  N  G  R  K  I  R  M  R  C  R  A  I  D  G  D  V  T  F

          970         980         990        1000        1010        1020
TGTCGCCCTAAATCTCCTGTTTATGTTGGTAATGGTGTGCATGCGAATCTTCACGTGGCA
C  R  P  K  S  P  V  Y  V  G  N  G  V  H  A  N  L  H  V  A

         1030        1040        1050        1060        1070        1080
TTTCACAGAAGCAGCTCGGAGAAAATTCATTCTAATGAAATTTCGTCGGATTCCATAGGC
F  H  R  S  S  S  E  K  I  H  S  N  E  I  S  S  D  S  I  G

         1090        1100        1110        1120        1130        1140
GTTCTTGGGTACCAGAAAACAGTAGATCACACCAAGGTTAATTCTAAGCTATCGCTATTT
V  L  G  Y  Q  K  T  V  D  H  T  K  V  N  S  K  L  S  L  F

         1150        1160
TTTGAAATCAAAAGCTGA
F  E  I  K  S  *
```

FIG. 7

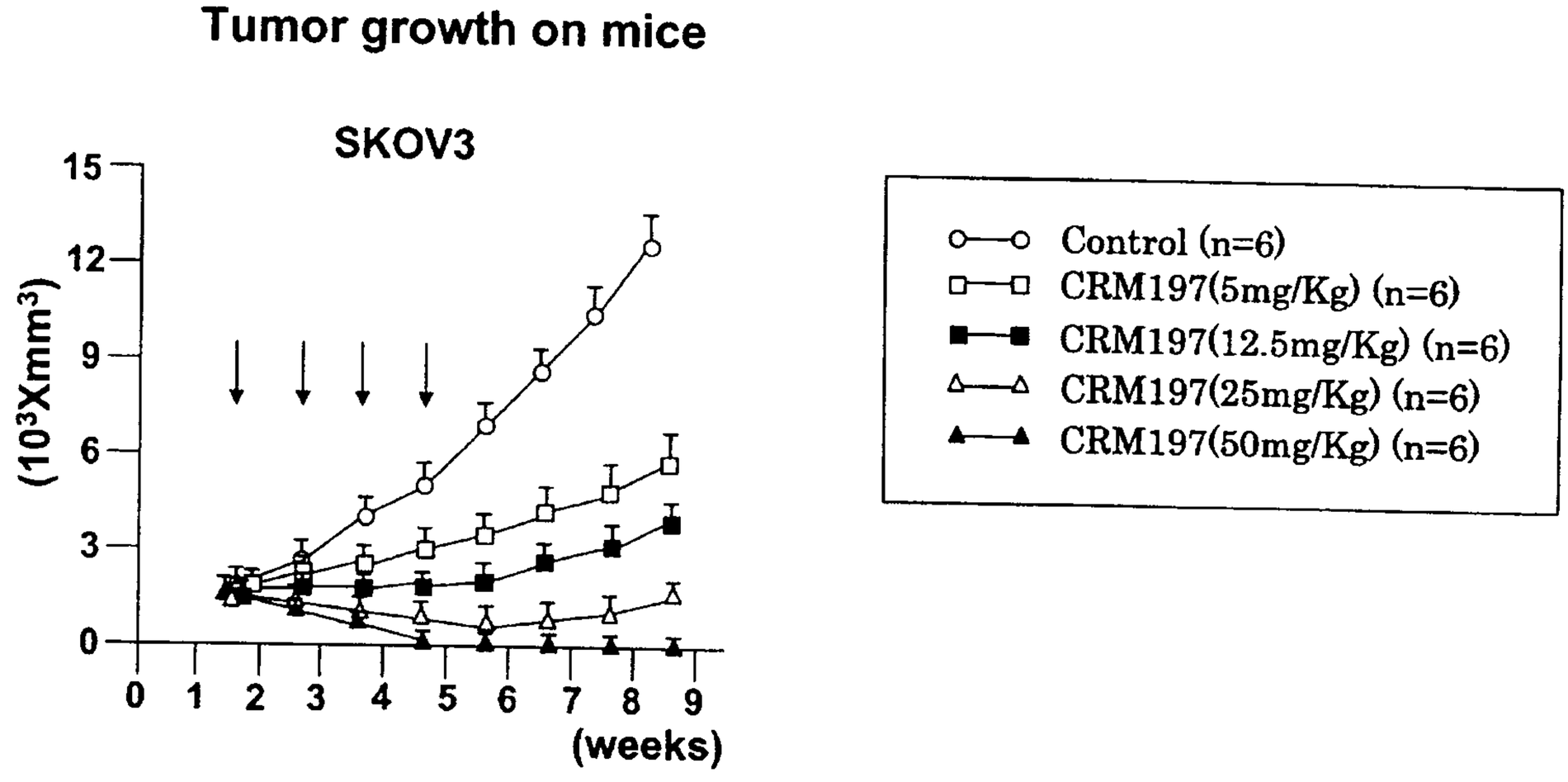

Tumor growth on mice
SKOV3
(10³Xmm³)
15
12
9
6
3
0
0 1 2 3 4 5 6 7 8 9
(weeks)
Control (n=6)
CRM197(5mg/Kg) (n=6)
CRM197(12.5mg/Kg) (n=6)
CRM197(25mg/Kg) (n=6)
CRM197(50mg/Kg) (n=6)

FIG. 8

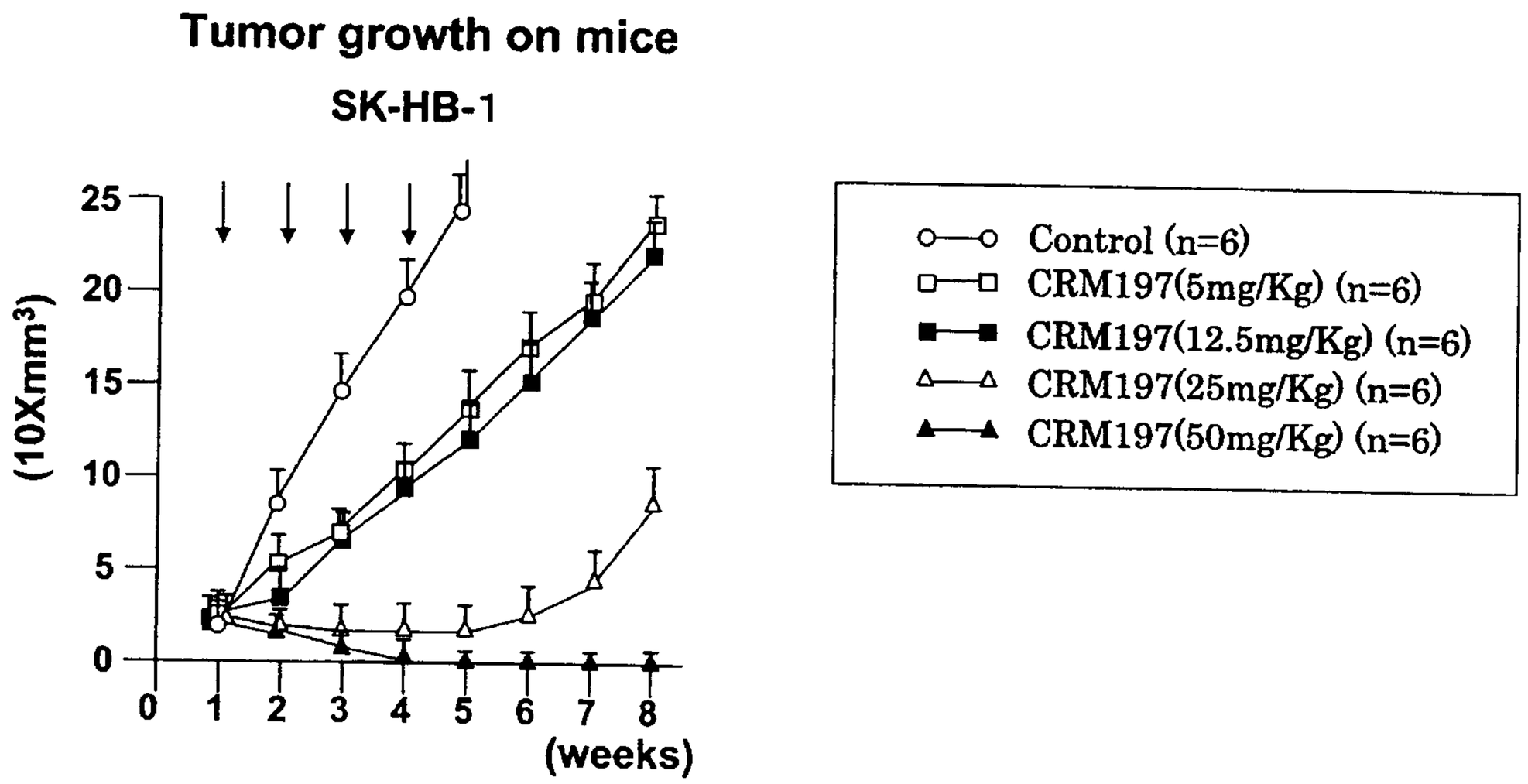

Tumor growth on mice
SK-HB-1
(10Xmm³)
25
20
15
10
5
0
0 1 2 3 4 5 6 7 8
(weeks)
Control (n=6)
CRM197(5mg/Kg) (n=6)
CRM197(12.5mg/Kg) (n=6)
CRM197(25mg/Kg) (n=6)
CRM197(50mg/Kg) (n=6)

FIG. 9

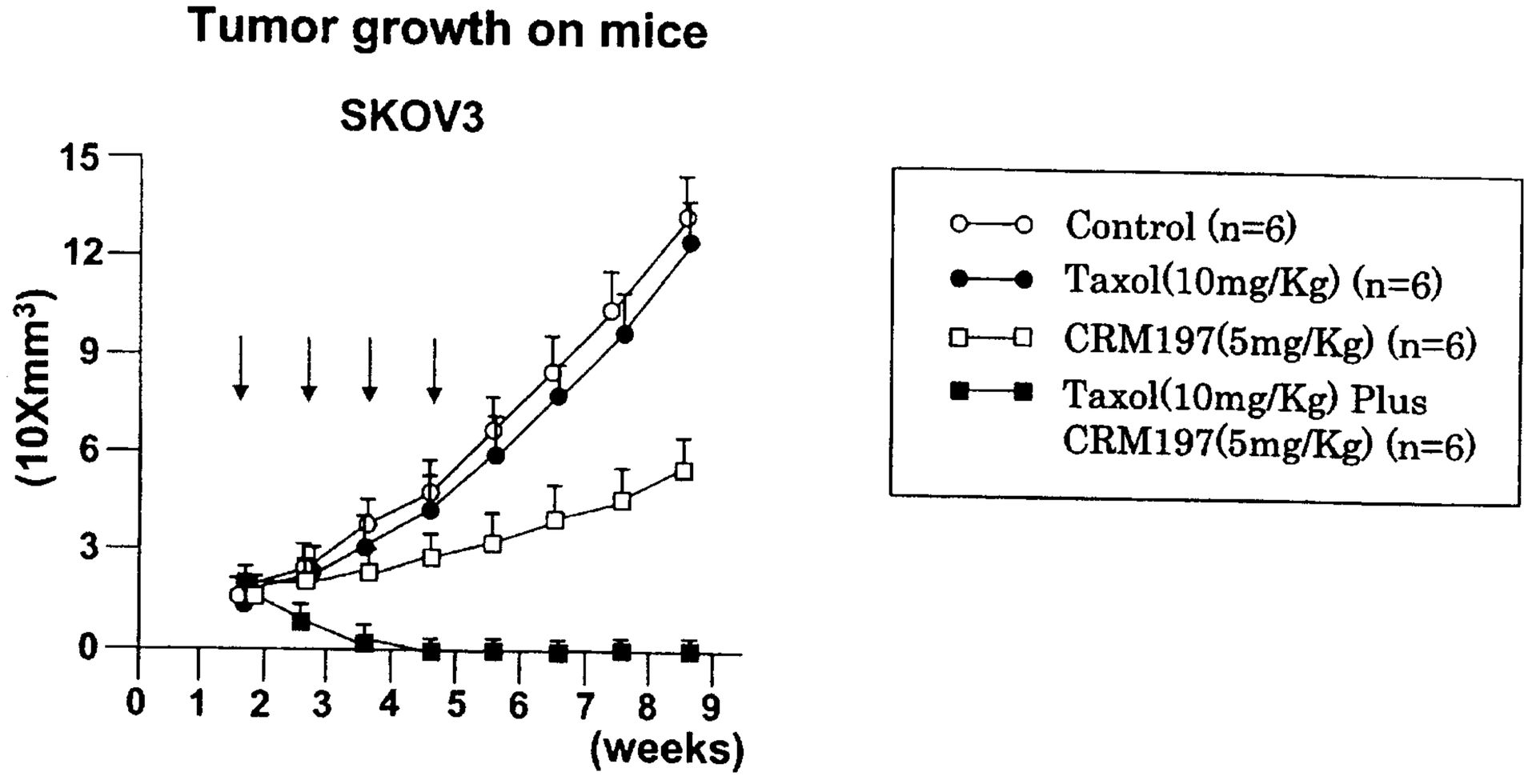

Tumor growth on mice
SKOV3
(10Xmm³)
15
12
9
6
3
0
0 1 2 3 4 5 6 7 8 9
(weeks)
Control (n=6)
Taxol(10mg/Kg) (n=6)
CRM197(5mg/Kg) (n=6)
Taxol(10mg/Kg) Plus
CRM197(5mg/Kg) (n=6)

FIG. 10

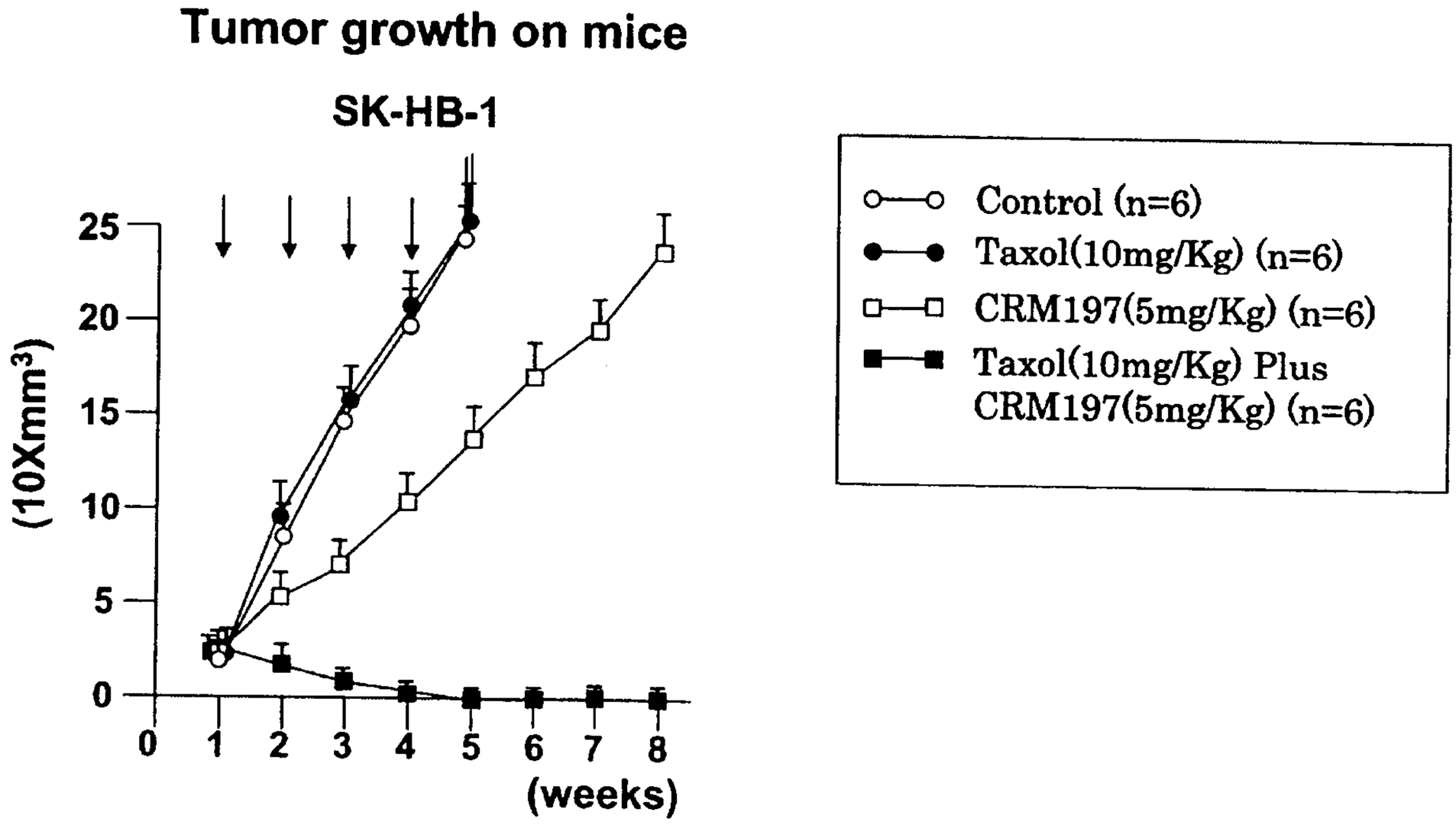
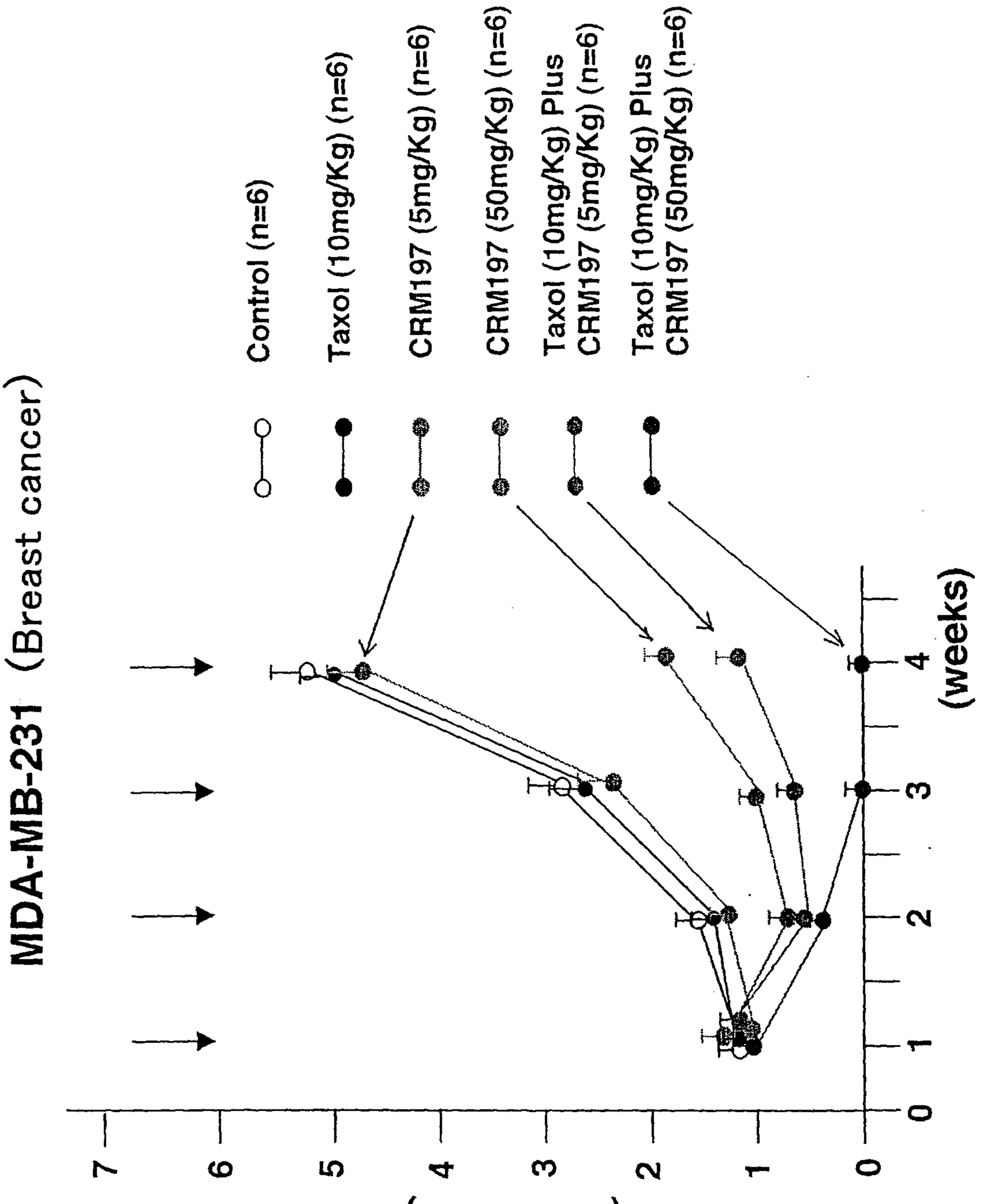

Tumor growth on mice
SK-HB-1
(10Xmm³)
25
20
15
10
5
0
0 1 2 3 4 5 6 7 8
(weeks)
Control (n=6)
Taxol(10mg/Kg) (n=6)
CRM197(5mg/Kg) (n=6)
Taxol(10mg/Kg) Plus
CRM197(5mg/Kg) (n=6)

FIG. 11


MDA-MB-231 (Breast cancer)
Control (n=6)
Taxol (10mg/Kg) (n=6)
CRM197 (5mg/Kg) (n=6)
CRM197 (50mg/Kg) (n=6)
Taxol (10mg/Kg) Plus
CRM197 (5mg/Kg) (n=6)
Taxol (10mg/Kg) Plus
CRM197 (50mg/Kg) (n=6)
0 1 2 3 4
(weeks)
0 1 2 3 4 5 6 7
(x 100 mm3)