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(54) Titre : COMPLEXES COMPRENANT DE L'ANGIOSTATINE ET SES FRAGMENTS, LEURS PROCEDES DE PREPARATION ET LEURS UTILISATIONS

(54) Title: A CONJUGATE COMPRISING ANGIOSTATIN OR ITS FRAGMENT, THE METHOD FOR PRODUCING THE CONJUGATE AND USE THEREOF

(57) **Abrégé/Abstract:**

A medicament useful for treating tumors or angiogenesis mediated diseases, medical compositions and kits comprising the medicament and a method for preparing the medicament are provided. The medicament contains complexes of a modifier and angiostatin or its fragments, which have longer in vivo half-life than non-modified angiostatin or its fragments. The modifier is selected from high molecular polymers, proteins or their fragments, polypeptides, small molecules and any other type of compounds.



Abstract

The present invention provides an anti-tumor or anti-angiogenesis medicament, the combination or kit containing the medicament, and the method for producing the same. The anti-tumor or anti-angiogenesis medicament contains a conjugate comprising a modifying agent and the angiostatin or its fragments, wherein the conjugate exhibits prolonged *in vivo* half-life as compared to an unmodified angiostatin or its fragments. The modifying agent is selected from the group consisting of macromolecular polymers, protein molecules or fragments thereof, peptides, small molecules, or chemical substances of any other forms.

A conjugate comprising angiostatin or its fragment, the method for producing the conjugate and use thereof

Field of the invention

The present invention relates to a conjugate comprising angiostatin and its fragments, preferable fragment K1-3002C a sustained-release formulation comprising the conjugate and a method for preparing them. The conjugate of the present innovation has no toxic side effects, exhibits higher biological activities and is more metabolically stable. The present invention also provides a pharmaceutical composition comprising the conjugate or the sustained-release formulation and a kit containing the conjugates. The present invention also relates to the use of the conjugate, the sustained-release formulation, the pharmaceutical composition and the kit for preventing, diagnosing, and treating tumors, for preparing antitumor medicaments and for preventing diseases related to neovascularization.

Background of the invention

Cancer is a common and frequently-occurring disease nowadays, as well as the number one killer threatening human life. Therefore, the research and development of anti-cancer drugs is a hot topic in China and abroad. Currently, the anti-cancer drugs commonly used for clinical treatments are traditional chemotherapy drugs, which can easily lead to resistance and have many toxic side effects during long-term usage. Therefore, their clinical applications have been greatly restricted.

In 1971, Folkman proposed the theory that the growth and migration of tumor cells depend on angiogenesis, the growth of new capillaries from existing blood vessels. Therefore, blocking tumor angiogenesis may inhibit tumor growth and metastasis. Taking tumor vascular endothelial cells as the target of cancer therapy provides a new strategy for cancer treatment, i.e. anti-angiogenesis therapy (Folkman J. N Engl J Med 1971;285: 1182 -1186).

Angiostatin is the endogenous angiogenesis inhibitor extracted by O'Reilly et al. from serum and urine of Lewis lung tumor-bearing mice. It is the N-terminal sequence of plasminogen starting from the 98th amino acids (using the initial Met as the first amino acid) (O'Reilly MS, Holmgren L, Shing Y, et al. Cell, 1994, 79: 315-328), including at least the first four Kringle structures of five, each of which consists of about 80 amino acids (Lerch P G, Riskli E E, Lergier W, et al. Eur J Biochem, 1980, 107: 7-13).

It has been shown that the fragments K1, K2, K3, K5, K1-3, K2-3 and K1-5 of recombinant human plasminogen can effectively inhibit bovine capillary endothelial cell (BCE) proliferation, while K4 almost has no such activity. The inhibitory effect of K1-3 on cell proliferation is significantly stronger than that of the initially-discovered angiostatin K1-4 (Cao Y, Ji RW, Davidson D, et al. J Biol Chem, 1996, 271(46): 29461-29467.). It has also been shown that K1-3 can not only suppress the growth and metastasis of mouse Lewis lung cancer, but also inhibit the growth of a variety of primary tumors such as mouse Lewis lung cancer primary tumor and hemangioendothelioma. The mechanism underlying the activities of angiostatin is that it suppresses the angiogenesis in tumor tissues by inhibiting the growth of vascular endothelial cells. Consequently, the tumor cannot obtain necessary nutrition and oxygen needed for its growth, which results in growth inhibition and starvation. O'Reilly pointed out that angiostatin is a fragment within endogenous plasminogen, which has specific effect on proliferating vesicular endothelial cells. Therefore, it will generally not cause immune response, as well as side effects such as loss of weight, reduced activities, loss of appetite and conditioned pathogen infection under long-term injection (Sim BKL, O'Reilly MS, et al. Cancer Res, 1997, 57: 1329-1334.). At the same time, it will not affect the liver and kidney function (Kirsch M, Strasser J, Allende R, et al. J Biol Chem, 1996, 271(46): 29461-29467). In contrast, most of commercially available chemotherapeutic drugs are targeted on tumor cells that are genetically instable and highly mutative, resulting in a high risk of resistance and thus decrease the efficacy. Until recently, the use of angiostatin in tumor treatment has shown promising results. For the above reasons, angiostatin is expected to become a main anti-cancer drug in 21st century.

Compared to traditional chemical drugs, polypeptide and protein drugs including angiostatin have the advantages of causing low toxicity/side effects or little drug resistance etc, and in order to increase bioavailability and decrease degradation *in vivo*, the protein drugs are usually administrated intravenously. However, the half-life of low molecular weight protein drugs is very short, not only because of the degradation, but also because of the quick elimination via kidney.

It has been shown that in blood, if the hydraulic radius of a protein is larger than that of albumin or if the molecular weight of a protein is greater than 66,000 Dalton, the protein can be reserved stably in circulation. However, protein of lower molecular weight will be quickly eliminated from the blood via glomeruli. As such, in order to

maintain the effective treatment concentration of low molecular weight proteins in blood, frequent injection or continuous infusion is required. Although treatment in such a way could achieve the therapeutic effects, it also causes many inconveniences and pains to the patients and increases the cost of treatment. Meanwhile, long term administration of some drugs may cause some side effects, for instance, immunological reactions.

As a protein drug, angiostatin also has the disadvantages of short half-life and high elimination rate *in vivo*. The purpose of the present invention is to improve metabolism characteristics of angiostatin, which would result in higher stability, longer half-life, and even better efficacy *in vivo*.

Using macromolecular polymer to modify proteins is a common method to change and control the dynamic characteristics of drugs such as half-life, immunological characteristics, and toxicological characteristics. The macromolecular polymer employed to modify proteins should have desirable water-solubility and biocompatibility, little or no immunogenicity and so forth. Polyethylene glycol (PEG) is a common protein-modified molecule. It is amphipathic, and can be dissolved not only in water, but also in most of the organic solvents. Since polyethylene glycol is non-toxic, non-immunogenic, and highly soluble in water, it has been approved as a macromolecular polymer for drug preparation by drug administrations in many countries including SFDA of China as well as FDA of the US. Coupling the protein with macromolecular polymers, for example, polyethylene glycol can increase the *in vivo* stability and decrease nonspecific adsorption and antigenicity of the protein. Once the molecular weight of the conjugate reaches a certain value, the eliminating rate by kidney can be reduced effectively, which is an effective measure to prolong the half-life of the protein drug *in vivo* (Frokjaer S. et al. Nat. Rev. Drug Discov. 2005 Apr 4(4): 298-306; Harris JM. et al. Nat. Rev. Drug Discov. 2003 Mar 2(3): 214-21). The amino groups used as the reaction site in the modification of PEG were mainly α -amino group of N-terminus of the protein and ϵ -amino group on the side chain of the lysine residue. The result of the reaction is the coupling of one protein molecule with one or multiple PEG molecules. The modification of ϵ -amino group on the side-chain of the lysine residue may result in modified isomers due to the non-specific reaction sites.

Recently, taking advantage of the difference of isoelectric points between α -amino group of N-terminus of the protein and ϵ -amino group on the side chain of

the lysine residue, polyethylene glycol modifying agents have been developed which specifically modify N-terminus of the protein and as a result, it is possible to obtain uniform modification products with modification at the identical site. Another protein site for modification with polyethylene glycol is the mercapto group of the cysteine residue. Generally, the number of the mercapto groups is fewer than that of the amino groups in a protein, and thus modification of the mercapto groups is more specific. Using genetic engineering techniques, now it is possible to introduce a cysteine at any position of a protein to serve as a specific modification site. However, introducing a cysteine as the modification site also has certain limitations because, for those proteins that already contain cysteine residues, this may cause a mispairing of disulfide bonds or make it difficult for such proteins to refold. In addition, the carboxyl group of the protein is also frequently used as a site for modification (Veronese FM et al. *Drug Discov. Today*. 2005 Nov 1;10(21):1451-8.). Modification techniques with polyethylene glycol have been successfully used in a plurality of protein drugs, including PEG-asparaginase (Graham ML *Adv. drug Deliv. Rev.* 55, 1293–1302 Avramis Vassilios I. et al. WO9939732 and US6689762), PEG-adenosine deaminase (Levy Y et al. *J. Pediatr.* 113, 312–317; Davis S et al. *ClinExp. Immunol.* 46: 649-652; Hershfield MS et al. *N Engl J Med* 316 : 589-596), PEG-interferons such as PEG-interferon $\alpha 2a$ and PEG-interferon $\alpha 2b$ etc. (Bailon P et al. *C. Bioconjug. Chem.* 12, 195–202, Wang YS et al. *Adv. Drug Deliv. Rev.* 54, 547–570, Meng Xiantai et al. WO2005077421, Van Vlasselaer Peter et al. WO2004076474, Ballon Pascal Sebastlan et al. US2004030101, Karasiewicz Robert et al. EP0593868) etc. The purpose of this lies in obtaining fragments of angiostatin or complex containing angiostatin with prolonged half-life and higher biological activities.

Other representative high molecular modifying agents include glucan, polysucrose, starch, polyalanine, copolymer of ethanedioic acid and the malonic acid, carboxymethyl cellulose, polyvinylpyrrolidone, poly 1,3-dioxolane, copolymer of ethylene and maleic hydrazide, poly sialic acid, cyclodextrin, etc.

Another method of prolonging the half-life of a protein drug is to couple or fuse it with a blood protein or a fragment thereof used as a carrier to increase the molecular weight of the protein drug. For example, Fc fragment of immunoglobulin may be coupled with the target protein to prolong the half-life of the latter. For example, this strategy has been used in the Notch 1 receptor protein (Kitajewsky Jan et al. WO2005111072), erythropoietin (EPO) (Gillies Stephen D et al. WO2005063808),

human somatropin (Kim Young Min et al. WO2005047337), etc. The plasma albumin is another commonly-used coupling carrier, which is used in the proteins such as some antibiotics, anti-inflammatory agents, and anti-oxidants (Ezrin Alan M et al. WO0117568, Otagiri Masaki et al. EP1571212), etc.

Sustained release formulation is another approach of prolonging the *in vivo* half-life of a protein drug. The protein drug is placed in a pharmaceutical carrier to allow the protein to be released from the carrier slowly, and thereby a stable *in vivo* drug concentration is maintained. The commonly used sustained release formulations comprise hydrogel, microcapsule, microballoon, micro-osmotic pump, liposome etc. (Peppas NA et al. Eur. J. Pharm. Biopharm. 50, 27–46 (2000); Packhaeuser CB et al. Eur. J. Pharm. Biopharm. 58, 445–455 (2004); Metselaar JM et al. Mini Rev. Med. Chem. 4, 319–329 (2002)). Liposome is an ultramicroscopic particle in the form of a hollow sphere with a bilayer membrane structure. The bilayer membrane is composed of amphipathic molecules, most of which are phosphatides, and forms a hydrophilic inner chamber. The hydrophilic protein drug is encapsulated in the inner chamber of the liposome and thus can be slowly released *in vivo* to maintain the concentration of the protein drug and prolong the half-life. Examples are nerve growth factor (Hou Xinpu et al. CN1616087) and hemoglobin (Farmer Martha C et al. US4911929) etc.

Summary

"K1", "K2", "K3", "K5", "K1-2", "K1-3", "K1-5", "K2-3", "K2-5", "K3-5" and "kringles1-3" mentioned in this invention, unless indicated otherwise, are fragments of plasminogen, and the sequence of human plasminogen is shown in SEQ ID NO:7 (refer to U.S. Patent NO:7, 157,556 and WO 02/26782 A2). "K1-3" here is the abbreviation of "Kringles1-3". Unless otherwise indicated, PEG is polyethylene glycol, preferably monomethyl polyethylene glycol (mPEG).

Another aspect of the invention is that, the inventor surprisingly discovered that a conjugate formed by modifying agents and angiostatin or its fragments has prolonged *in vivo* half life as compared to the unmodified angiostatin or its fragments. The agents used for modification are selected from macromolecular polymers, protein molecules or its fragments, peptides, small molecules or chemicals of other forms. According to this invention, the conjugate not only maintains its original activity of inhibiting angiogenesis, but also exhibits higher biological activity and longer *in vivo* half life. Moreover, it does not exert toxic side effects on cells and animals, and even

for long time use it does not result in side effects such as resistance. In addition, Henkin et al. reports that the conjugate formed by PEG and K5 maintains the original biological activity of K5, and its half life increases to 5.53h (WO 02/26782 A2). Surprisingly, the half-life of the conjugate formed by PEG and K1-3 mentioned in this invention increases to 81.5h, much higher than that of the conjugate formed by PEG and K5 (refer to the example 4). For clinical application purpose, a longer delivery interval or a smaller dose of the drug is of great importance to patients, whether out of economic or mental health consideration.

In a preferred embodiment, the angiostatin of the present invention is of human, mice or other mammal origin. Preferably, the angiostatin is human angiostatin.

In another preferred embodiment, the angiostatin of the present invention is recombinant angiostatin. More preferably, the angiostatin is human recombinant angiostatin.

In one preferred embodiment, the fragments of angiostatin are selected from angiostatin K1, K2, K3, K5, K1-2, K1-3, K1-5, K2-3, K2-5 and K3-5. More preferably, the fragments of angiostatin mentioned here is angiostatin K1 having the sequence shown in SEQ ID NO:3, angiostatin K2 having the sequence shown in SEQ ID NO:4 or angiostatin K3 having the sequence shown in SEQ ID NO:5. Most preferably, the fragments of angiostatin mentioned here is angiostatin K1-3 having the sequence shown in SEQ ID NO:1. When the angiostatin mentioned is recombinant angiostatin K1-3 expressed by *E.coli*, it has the sequence shown in SEQ ID NO:2, and the N-terminal Met is randomly deleted when expressed in *E.coli*.

In one preferred embodiment, the angiostatin or its fragments of the present invention also includes the biologically active mutant, derivative, isomer or the combination thereof. In one preferred embodiment, the derivative of angiostatin or its fragment is formed by adding a peptide of 1-15 amino acids to the N or C terminal of the angiostatin or its fragments. In a further preferred embodiment, the derivative of angiostatin or its fragments is formed by adding a 7-amino-acid peptide MHHHHHHH containing a His-tag to the N-terminal of human angiostatin. This derivative has the sequence shown in SEQ ID NO:6 and its N-terminal Met are randomly deleted when expressed in *E.coli*. In another preferred embodiment, the derivative of angiostatin or its fragments is formed by adding the peptide MGGSHHHHHH or MGGSHHHHHHH to the C terminal of angiostatin, and its N-terminal Met is randomly deleted when expressed in *E.coli*.

In one of the embodiments of this invention, the modifying agents in the conjugate of the present invention are linked to angiostatin or its fragments by covalent bonds. In another embodiment of this invention, the modifying agents in the conjugate are linked to angiostatin or its fragments by non-covalent bonds. Preferably, the modifying agents are linked to angiostatin or its fragments through chemical coupling or fusion and expression. The macromolecular polymers as mentioned above can be biologically active or not. The suitable macromolecular polymers include, but not limited to, polyenol compounds, polyether compounds, polyvinylpyrrolidone, poly amino acids, copolymer of divinyl ether and maleic anhydride, N-(2-Hydroxypropyl)-methacrylamide, dextran and its derivatives like dextran sulfate, cellulose and its derivatives (including methyl-cellulose and carboxymethyl cellulose), amylum and its derivatives, poly-sucrose, polyethoxypolyol, heparin or its fragment, polyalkylene glycol and its derivatives, copolymers of polyalkylene glycol and its derivatives, poly(vinyl ethyl ether), a,p-poly((2-hydroxyethyl)-DL-aspartamide, polycarboxylates, poly oxyethylene-oxymethylenes, polyacryloyl morpholines, copolymer of amino compounds and oxyalkylene, poly hyaluronic acid, polyoxiranes, copolymer of oxalic acid and malonic acid, Poly 1,3 - methylene glycol ether, copolymer of ethylene and maleic hydrazide, poly sialic acid, cyclodextrin and etc.

The polyethers as mentioned above include, but are not limited to, poly alkylene glycol ($\text{HO}((\text{CH}_2)_x\text{O})_n\text{H}$), polypropylene glycol, polyoxyethylene ($\text{HO}((\text{CH}_2)_2\text{O})_n\text{H}$), polyvinyl alcohol ($((\text{CH}_2\text{CHOH})_n)$) and derivatives thereof. The polyethylene alcohols as mentioned above include, but are not limited to, polyethylene glycol (monomethyl polyethylene glycol, hydroxypolyethylene glycol), polyvinyl alcohol, polypropylene alcohol, polybutene alcohol and their derivatives. In a preferred embodiment, the modifying agent is polyethylene glycol. In a more preferred embodiment, polyethylene glycol is monomethyl polyethylene glycol.

The polyethylene glycol (PEG) of present invention is either linear or branched. Preferably, the average molecular weight of the PEG used herein is between 1,000 to 100,000 Daltons. More preferably, the molecular weight of PEG used herein is between 5,000 to 40,000 Daltons. Most preferably, the PEG used herein is 20 kDa monomethyl polyethylene glycol.

In one embodiment of the present invention, the polyethylene glycol (PEG)-modified angiostatin is characterized in that one angiostatin molecule is covalently coupled to one or more PEG molecules. Preferably, the modification is site

specific and will not influence the bioactivity of angiostatin or its fragment. This modified product maintains the antiangiogenic activity and shows higher metabolic stability and longer half-life in blood than the unmodified angiostatin, and therefore it can be used as an antiangiogenic or anti-tumor conjugate. Preferably, the coupling site of angiostatin may be at the N-terminal α -amino group, the ϵ -amino group on the side chain of a lysine residue, the mercapto group of on the side chain of a cysteine residue, the carboxyl group on the side chain of an aspartate residue, the carboxyl group on the side chain of a glutamate residue, or any combination thereof. More preferably, the coupling site of angiostatin or its fragment used herein is selected from the N-terminal α -amino group of said angiostatin fragment K1-3 or the ϵ -amino group on the side chain of a lysine residue corresponding to the 2nd, 7th, 15th, 17th, 24th, 69th, 94th, 97th, 121st, 125th, 128th, 129th, 150th, 175th, 215th, 228th, 246th lysine residue as indicated in SEQ ID NO:1 or any combination thereof. More preferably, one angiostatin molecule or its fragment is coupled with one polyethylene glycol molecule at the N-terminal α -amino group of said angiostatin molecule. More preferably, in the conjugate of the present invention, one recombinant angiostatin fragment K1-3 shown as SEQ ID NO:2 is coupled with one polyethylene glycol molecule. The coupling site is the N-terminal α -amino group of the angiostatin fragment K1-3. Most preferably, in the conjugate of the present invention, one recombinant angiostatin fragment K1-3 shown as SEQ ID NO:2 is coupled with one 20 kDa monomethyl polyethylene glycol. The coupling site is the N-terminal α -amino group of the angiostatin fragment K1-3. In a preferred embodiment, the present invention uses PEG to modify the N-terminal α -amino group of K1-3 and the product obtained has inhibitory activities on the proliferation, migration of vascular endothelial cells, the proliferation of tumor cells in mice, and the biological activity of the tumor growth in mice.

In one embodiment of the present invention, the conjugate is one angiostatin molecule covalently coupled to one or more polyethylene glycol molecules at the mercapto groups on the side chains of additional cysteine residues of said angiostatin molecule, wherein the additional cysteine residues are introduced by adding cysteines or peptide chains containing cysteines to the N-terminus, C-terminus, or the internal region of said angiostatin molecule.

According to the present invention, the poly amino acids include, but not limited to, polymers of one type of amino acid, copolymers of two or more types of amino acids, for example, polyalanine.

According to the present invention, preferred protein molecule used as a modifying carrier is a protein naturally occurring or a fragment thereof, which includes, but not limited to, thyroxine-binding protein, transthyretin, transferrin, fibrinogen, immunoglobulin, albumin and fragments thereof. Preferably carrier proteins are human proteins. A fragment of a protein as mentioned above refers to a part of the protein that keeps the function of a carrier protein. In one embodiment of the present invention, angiostatin or its fragment is coupled to albumin, characterized in that one angiostatin molecule or its fragment is linked to one or more albumin molecules. The conjugate may be obtained via chemical modification, fusion expression, or other methods. Preferably, the albumin is human serum albumin or its fragment. In another preferred embodiment, the conjugate is one angiostatin molecule or its fragment linked to Fc fragment of human immunoglobulin IgG. It is characterized in that one angiostatin molecule is covalently coupled to one or more Fc fragment of human immunoglobulin IgG. The conjugate may be obtained either via chemical modification, or via fusion expression, or via other methods. Preferably, the Fc fragment is a fragment of the Fc region of human immunoglobulin IgG.

The modifying agent in the present invention also includes a small molecule or a small peptide or other compound. In one embodiment of the present invention, the conjugate is an angiostatin molecule or its fragment modified by a small molecule or a small peptide or other compound. It is characterized in that the product has the activity to react with or bind to other molecules or component of the body. The reactivity mentioned can be of a reactively active group, capable of reacting with amino, carboxyl or mercapto group to form a covalent bond. In another specific embodiment, the active group of the product is maleimide, which is capable of reacting with a mercapto group of a blood component, such as albumin. In a preferred embodiment, the conjugate is the product of the modification of an angiostatin molecule or its fragment by glycosylation, phosphorylation or acylation. The modification sites may be amino acid residues originally contained the protein or amino acid residues generated via mutation. Although the molecular weight of the conjugate is not very large, the half-life of the conjugate becomes longer since the modification changes the characteristics of K1-3.

Angiostatin or its fragment conjugates is directly or indirectly linked to the modifying agent via a covalent bond. Direct linking means that an amino acid of angiostatin or its fragment is directly linked to an amino acid of the carrier protein, via

peptide bond or a disulfide bridge. Indirect linking means that angiostatin or its fragment conjugates is linked to the carrier protein via one or more chemical groups. In another embodiment of the present invention, the conjugate comprising angiostatin or its fragment is a conjugate formed via non-covalent interaction between angiostatin or its fragment and a modifying agent such as protein and small molecule.

Another embodiment of the present invention relates to a sustained-release formulation comprising angiostatin or its fragment, preferably fragment K1-3, or an angiostatin containing conjugate and a bio-compatible substance. The sustained-release formulation may be in a form selected from the group consisting of microcapsule, hydrogel, microsphere, micro-osmotic pump or liposome.

The present invention also provides a pharmaceutical composition comprising a conjugate or sustained-release formulation of the present invention and a pharmaceutically acceptable carrier, wherein the conjugate is formed by a modifying agent and angiostatin or its fragment, preferably angiostatin fragment K1-3. The pharmaceutically acceptable carriers used in this invention include carriers, excipients, or stabilizers, which are nontoxic to the cells or mammals to be contacted at selected doses and concentrations. A commonly used physiologically acceptable carrier is an aqueous pH buffer solution. Examples of physiologically acceptable carriers include solutions such as phosphate buffer solution, citrate buffer solution and other organic acid buffer solution; anti-oxidants including ascorbic acid; polypeptides with low molecular weight (no more than 10 residues); proteins such as serum albumin, glutin or immunoglobulin; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharide, disaccharide and other carbohydrates including glucose, mannose or dextrin; chelating agents such as EDTA; sugar alcohol such as mannitol or sorbitol; salt-forming counter ion such as sodium ion; and/or nonionic surfactant such as TWEEN®, PEG and PLURONICS®. Preferably, the excipients are sterilized and usually free of undesired substance. These compositions can be sterilized by routine sterilizing techniques.

The present invention also provides kits comprising the conjugate, composition or sustained-release formulation of the present invention and instruction for the use thereof. The complex is composed of a modifying agent and angiostatin or its fragment, preferably PEGylated angiostatin fragment K1-3.

The present invention also relates to a method of preparing the conjugate of the present invention, which is formed by a modifying agent and angiostatin or its

fragment, preferably fragment K1-3. Specifically, the present invention provides a method of preparing a PEGylated angiostatin or the fragment thereof comprising mixing the activated PEG with angiostatin or its fragment, allowing the reaction under proper conditions including solution, temperature, pH, and reaction molar ratio, and purifying the coupled product by using cation-exchange column or molecular sieve. Preferably, the pH of this reaction is within 3 to 10. Preferably pH 5 to 7, the molar ratio of PEG and angiostatin is 1:1 to 10:1.

The present invention also provides the use of the aforementioned angiostatin-containing conjugate, formed by a modifying agent and angiostatin or its fragment preferably fragment K1-3, the sustained-release formulation, the pharmaceutical composition or the kit of the present invention in the prevention, diagnosis and treatment of tumor. The tumors that are suitable to be treated with the method of the invention include, but not limited to, lung cancer, neuroendocrine tumor, colon cancer, bone cancer, liver cancer, gastric cancer, pancreatic cancer, oral cancer, breast cancer, prostate cancer, lymphoma, esophagus cancer, nasopharyngeal carcinoma, cervical cancer, sarcoma, renal carcinoma, biliary cancer, malignant melanoma, et al. The present invention also provides the use of the aforementioned conjugate, sustained-release formulation or pharmaceutical composition comprising the angiostatin or its fragment preferably K1-3, in the preparation of a medicament for the treatment of aforementioned tumors. The administration route is selected from the group consisting of intravenous injection, intravenous infusion, subcutaneous injection, intramuscular injection, intraperitoneal injection, percutaneous absorption, subcutaneous embedment, liver arterial injection, oral administration, nasal administration, oral mucosa administration, ocular administration, rectal administration, vaginal delivery, or other clinically acceptable routes. The administration time is every day to every 21 days, preferably every day to every week.

The present invention also provides the use of the aforementioned conjugate, sustained-release formulation, pharmaceutical composition, or kit for the prevention, diagnosis, or treatment of a non-cancerous disease characterized by, but not limited to, tissue or organ pathological changes caused by abnormal neovascularization, or in preparation of a medicament for the treatment of such a disease, wherein the conjugate is formed by a modifying agent and angiostatin or its fragment, preferably said conjugate is angiostatin fragment K1-3 modified by PEG. According to the present invention, the administration route is selected from the group consisting of

intravenous injection, intravenous infusion, subcutaneous injection, intramuscular injection, intraperitoneal injection, percutaneous absorption, subcutaneous embedment, liver arterial injection, oral administration, nasal administration, oral mucosa administration, ocular administration, rectal administration, vaginal delivery, or other clinically acceptable routes. The administration frequency is selected from daily to every 21 days, preferably daily to weekly.

The present invention also provides a method for prolonging the half-life of angiostatin or its fragments, preferably fragment K1-3, comprising the step of forming a conjugate between angiostatin or its fragments and a modifying agent capable of prolonging in vivo half-life of angiostatin and optionally the step of preparing a sustained-release formulation containing the aforementioned conjugate and biocompatible substances. Our experimental data show that, a product of the preferred embodiment in the present invention, i.e., a conjugate formed by coupling PEG to the N-terminus of K1-3, has the activity of inhibiting the migration of endothelial cell and tumor growth in mice. This conjugate showed significantly higher activity as compared with non-modified K1-3. Furthermore, pharmacokinetic study revealed that the in vivo metabolism of this modified product was effectively slowed down, and its in vivo half-life was prolonged.

Unless otherwise specified, the angiostatin or its fragments as used herein can be a wild-type angiostatin, i.e., the naturally occurring form; or a biologically active mutant, fragment, isomer, or derivative thereof, or their combinations. Angiostatin or its fragments can be obtained upon but not limited to expression in animal cells, or can be obtained by fermentation and further purification from yeasts or *E. coli*. The amino acid sequence of the wild-type human angiostatin fragment K1-3 expressed in animal cells or yeasts is shown in SEQ ID NO.1. The recombinant human angiostatin or its fragments expressed in animal cells or yeasts may either have an amino acid sequence as shown in SEQ ID NO.1, or have the sequence of SEQ ID NO.1 with no more than 10 amino acids added to or deleted from its N-terminus. The amino acid sequence of the recombinant human K1-3 expressed in *E. coli* is shown in SEQ ID NO.2, but sometimes the N-terminal Met will be deleted after expression.

A mutant of K1-3 refers to a K1-3 molecule obtained by introducing amino acid replacement, deletion, or addition. The fragment of K1-3 refers to a part of the sequence of SEQ ID NO.1 or SEQ ID NO.2. The fragment may be obtained through, among others, enzymatic cleavage, genetic engineering expression, or polypeptide

synthesis. Preferably, the mutant have a 60% amino acid sequence homology to SEQ ID NO:1, or 70% amino acid sequence homology, more desirably 80% or even 90% amino acid sequence homology, especially 95% amino acid sequence homology. A fragment refers to a part of the sequence of SEQ ID NO:1. For instance, the fragment with the sequence of SEQ ID NO:3, SEQ ID NO:4, or SEQ ID NO:5 was reported to have the activity to inhibit endothelial cell proliferation (Cattaneo MG et al. THE JOURNAL OF BIOLOGICAL CHEMISTRY. Vol. 271, No. 46, Issue of November 15, pp. 29461 –29467, 1996). An isomer of K1-3 refers to a molecule with the same amino acid sequence or chemical formula as SEQ ID NO:1 but with different conformations, including difference in the secondary or tertiary protein structure, or changed optical activity in regional amino acids. The isomer may be a naturally occurring mutant or obtained via artificial design. A derivative of K1-3 refers to a product generated by modification of the SEQ ID NO:1. Modification refers to covalently attaching one or more small molecules, such as phosphoric acid molecules or carbohydrate molecules, or oligopeptides with less than 30 amino acids, to the protein at any amino acid of the protein. The combination of the active mutants, fragments, isomers or derivatives of K1-3 means that the product may simultaneously have two or more of the modifications mentioned above, for example without limitation, a mutant of a fragment or a modified mutant, et al.

Brief description of the drawings

FIG. 1 depicts the amino acid sequence of angiostatin fragments K1-3 (SEQ ID NO:1)

FIG. 2 depicts the amino acid sequence of angiostatin fragments K1-3 (SEQ ID NO:2) expressed in a strain of *E.coli*. The Met residue in the N-terminal may sometimes be deleted after expression.

FIG. 3 depicts the amino acid sequence of angiostatin k 1 (SEQ ID NO:3).

FIG. 4 depicts the amino acid sequence of angiostatin k 2 (SEQ ID NO:4).

FIG. 5 depicts the amino acid sequence of angiostatin k 3 (SEQ ID NO:5).

FIG. 6 depicts a biologically active fragment of K1-3 with additional amino acids at the N-terminus (SEQ ID NO: 6). N-terminal Met may sometimes be partially deleted after expression.

FIG. 7 shows the coupling of PEG with the N-terminus of K1-3 having the sequence of SEQ ID NO:2 at 4□ for 10h. The post-modification solutions are

subjected to SDS-PAGE detection. Lane 1, protein marker; lane 2, K1-3; lanes 3~8, K1-3 modified with PEG in a 1:5 molar ratio (K1-3:PEG) at pH 5.0, 5.5, 6.0, 6.5, 7.0, 8.0 respectively; and lanes 9-12: K1-3 modified with 20kDa PEG in a molar ratio (K1-3:PEG) of 1:1, 1:2, 1:5 and 1:10 respectively at pH 5.5.

FIG. 8 shows the coupling of PEG with the N-terminus of K1-3 having the sequence of SEQ ID NO:2 at room temperature for 10h. The post-modification solutions are subjected to SDS-PAGE detection. Lane 1, protein marker; lane 2, K1-3; lane 3~8, K1-3 modified with PEG in a 1:5 molar ratio at the pH of 5.0, 5.5, 6.0, 6.5, 7.0, 8.0 respectively; and lane 9~12, K1-3 modified with PEG in a molar ratio (K1-3:PEG) of 1:1, 1:2, 1:5 and 1:10 respectively at pH 5.5.

FIG. 9 shows the coupling of PEG to the N-terminus of K1-3 having the sequence of SEQ ID NO:2. The pre- and post-modification solutions are subjected to SDS-PAGE detection. Lane 1, protein marker; lane 2, K1-3; and lane 3, K1-3 modified with PEG.

FIG. 10 shows the cation-exchange chromatography SP purification of K1-3 having the sequence of SEQ ID NO:2 modified at the N-terminus with PEG. The protein are bound to the cation-exchange column SP, eluted with solution of a sodium chloride gradient, and subjected to reduced SDS-PAGE detection. Lane 1, protein marker; lane 2, before purification; lane 3, flow through; lane 4-13: collected elution fractions.

FIG. 11 shows the result of the *in vivo* metabolism experiment of the coupling product of PEG to the N-terminus of K1-3. K1-3: K1-3 having the sequence SEQ ID NO:2; PEG-K1-3: K1-3 having the sequence of SEQ ID NO:2 which is modified with PEG.

FIG. 12 shows the activity of the coupling product of PEG to the N-terminus of K1-3 of inhibiting the migration of human umbilical vein endothelium cells (HUVECs). K1-3: K1-3 having the sequence SEQ ID NO:2; PEG-K1-3: K1-3 having the sequence of SEQ ID NO:2 which is modified with PEG.

FIG. 13 shows the activity of the coupling product of PEG to the N-terminus of K1-3 of inhibiting the self-regeneration of HUVECs. K1-3: K1-3 having the sequence SEQ ID NO:2; PEG-K1-3: K1-3 having the sequence of SEQ ID NO:2 which is modified with PEG.

FIG. 14 shows the activity of the coupling product of PEG to the N-terminus of K1-3 of inhibiting the metastasis of HUVECs. K1-3: K1-3 having the sequence SEQ

ID NO:2; PEG-K1-3: K1-3 having the sequence of SEQ ID NO:2 which is modified with PEG.

FIG. 15 shows the activity of the coupling product of PEG to the N-terminus of K1-3 of inhibiting the metastasis of human dermal microvascular endothelial cells (HMECs). K1-3: K1-3 having the sequence SEQ ID NO:2; PEG-K1-3: K1-3 having the sequence of SEQ ID NO:2 which is modified with PEG.

FIG. 16 shows the activity of the coupling product of PEG to the N-terminus of K1-3 of inhibiting the metastasis of tumor cells (Hela). K1-3: K1-3 having the sequence SEQ ID NO:2; PEG-K1-3: K1-3 having the sequence of SEQ ID NO:2 which is modified with PEG.

FIG. 17 shows the activity of the coupling product of PEG to the N-terminus of K1-3 of inhibiting mouse melanoma cells (B16). K1-3-4.5-1: K1-3 having the sequence of SEQ ID NO:2, 4.5mg/kg body weight, administered daily; PEG-, K1-3-1.5-1: K1-3 having the sequence of SEQ ID NO:2 which is modified with PEG, 1.5mg/kg body weight, administered daily; PEG- K1-3-4.5-1 K1-3 having the sequence of SEQ ID NO:2 which is modified with PEG, 4.5mg/kg body weight, administered daily; PEG- K1-3-4.5-3 K1-3 having the sequence of SEQ ID NO:2 which is modified with PEG, 4.5mg/kg body weight, administered every 3 days; PEG- K1-3-4.5-3 K1-6 comprising the sequence of SEQ ID NO:2 which is modified with PEG, 4.5mg/kg body weight, administered every 6 days; PEG- K1-3-4.5-3 K1-9 comprising the sequence of SEQ ID NO:2 which is modified with PEG, 4.5mg/kg body weight, administered every 9 days.

FIG. 18 shows the activity of the coupling product of PEG to the N-terminus of K1-3 of inhibiting mouse liver cancer cells (H22). K1-3-4.5-1: K1-3 having the sequence of SEQ ID NO:2, 4.5mg/kg body weight, administered daily; PEG-, K1-3-1.5-1: K1-3 having the sequence of SEQ ID NO:2 which is modified with PEG, 1.5mg/kg body weight, administered daily; PEG- K1-3-4.5-1 K1-3 having the sequence of SEQ ID NO:2 which is modified with PEG, 4.5mg/kg body weight, administered daily; PEG- K1-3-4.5-3 K1-3 having the sequence of SEQ ID NO:2 which is modified with PEG, 4.5mg/kg body weight, administered every 3 days; PEG- K1-3-4.5-3 K1-7 comprising the sequence of SEQ ID NO:2 which is modified with PEG, 4.5mg/kg body weight, administered every week.

Examples

To further demonstrate the present invention, the following examples are provided. These examples are provided in order to set illustration but not specific limitation to the present invention. The technicians in this field are aware of the fact that this present invention includes the technical solutions derived from those in the following examples without any innovative effort.

Unless otherwise specified, the PEG as used herein is 082M0P01 MPEG-BUTYRALD-20K, NETKTARTM, Lot#: PT-03G-05.

Example 1: Coupling of PEG to the N-terminus of K1-3 under different conditions.

The K1-3 (Protgen Ltd., China) used in this example has the sequence of SEQ ID NO.2. Specifically, the K1-3 was dialyzed into 30 mM sodium acetate solution (Sino Pharm Chemical Reagent Co., Ltd, China), pH 5.5. Protein concentration was determined by measuring absorbance at 280 nm using a UV spectrophotometer (8453, Agilent Technologies), and then was adjusted to 1 mg/ml. PEG which specifically modifies the N-terminus of protein was used to couple with K1-3 covalently. In one experiment, 20 kDa PEG (solid) was added to the K1-3 containing solution obtained after dialysis (containing 10 mg protein) at molar ratios (PEG:K1-3) of 1:1, 2:1, 5:1, 10:1, and the mixed solution was stirred at room temperature until PEG solid dissolved completely. CH₃BNNa (Sigma) was added as reductant to reach a final concentration of 20 mM, and finally the pH value of the solution was adjusted to 5.5. In another experiment, 20 kDa PEG (solid) was added to the K1-3 containing solution obtained after dialysis (containing 10 mg protein) at a molar ratio (PEG:K1-3) of 5:1, and the mixed solution was stirred at room temperature until PEG solid dissolved completely. CH₃BNNa (Sigma) was added as reductant to reach a final concentration of 20 mM, and finally the pH value of the solution was adjusted to 5.0, 5.5, 6.0, 6.5, 7.0, 8.0. After resting at 4°C for different time courses, the following result was observed: one PEG molecule was covalently linked to one K1-3 molecule at N-terminal α -amino group of K1-3. Only a small amount of K1-3 was non-specifically modified at multiple sites. The optimal condition for the interaction between PEG and K1-3 is thereby determined. Electrophoresis analysis results of the solutions before and after the interaction are shown in Figures 7-8.

Example 2: Coupling of PEG to the N-terminus of K1-3.

The K1-3 used in this example has the sequence of SEQ ID NO.2. Specifically,

the K1-3 was dialyzed into 30 mM sodium acetate solution, pH 5.5. Protein concentration was determined by measuring absorbance at 280 nm using a UV spectrophotometer, and then was adjusted to 1 mg/ml. PEG which specifically modifies the N-terminus of protein was used for covalently coupling with the K1-3. Specifically, 100 mg 20 kDa PEG (solid) was added to 20 ml solution obtained after dialysis (containing 10 mg protein) at a molar ratio (PEG:K1-3) of 5:1, and the mixed solution was stirred at room temperature until PEG solid dissolved completely. CH₃BNNa (Sigma) was added as reductant to reach a final concentration of 20 mM, and finally the pH value of the solution was adjusted to 5.5. After resting at room temperature for 6 hours, more than 80% of K1-3 was modified with PEG at a single site, which means one PEG molecule was covalently linked to one K1-3 molecule at N-terminal α -amino group of K1-3. Only a small amount of K1-3 was non-specifically modified at multiple sites. The reaction solution can be purified directly through chromatography using a column. Electrophoresis analysis results of the solutions before and after the reaction are shown in Figure 9.

Example 3: Purification of K1-3 modified with 20 kDa PEG at N-terminus through cation-exchange SP column

The K1-3 used in this example has the sequence of SEQ ID NO.2. Specifically, K1-3 modified with 20 kDa PEG was purified through SP chromatography column (Amersham). The pH value of the mixed solution obtained after the coupling reaction was adjusted to 5.0. The sample was loaded onto the column pre-equilibrated by a buffer containing 20 mM sodium acetate, pH 5.0. Then gradient elution was performed with buffer containing 20 mM sodium acetate, 1 M NaCl, pH 5.0. The peak of PEG which did not react with K1-3 appeared during penetration and washing since PEG is extremely low charged. The elution peaks appeared in the following order: multi-modified K1-3, mono-modified K1-3, and non-modified K1-3. Different fractions can be collected according to absorbance at 280 nm. The result of purification is shown in Figure 10.

Example 4: Prolonged efficacy of K1-3 with PEG coupled to its N-terminus in the blood.

The K1-3 used in this example has the sequence of SEQ ID NO.2. The efficacy of tK1-3 with PEG coupled to its N-terminus was evaluated by measuring the *in vivo*

half-life of K1-3 with or without PEG modification in Wistar rat (Vitalriver Experimental Animal Center), respectively. Two healthy Wistar rat (about 200 g) were injected intravenously with K1-3 and K1-3 modified with PEG, respectively, at a dose of 4.5 mg/kg body weight. Blood samples were taken from eye sockets of the rats at 0, 5, 10, 30 minutes, 1, 2, 4, 8, 16, 24, 36, 48, 72, 96, 120 and 144 hours. Plasma was then collected and the concentration of K1-3 with or without PEG modification was measured by sandwich ELISA, respectively. As revealed by the pharmacokinetic analysis, upon modification with PEG, the half-life of K1-3 was increased from 12.2 hours to 81.5 hours in rats. These results demonstrate that coupling of high molecular weight PEG to K1-3 can effectively increase the *in vivo* half-life of K1-3 and result in prolonged efficacy. Results are indicated in Figure 11.

Example 5: The activity of K1-3 with PEG coupled to its N-terminus of inhibiting the migration of Human Umbilical Vein Endothelial Cell (HUVEC).

The K1-3 used in this example has the sequence of SEQ ID NO.2. HUVECs (Protgen, Beijing) were cultured in the M199 medium (Hyclone) containing 20% fetal bovine serum (Hyclone) to exponential phase. After starved for 12 hours, cells were added into trans-well inserts (Millipore, USA) at the concentration of 10,000 cells per well for measurement of cell migration. Mediums for migration assay included M199 medium with 5% serum. For the treatment groups, K1-3 with or without modification were added to the cells at 0.004, 0.04, 0.4 and 4 $\mu\text{g/ml}$. After culturing for 8 hours at 37°C, the cells in the trans-well inserts were fixed with 1% glutaraldehyde (Beijing chemical plant, China). The upper layer of cells on the trans-well membranes, which did not migrate, were removed, and the lower layer of cells were stained with hematoxylin (Sino Pharm Chemical Reagent Co., Ltd, China). The migrated cell number was counted under microscope (Olympus). Cells in three different fields in one plate treatment were counted and then the inhibition rate was calculated. At the concentration of 0.04-4 $\mu\text{g/ml}$, the inhibitory effect of cell migration improves with the increase of protein concentration. The results revealed that the inhibition rates on cells migration were 43%, 68% and 76% for K1-3 with the sequence of SEQ ID NO.2, while 58%、65%、69% for modified K1-3. The above results demonstrate that K1-3 modified with PEG will maintain its biological activity. The experimental results are shown in Figure 12.

Example 6: The activity of K1-3 with PEG coupled to its N-terminus of inhibiting wound healing of Human Umbilical Vein Endothelial Cell (HUVEC).

The K1-3 used in this example has the sequence of SEQ ID NO.2. 2×10^5 /ml HUVEC were inoculated in 12-well plates (Corning) and cultured in the M199 medium (Hyclone) containing 20% fetal bovine serum (Hyclone) to exponential phase. After starved for 12 hours, crossed wounds were created in each well. Suspending cells were washed away. Experimental groups were as follows: negative control (PBS), positive control (K1-3 at the concentration of 0.04, 0.4 and 4 μ g/ml) and treatment group (K1-3 with PEG at the concentration of 0.04, 0.4 and 4 μ g/ml). After culturing for 16 hours at 37°C, the amounts of migrated cells were detected. At the concentration of 0.04-4 μ g/ml, the inhibitory effect of cell migration was improved with the increase of protein concentration. The results revealed that the inhibition rates on cells migration were 0, 20% and 25% for K1-3, while 35%, 40% and 105% for modified K1-3. The above results demonstrate that the modification of K1-3 with PEG will not only maintain but also enhance its biological activity. 4 μ g/ml K1-3 modified with PEG resulted in some cell crimple and death. The experimental results are shown in Figure 13.

Example 7: The activity of K1-3 with PEG coupled to its N-terminus of inhibiting proliferation of Human Umbilical Vein Endothelial Cells (HUVECs).

The K1-3 used in this example has the sequence of SEQ ID NO.2. HUVECs were starved overnight, inoculated in 96-well plates at the concentration of 2×10^4 /ml and cultured in the M199 medium (Hyclone) containing 20% fetal bovine serum (Hyclone) to exponential phase. After starved for 12 hours, cells were treated with K1-3 and K1-3 with PEG. Experimental groups were as follows: negative control (PBS), positive control (K1-3 at the concentration of 0.04, 0.4 and 4 μ g/ml) and treatment group (K1-3 with PEG at the concentration of 0.04, 0.4 and 4 μ g/ml). After culturing for 48 hours at 37 °C, cells were incubated with MTT (Amresco) for 4 hours at 37°C. Then DMSO (Shenggong, China) was added and absorption at 570 nm was measured by Microplate Reader (Model 550, Biorad). Results are indicated in Figure 14.

Example 8: The activity of K1-3 with PEG coupled to its N-terminus of inhibiting the proliferation of vascular endothelial cells

K1-3 used in this example is the K1-3 having the sequence of SEQ ID NO.2. Specifically, the starved human microvascular endothelial cells (HMEC) were added into 96-well plates at 20,000 cells per well, then cultured in the DMEM medium (Hyclone) containing 10% serum to exponential phase. After starving for 12 hours, cells were treated with different concentrations of modified or unmodified K1-3 for measurement of cell proliferation. Experimental groups were as follows: negative control group (PBS), positive control groups (0.04, 0.4, and 4 $\mu\text{g/ml}$ K1-3), and treatment group (0.04, 0.4, and 4 $\mu\text{g/ml}$ K1-3 with PEG coupled to its N-terminus). After culturing for 48 hours at 37 °C, the cells in the plates were treated with MTT for 4 h at 37 °C. Then DMSO was added, and the absorption was measured at 570 nm by microplate spectrophotometer. The experimental results are shown in Figure 15.

Example 9: The activity of K1-3 with PEG coupled to its N-terminus of inhibiting the proliferation of tumor cells (human cervical cancer, HeLa)

K1-3 used in this example is the K1-3 having the sequence of SEQ ID NO.2. Specifically, HeLa (ATCC# CCL-2, USA) were added into 96-well plates at 20,000 cells per well, then cultured in the DMEM medium (Hyclone) containing 10% serum to exponential phase. After starving for 12 hours, cells were treated with different concentrations of modified or unmodified K1-3 for measurement of cell proliferation. Experimental groups were as follows: negative control group (PBS), positive control groups (0.4, 4, 40, and 150 $\mu\text{g/ml}$ K1-3), and treatment group (0.4, 4, 40, and 150 $\mu\text{g/ml}$ K1-3 with PEG coupled to its N-terminus). After culturing for 48 hours at 37 °C, the cells in the plates were treated with MTT for 4 h at 37 °C. Then DMSO was added, and the absorption was measured at 570 nm by microplate spectrophotometer. The results revealed that K1-3 with PEG coupled to its N-terminus was more potent than unmodified K1-3 in the inhibition of the tumor cell proliferation. The results also showed that, compared with the concentrations of 0.4, 4, and 150 $\mu\text{g/ml}$, K1-3 and K1-3 with PEG coupled to its N-terminus showed the highest anti-proliferation effect on tumor cells at the concentration of 40 $\mu\text{g/ml}$. The experimental results are shown in Figure 16.

Example 10: Activity of K1-3 with PEG coupled to its N-terminus in the treatment of late-stage B16 melanoma mice tumor model.

K1-3 used in this example is K1-3 having the sequence of SEQ ID NO.2.

Experiments were performed to observe the *in vivo* inhibitory activity of K1-3 with PEG coupled to its N-terminus on late-stage B16 tumor. C57B/L mice of about 20 g were used in the experiment (Beijing Vital River Laboratory Animal Co. Ltd.). Mice were inoculated at back with B16 cells (ATCC# CRL-6475, USA), 1×10^6 cells per mouse. The mice were then randomly grouped after the tumor volume reaches 1 cm^3 , 8 mice per group, including the negative control group (sodium acetate), the positive control group (K1-3, 4.5 mg/kg body weight, administered daily), three treatment groups (K1-3 with PEG coupled to its N-terminus, 1.5 mg/kg body weight, administered daily, or 4.5 mg/kg body weight, administered daily, every 3 days, every 6 days, or every 9 days, respectively). Administration was performed by subcutaneous injection and lasted for 10 days. The mice were sacrificed on day 11, the weight of tumor was measured, and the efficacy was evaluated by tumor inhibition rate. For K1-3 having the sequence of SEQ ID NO.2 with PEG coupled to its N-terminus, the experimental results revealed that the tumor inhibition rate of the positive control group and the groups treated with 1.5 mg/kg body weight daily was 45% and 28%, respectively, whereas the tumor inhibition rates were 60%, 34%, 25%, and 24% for the groups treated with 4.5 mg/kg body weight, daily, every 3 days, every 6 days, or every 9 days, respectively. The results demonstrate that the anti-tumor activity of K1-3 with PEG coupled to its N-terminus is superior to that of the unmodified K1-3. Furthermore, it can maintain its superior efficacy even when administered at a low concentration. The experimental results are shown in Figure 17.

Example 11: Activity of K1-3 with PEG coupled to its N-terminus in the treatment of early-stage H22 hepatocellular carcinoma mice tumor model.

K1-3 used in this example is K1-3 having the sequence of SEQ ID NO.2. Experiments were performed to observe the *in vivo* inhibitory activity of K1-3 with PEG coupled to its N-terminus on early-stage H22 tumor. Balb/c mice of about 20 g were used in the experiment (Beijing Vital River Laboratory Animal Co. Ltd.). Mice were inoculated at back with H22 cells, 1×10^6 cells per mouse. The mice were then randomly grouped after the tumor volume reaches 1 cm^3 , 8 mice per group, including the negative control group (sodium acetate), the positive control group (K1-3, 4.5 mg/kg body weight, administered daily), three treatment groups (K1-3 with PEG coupled to its N-terminus, 1.5 mg/kg body weight, administered daily, or 4.5 mg/kg body weight, administered daily, twice per week, or weekly, respectively). The

administration was performed by subcutaneous injection and lasted for 3 weeks. The mice were sacrificed on day 22, the weight of tumor was measured, and the efficacy was evaluated by tumor inhibition rate. For K1-3 having the sequence of SEQ ID NO.2 with PEG coupled to its N-terminus, the experimental results revealed that the tumor inhibition rate of the positive control group and the groups treated with 1.5 mg/kg body weight daily was 51% and 54%, respectively, whereas the tumor inhibition rates were 60%, 84%, and 50% for the groups treated with 4.5 mg/kg body weight, daily, twice per week, and weekly, respectively. The results demonstrate that the anti-tumor activity of K1-3 with PEG coupled to its N-terminus is superior to that of the unmodified K1-3. Furthermore, the efficacy of K1-3 at a low concentration is superior in inhibiting the growth of liver cancer cells than that of K1-3 at a higher concentration. Since the *in vivo* half life of K1-3 with PEG coupled to its N-terminus are longer than that of unmodified K1-3 of the same dosage, the biological activity K1-3 with PEG coupled to its N-terminus is higher than that of the unmodified K1-3 of the same concentration when administration intervals are extended. Administrations at twice per week showed the highest anti-tumor activity, up to more than 80%. The experimental results are shown in Figure 18.

Example 12: The *in vivo* anti-tumor activity of a conjugate of PEG and K1-3 (PEG-K1-3) in NCI-H226 NSCLC xenograft model.

The recombinant protein K1-3 used in this example has a sequence of SEQ ID NO:2. Female Balb/c nu/nu mice aged 6-8 weeks of about 18-20g weight (institute of laboratory animal sciences, CAMS) were used in this experiment. Mice were subcutaneously inoculated on the right side with NCI-H226 cells (human pancreatic cancer cell line, derived from ATCC, ATCC number CRL-5826). When the tumor volume reached about 80-125 mm³, the animals were divided into 3 groups randomly (6 per group). The treatment was administered according to the following Table 1. The body weight and tumor volume of the animals were measured twice a week. Sacrifice the animals on day 28, collect the tumors and take photographs.

Table 1: Grouping of animals and the corresponding treatments.

Group	Sample size	drug name	dosage	drug volume	administration route	administration time
1	6	Vector	0 mg/kg	5 µl/g	i.v.	Twice a week * 3 weeks

2	6	PEG-K1-3	4.5 mg/kg	5 μ l/g	i.v.	Twice a week * 3 weeks
3	6	Taxol	20 mg/kg	10 μ l/g	i.v.	Twice a week * 3 weeks

Remark: Taxol was purchased from Beijing Union Pharmaceutical Factory. Lot number 060408.

The results revealed that, as is indicated in Fig 19A, on Day 22 and Day 25, the average body weight of the animals in the group treated with 4.5 mg/kg PEG-K1-3 (Group 2) is significantly different from that of the animals in the control group (Group 1). The body weights of Group 2 are significantly heavier than that of Group 1, single variant ANOVA, * $p < 0.05$. On Day 11 and Day 25, the average body weight of the animals in the group treated with 20 mg/kg taxol (Group 3) is significantly different from that of the animals in the control group. The average body weight of animals in Group 3 is significantly lower than that of the animals in Group 1, single variant ANOVA, * $p < 0.05$.

As is shown in Figure 19B, compared with the control group, tumor burden of those animals treated with 4.5 mg/kg PEG-K1-3 (Group 2) is significantly decreased ($p < 0.05$). Tumor burden of animals in Group 3 show significant difference in Day 11, Day 15, Day 18. Furthermore, in Day 4, Day 11, Day 18 and Day 25, the T/C ratios of Group 2 (4.5mg/kg PEG-K1-3 i.v. * 21 days) are 73%, 63%, 62% and 66%, respectively. While T/C ratios of Group 3 (20mg/kg Taxol) are 69%, 39%, 28% and 18%, respectively.

As is shown in Figure 19C, the tumor burden 21 days after treatment is calculated. Compared to the control group (Group 1), tumor inhibition rate in animals treated with 4.5 mg/kg PEG-K1-3 (Group 2) and animals treated with 20mg/kg Taxol (Group 3) are 17.18% and 84.43%, respectively. Tumor burden in animals treated with 4.5 mg/kg PEG-K1-3 and the animals in the control group show significant difference compared to animals treated with Taxol. ($P = 0.008$ and $P = 0.026$, respectively)

Sequence Listing

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<213> Homo sapiens

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Thr Cys Gln Lys Trp Ser Ser Thr Ser Pro His Arg Pro Arg Phe Ser
 35 40 45

Pro Ala Thr His Pro Ser Glu Gly Leu Glu Glu Asn Tyr Cys Arg Asn
 50 55 60

Pro Asp Asn Asp Pro Gln Gly Pro Trp Cys Tyr Thr Thr Asp Pro Glu

65	70	75	80
Lys Arg Tyr Asp Tyr Cys Asp Ile Leu Glu Cys Glu Glu Glu Cys Met			
	85	90	95
His Cys Ser Gly Glu Asn Tyr Asp Gly Lys Ile Ser Lys Thr Met Ser			
	100	105	110
Gly Leu Glu Cys Gln Ala Trp Asp Ser Gln Ser Pro His Ala His Gly			
	115	120	125
Tyr Ile Pro Ser Lys Phe Pro Asn Lys Asn Leu Lys Lys Asn Tyr Cys			
	130	135	140
Arg Asn Pro Asp Arg Glu Leu Arg Pro Trp Cys Phe Thr Thr Asp Pro			
	145	150	155
Asn Lys Arg Trp Glu Leu Cys Asp Ile Pro Arg Cys Thr Thr Pro Pro			
	165	170	175
Pro Ser Ser Gly Pro Thr Tyr Gln Cys Leu Lys Gly Thr Gly Glu Asn			
	180	185	190
Tyr Arg Gly Asn Val Ala Val Thr Val Ser Gly His Thr Cys Gln His			
	195	200	205
Trp Ser Ala Gln Thr Pro His Thr His Asn Arg Thr Pro Glu Asn Phe			
	210	215	220
Pro Cys Lys Asn Leu Asp Glu Asn Tyr Cys Arg Asn Pro Asp Gly Lys			
	225	230	235
Arg Ala Pro Trp Cys His Thr Thr Asn Ser Gln Val Arg Trp Glu Tyr			

245

250

255

Cys Lys Ile Pro Ser Cys Asp Ser Ser Pro
 260 265

<210> 7

<211> 715

<212> PRT

<213> Homo sapiens

<400> 7

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Ala Gly Gly Thr Lys Ser Ala Glu Glu Glu His Tyr Gly Tyr Ala Ser
 35 40 45

Ser Asn Val Ser Pro Ala Leu Pro Leu Pro Thr Ala His Ser Thr Leu
 50 55 60

Pro Ala Pro Cys His Asn Leu Gln Thr Ser Thr Pro Gly Ile Ile Pro
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Pro Ala Asp His Pro Ser Gly Tyr Gly Ala Ala Leu Asp Gly Gly Pro
 85 90 95

Ala Gly Tyr Phe Leu Ser Ser Gly His Thr Arg Pro Asp Gly Ala Pro
 100 105 110

Ala Leu Glu Ser Pro Arg Ile Glu Ile Thr Ser Cys Leu Gly Leu Tyr
 115 120 125

His Asn Asn Asn Gln Phe Phe His Asp Val Glu Val Glu Asp Val Leu
 130 135 140

Pro Ser Ser Lys Arg Ser Pro Ser Thr Ala Thr Leu Ser Leu Pro Ser
 145 150 155 160

Leu Glu Ala Tyr Arg Asp Pro Ser Cys Leu Ser Pro Ala Ser Ser Leu
 165 170 175

Ser Ser Arg Ser Cys Asn Ser Glu Ala Ser Ser Tyr Glu Ser Asn Tyr
 180 185 190

Ser Tyr Pro Tyr Ala Ser Pro Gln Thr Ser Pro Trp Gln Ser Pro Cys
 195 200 205

Val Ser Pro Lys Thr Thr Asp Pro Glu Glu Gly Phe Pro Arg Gly Leu
 210 215 220

Gly Ala Cys Thr Leu Leu Gly Ser Pro Arg His Ser Pro Ser Thr Ser
 225 230 235 240

Pro Arg Ala Ser Val Thr Glu Glu Ser Trp Leu Gly Ala Arg Ser Ser
 245 250 255

Arg Pro Ala Ser Pro Cys Asn Lys Arg Lys Tyr Ser Leu Asn Gly Arg
 260 265 270

Gln Pro Pro Tyr Ser Pro His His Ser Pro Thr Pro Ser Pro His Gly
 275 280 285

Ser Pro Arg Val Ser Val Thr Asp Asp Ser Trp Leu Gly Asn Thr Thr
 290 295 300

Gln Tyr Thr Ser Ser Ala Ile Val Ala Ala Ile Asn Ala Leu Thr Thr
 305 310 315 320

Asp Ser Ser Leu Asp Leu Gly Asp Gly Val Pro Val Lys Ser Arg Lys
 325 330 335

Thr Thr Leu Glu Gln Pro Pro Ser Val Ala Leu Lys Val Glu Pro Val
 340 345 350

Gly Glu Asp Leu Gly Ser Pro Pro Pro Pro Ala Asp Phe Ala Pro Glu
 355 360 365

Asp Tyr Ser Ser Phe Gln His Ile Arg Lys Gly Gly Phe Cys Asp Gln
 370 375 380

Tyr Leu Ala Val Pro Gln His Pro Tyr Gln Trp Ala Lys Pro Lys Pro
 385 390 395 400

Leu Ser Pro Thr Ser Tyr Met Ser Pro Thr Leu Pro Ala Leu Asp Trp
 405 410 415

Gln Leu Pro Ser His Ser Gly Pro Tyr Glu Leu Arg Ile Glu Val Gln
 420 425 430

Pro Lys Ser His His Arg Ala His Tyr Glu Thr Glu Gly Ser Arg Gly
 435 440 445

Ala Val Lys Ala Ser Ala Gly Gly His Pro Ile Val Gln Leu His Gly
 450 455 460

Tyr Leu Glu Asn Glu Pro Leu Met Leu Gln Leu Phe Ile Gly Thr Ala
 465 470 475 480

Asp Asp Arg Leu Leu Arg Pro His Ala Phe Tyr Gln Val His Arg Ile
 485 490 495

Thr Gly Lys Thr Val Ser Thr Thr Ser His Glu Ala Ile Leu Ser Asn
 500 505 510

Thr Lys Val Leu Glu Ile Pro Leu Leu Pro Glu Asn Ser Met Arg Ala
 515 520 525

Val Ile Asp Cys Ala Gly Ile Leu Lys Leu Arg Asn Ser Asp Ile Glu
 530 535 540

Leu Arg Lys Gly Glu Thr Asp Ile Gly Arg Lys Asn Thr Arg Val Arg
 545 550 555 560

Leu Val Phe Arg Val His Val Pro Gln Pro Ser Gly Arg Thr Leu Ser
 565 570 575

Leu Gln Val Ala Ser Asn Pro Ile Glu Cys Ser Gln Arg Ser Ala Gln
 580 585 590

Glu Leu Pro Leu Val Glu Lys Gln Ser Thr Asp Ser Tyr Pro Val Val
 595 600 605

Gly Gly Lys Lys Met Val Leu Ser Gly His Asn Phe Leu Gln Asp Ser
 610 615 620

Lys Val Ile Phe Val Glu Lys Ala Pro Asp Gly His His Val Trp Glu
 625 630 635 640

Met Glu Ala Lys Thr Asp Arg Asp Leu Cys Lys Pro Asn Ser Leu Val
 645 650 655

Val Glu Ile Pro Pro Phe Arg Asn Gln Arg Ile Thr Ser Pro Val His
 660 665 670

Val Ser Phe Tyr Val Cys Asn Gly Lys Arg Lys Arg Ser Gln Tyr Gln
 675 680 685

Arg Phe Thr Tyr Leu Pro Ala Asn Gly Asn Ala Ile Phe Leu Thr Val
 690 695 700

Ser Arg Glu His Glu Arg Val Gly Cys Phe Phe
 705 710 715

Claims

1. A conjugate of polyethylene glycol and angiostatin fragment K1-3, wherein the conjugate exhibits prolonged *in vivo* half-life and enhanced *in vivo* biological activity as compared to unmodified angiostatin fragment K1-3, and wherein the conjugate is formed by coupling one angiostatin fragment K1-3 with one polyethylene glycol molecule at the N-terminal α -amino group of the angiostatin fragment K1-3, and said angiostatin fragment K1-3 is the K1-3 fragment of plasminogen, or its biologically active fragment, mutant, derivative, isomer or the combination thereof.

2. The conjugate of claim 1, wherein said angiostatin fragment K1-3 is of mammal origin, particularly of human or murine origin.

3. The conjugate of claim 2, wherein said angiostatin fragment K1-3 is a human angiostatin fragment K1-3 having a sequence as shown in SEQ ID NO:1, or its biologically active fragment, mutant, derivative, isomer or the combination thereof.

4. The conjugate of claim 1, wherein said angiostatin fragment K1-3 is a recombinant angiostatin fragment K1-3.

5. The conjugate of claim 4, wherein said recombinant angiostatin fragment K1-3 is a recombinant human angiostatin fragment K1-3 having a sequence as shown in SEQ ID NO:2, or its biologically active fragment, mutant, derivative or isomer, or the combination thereof.

6. The conjugate of claim 5, wherein said recombinant angiostatin fragment K1-3 is expressed by *E. coli* and the N-terminal Met can be randomly deleted when expressed in *E. coli*.

7. The conjugate of any one of claims 1 to 6, wherein the derivative is formed by adding a peptide chain of 1-15 amino acids to the N-terminal or C-terminal of said angiostatin fragment K1-3.

8. The conjugate of claim 7, wherein the derivative is formed by adding a peptide chain having the amino acid sequence selected from a group consisting of MHHHHHHH, MGGSHHHHHH and MGGSHHHHHHH to the N-terminal of said angiostatin fragment K1-3, and wherein the N-terminal Met of said angiostatin fragment K1-3 is randomly deleted when expressed in *E. coli*.

9. The conjugate of any one of claims 1-6, wherein said polyethylene glycol is monomethyl polyethylene glycol.

10. The conjugate of claim 9, wherein said polyethylene glycol is monomethoxy polyethylene glycol butyraldehyde.

11. The conjugate of any one of claims 1-6, wherein said polyethylene glycol is linear or branched.

12. The conjugate of any one of claims 1 to 11, wherein said polyethylene glycol molecule has a molecular weight of 1,000 to 100,000 Daltons, preferably 5,000 to 40,000 Daltons, and more preferably 20 kDa .

13. The conjugate of claim 12, wherein one recombinant human angiostatin fragment K1-3 having a sequence as shown in SEQ ID NO:2 is coupled with one monomethyl polyethylene glycol molecule of 20 kDa.

14. The conjugate of claim 13, wherein one recombinant human angiostatin fragment K1-3 having a sequence as shown in SEQ ID NO:2 is coupled with one monomethoxy polyethylene glycol butyraldehyde molecule of 20 kDa.

15. A sustained-release formulation comprising the conjugate of any one of claims 1 to 14 and a bio-compatible substance.

16. The sustained-release formulation of claim 15, wherein said sustained-release formulation is selected from the group consisting of a microcapsule, a hydrogel, a microsphere, a micro-osmotic pump or a liposome.

17. A pharmaceutical composition comprising the conjugate of any one of claims 1 to 14 or the sustained-release formulation of claims 15 or 16 and a pharmaceutically acceptable carrier.

18. Use of the conjugate of any one of claims 1 to 14, the sustained-release formulation of claims 15 or 16 or the pharmaceutical composition of claim 17 in the preparation of anti-tumor medicaments.

19. The use of claim 18, wherein said tumor is selected from a group consisting of lung cancer, neuroendocrine tumor, colon cancer, bone cancer, liver cancer, gastric cancer, pancreatic cancer, oral cancer, breast cancer, prostate cancer, lymphoma, esophagus cancer, nasopharyngeal carcinoma, cervical cancer, sarcoma, renal carcinoma, biliary cancer, malignant melanoma, and other tumors.

20. Use of the conjugate of any one of claims 1-14, the sustained-release formulation of claims 15 or 16 or the pharmaceutical composition of claim 17 in the preparation of medicaments for treating non-tumor diseases characterized by pathological changes in human tissues or organs caused by abnormal neovascularization.

21. The use of any one of claims 18 to 20, wherein the medicament is administered by intravenous injection, intravenous infusion, subcutaneous injection,

intramuscular injection, intraperitoneal injection, subcutaneous embedment, percutaneous absorption, liver arterial injection, oral administration, nasal administration, oral mucosa administration, ocular administration, rectal administration, vaginal delivery, or other clinically acceptable routes.

22. A method to prolong the *in vivo* half-life and enhance *in vivo* biological activity of the angiostatin fragment K1-3, comprising the step of forming the conjugate of any one of claims 1-14 by using angiostatin fragment K1-3 and polyethylene glycol.

Application number/numéro de demande: CN 2008 000067

Figures: 10

Pages: 4

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CKTGNGKNYRGTMSTKNGITCQKWSSTSPHRPRFSPATHPS
EGLEENYCRNPDNDPQGPWCYTDDPEKRYDYCDILECEEEEC
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SGPTYQCLKGTGENYRGNAVTVSGHTCQHWSAQTPHPTHN
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PSC

FIG. 1

(M)VYLSECKTGNGKNYRGTMSTKNGITCQKWSSTSPHRPR
F
SPATHPSEGLEENYCRNPDNDPQGPWCYTDDPEKRYDYCDIL
ECEEECMHCSGENYDYGKISKTMSTGLECQAWDSQSPHAHGYI
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TTPPP
SSGPTYQCLKGTGENYRGNAVTVSGHTCQHWSAQTPHPTH
NRTPENFPCKNLDENYCRNPDGKRAPWCHTTNSQVRWEYC
KIPSCDSSP

FIG. 2

CKTGNGKNYRGTMSTKNGITCQKWSSTSPHRPRFSPATHPS
EGLEENYCRNPDNDPQGPWCYTDDPE KRYDYCDILEC

FIG. 3

CMHCSGENYDYGKISKTMSTGLECQAWDSQSPHAHGYIPSKFP
NKNLKKKNYCRNPDRELRPWCFTTDPNKRWELCDIPRC

FIG. 4

CLKGTGENYRGNAVTVSGHTCQHWSAQTPHTHNRTPENFP
CKNLDENYCRNPDGKRAPWCHTTNSQVRWEYCKIPSC

FIG. 5

(M)HHHHHHVYLSECKTGNGKNYRGTMSTKNGITCQKWSS
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FIG. 6

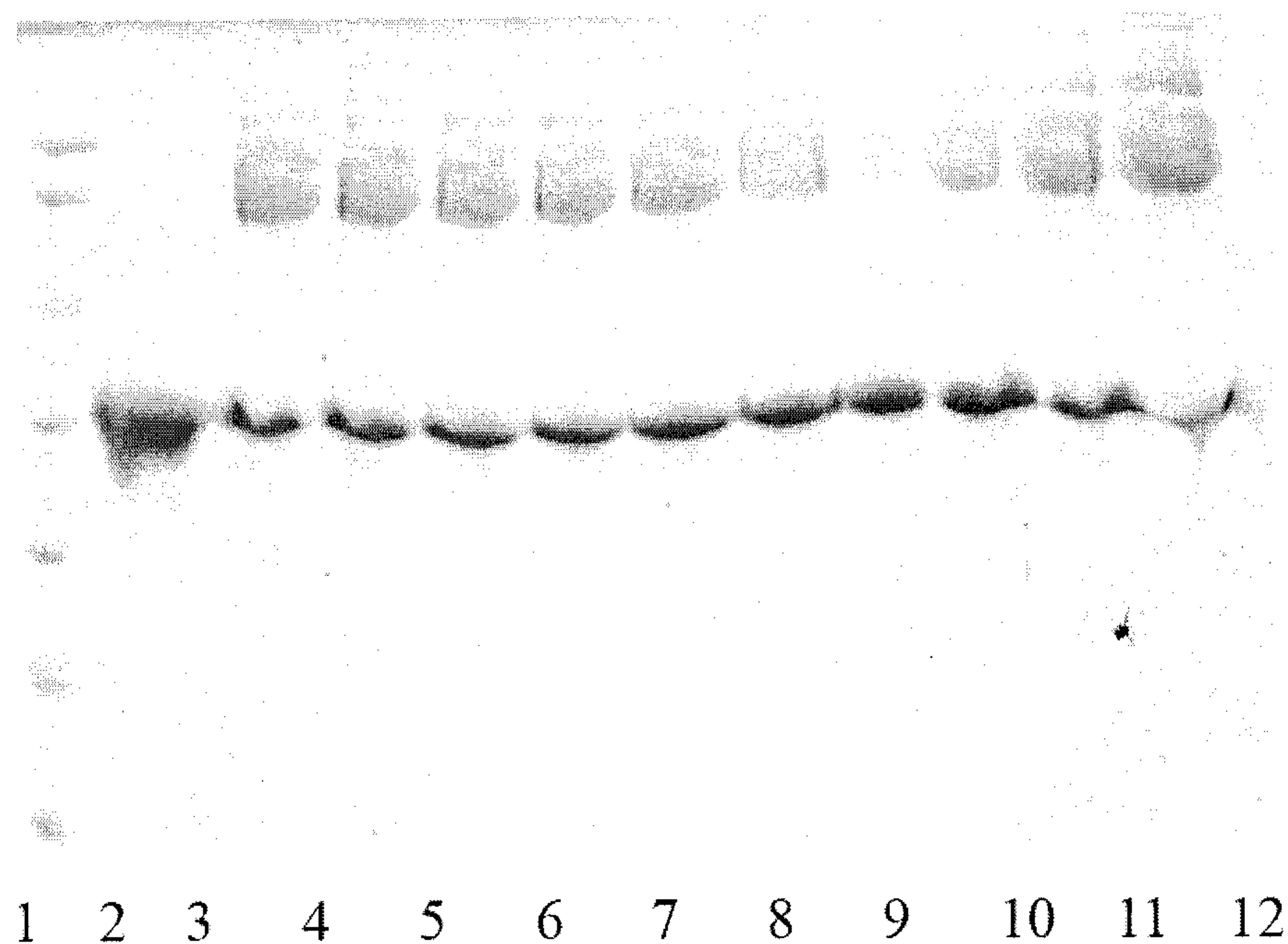


FIG. 7

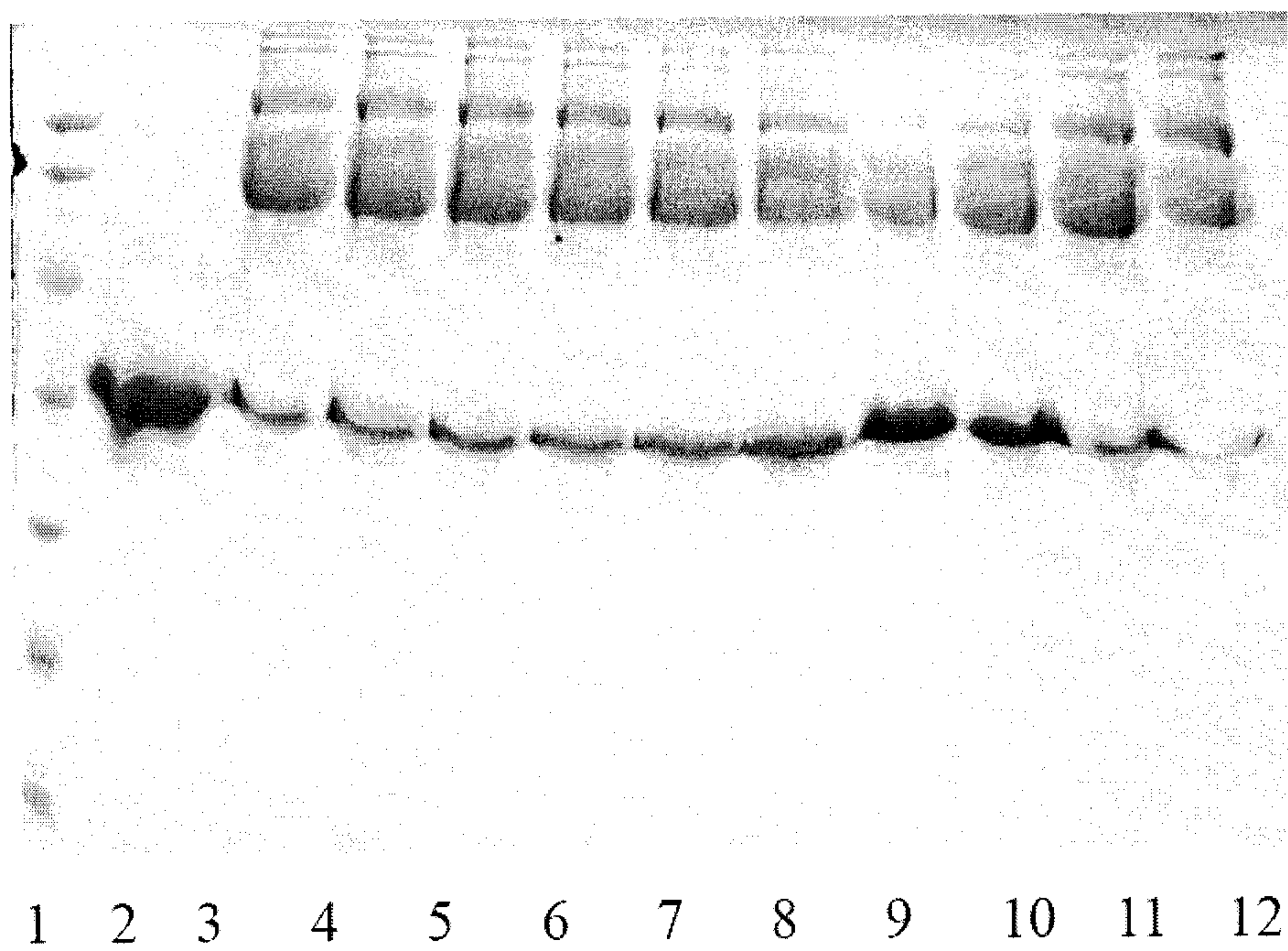


FIG. 8

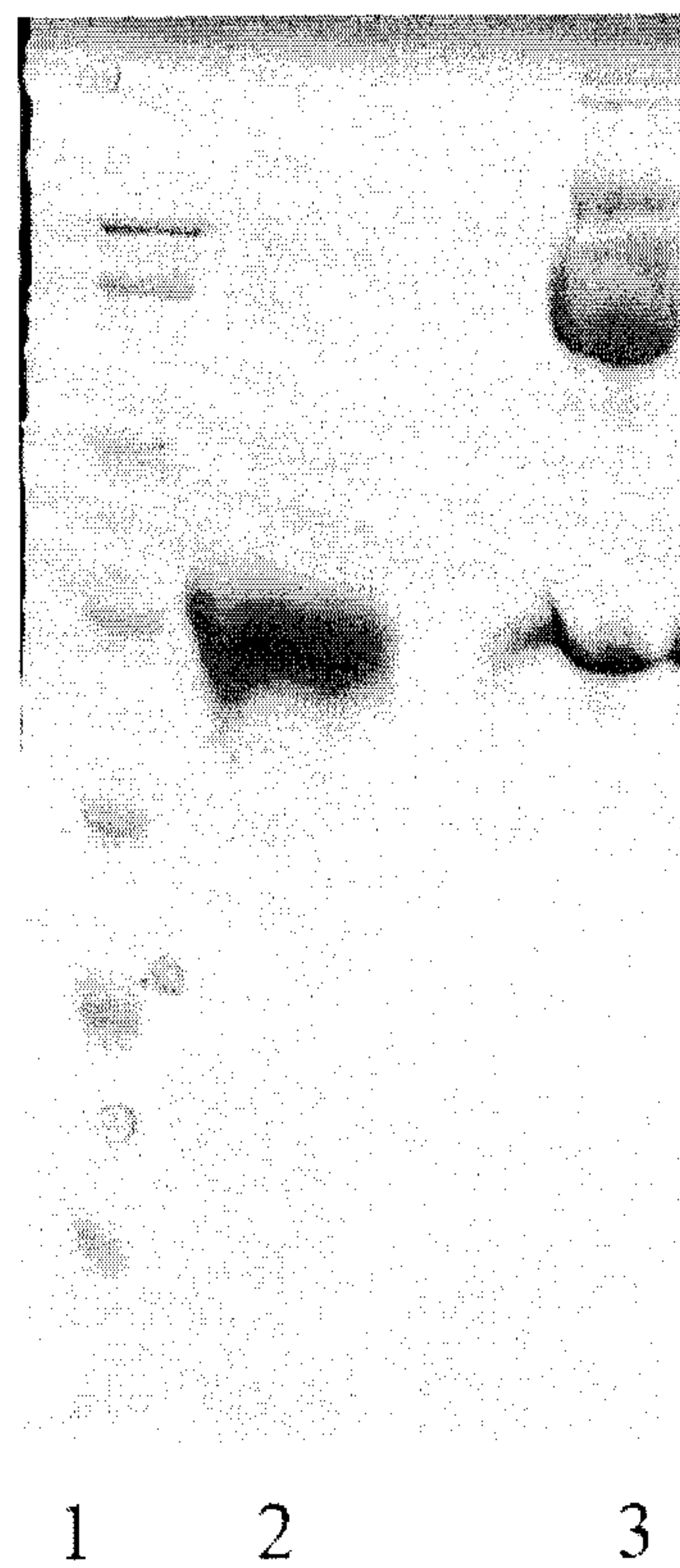


FIG. 9

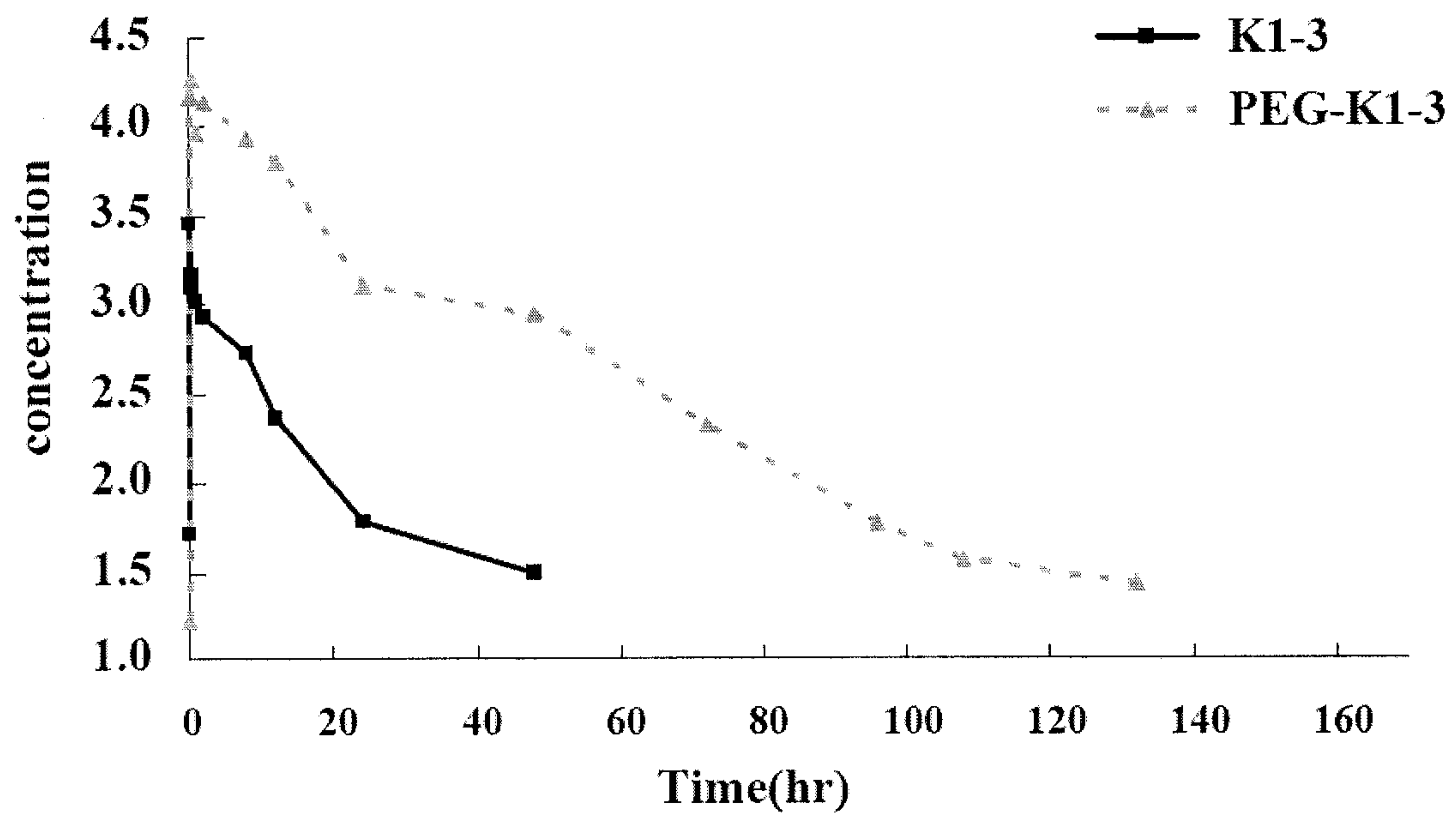


FIG. 11

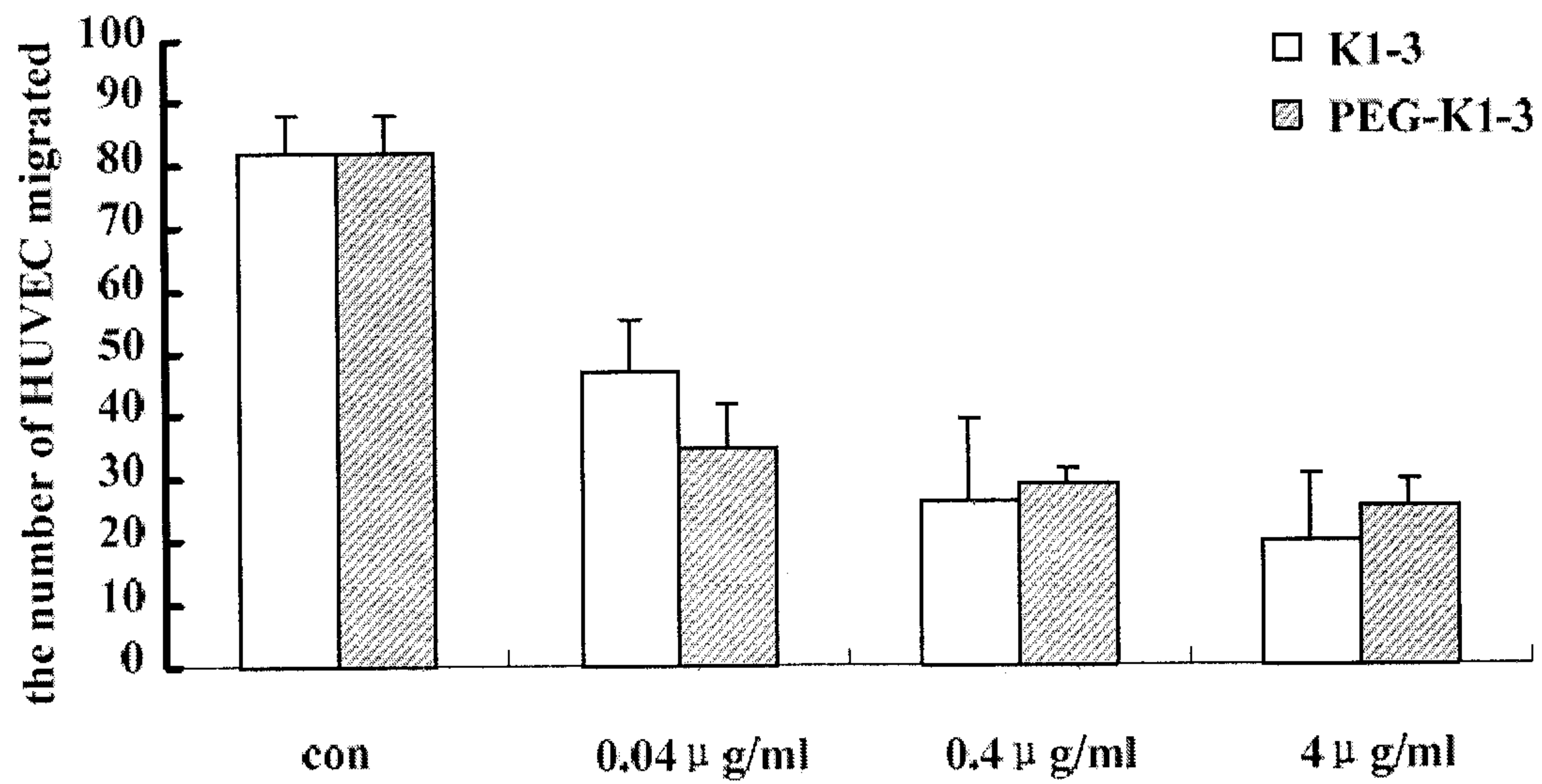


FIG. 12

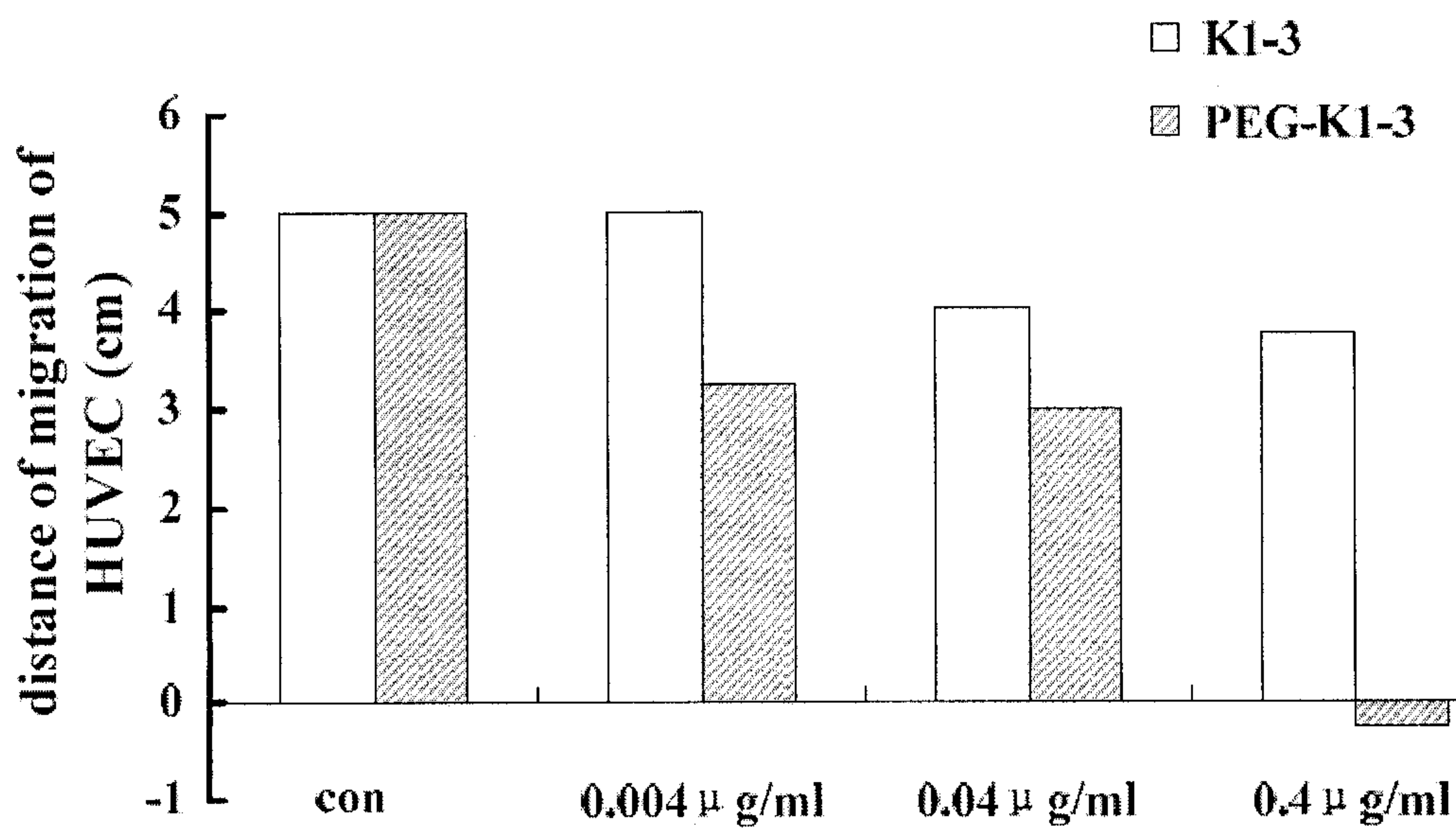


FIG. 13

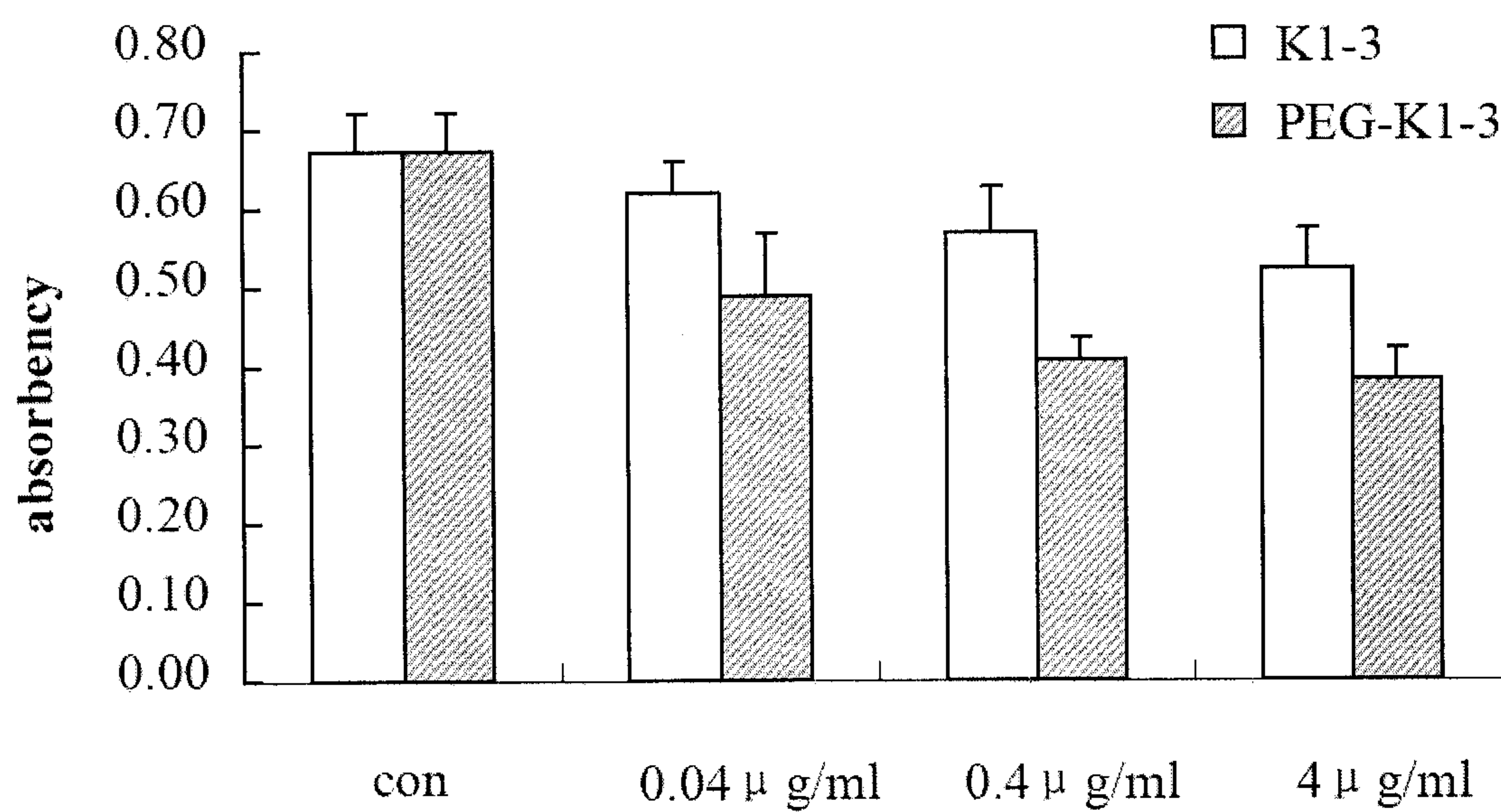


FIG. 14

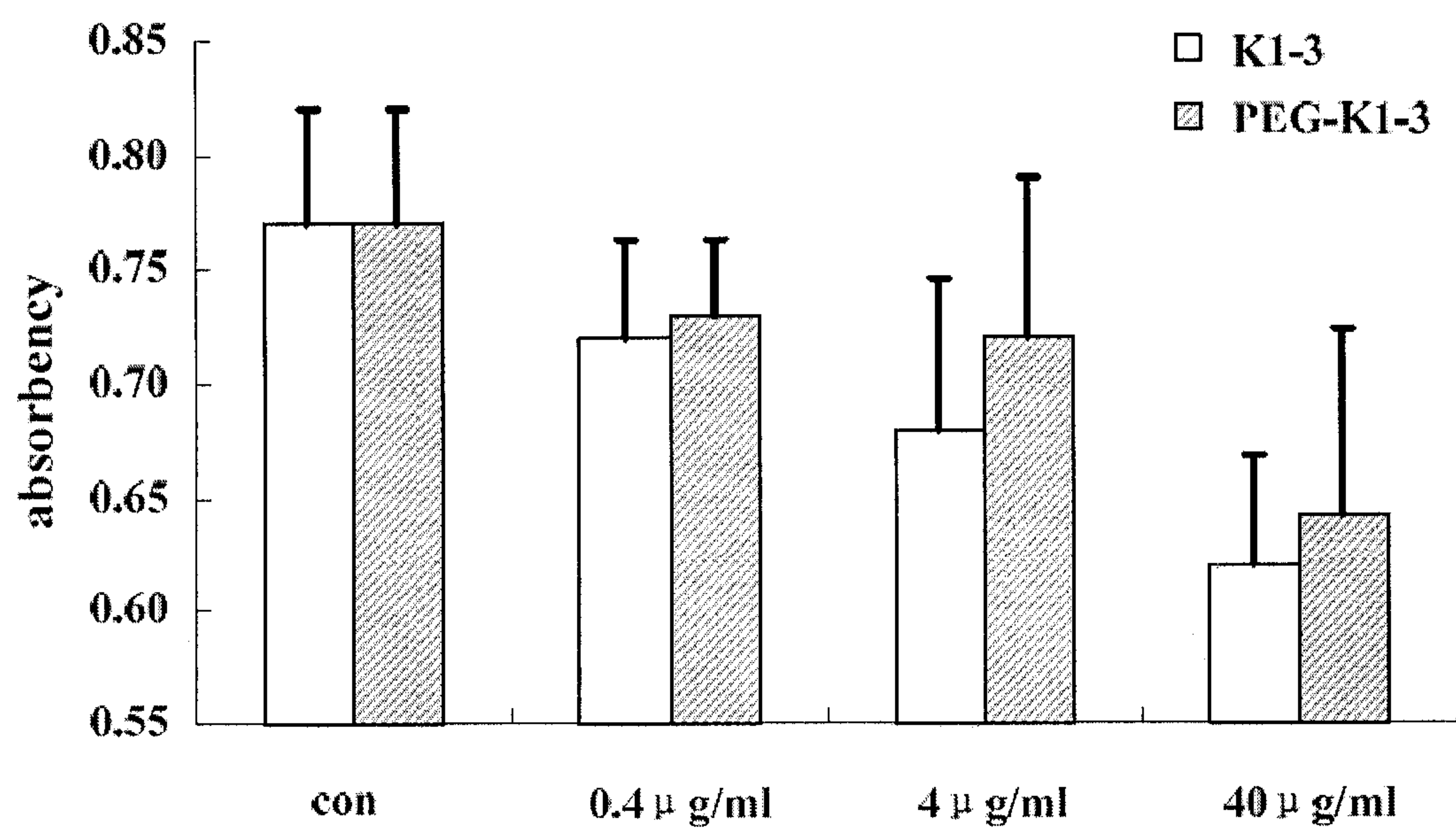


FIG. 15

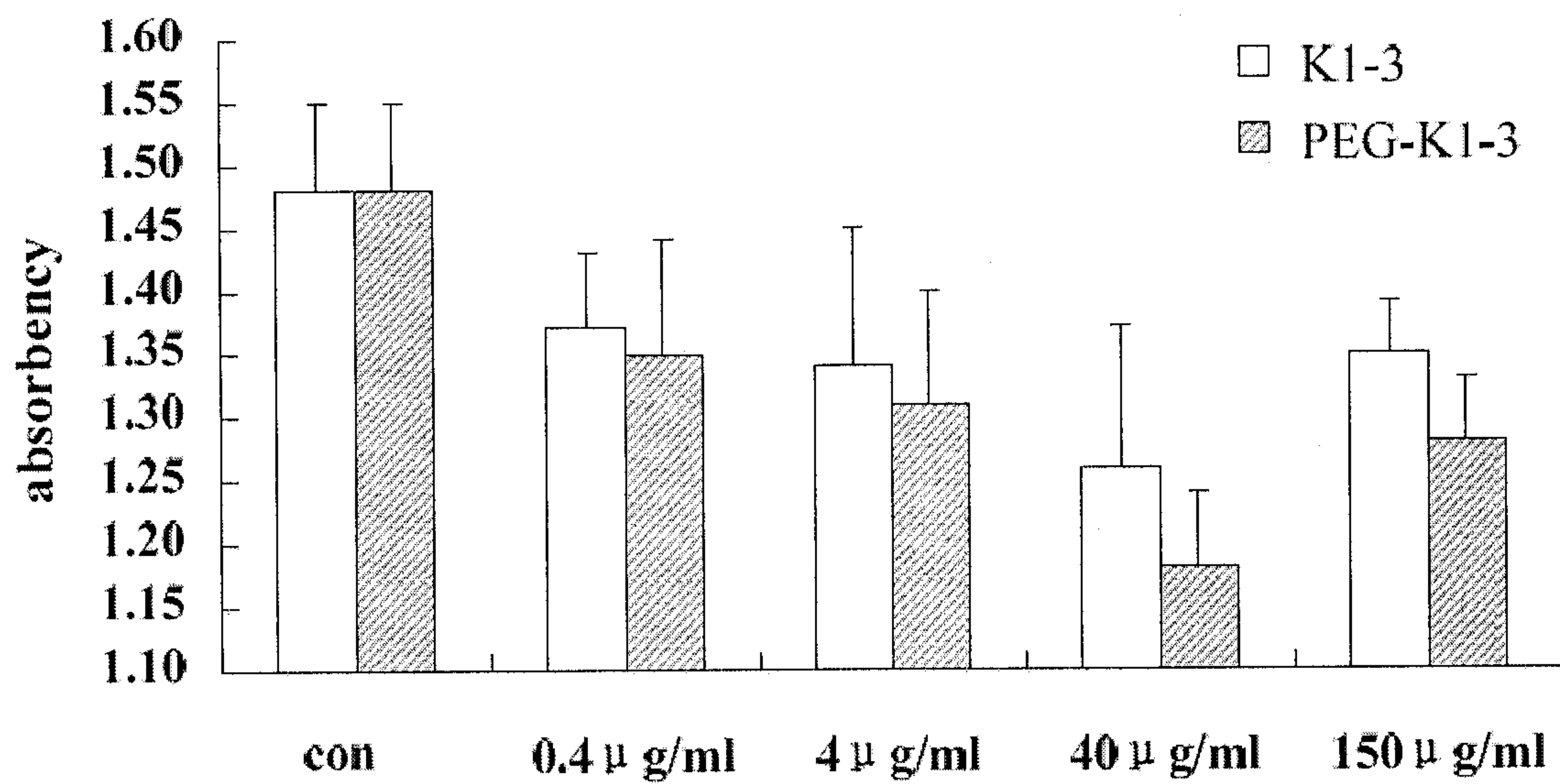


FIG. 16

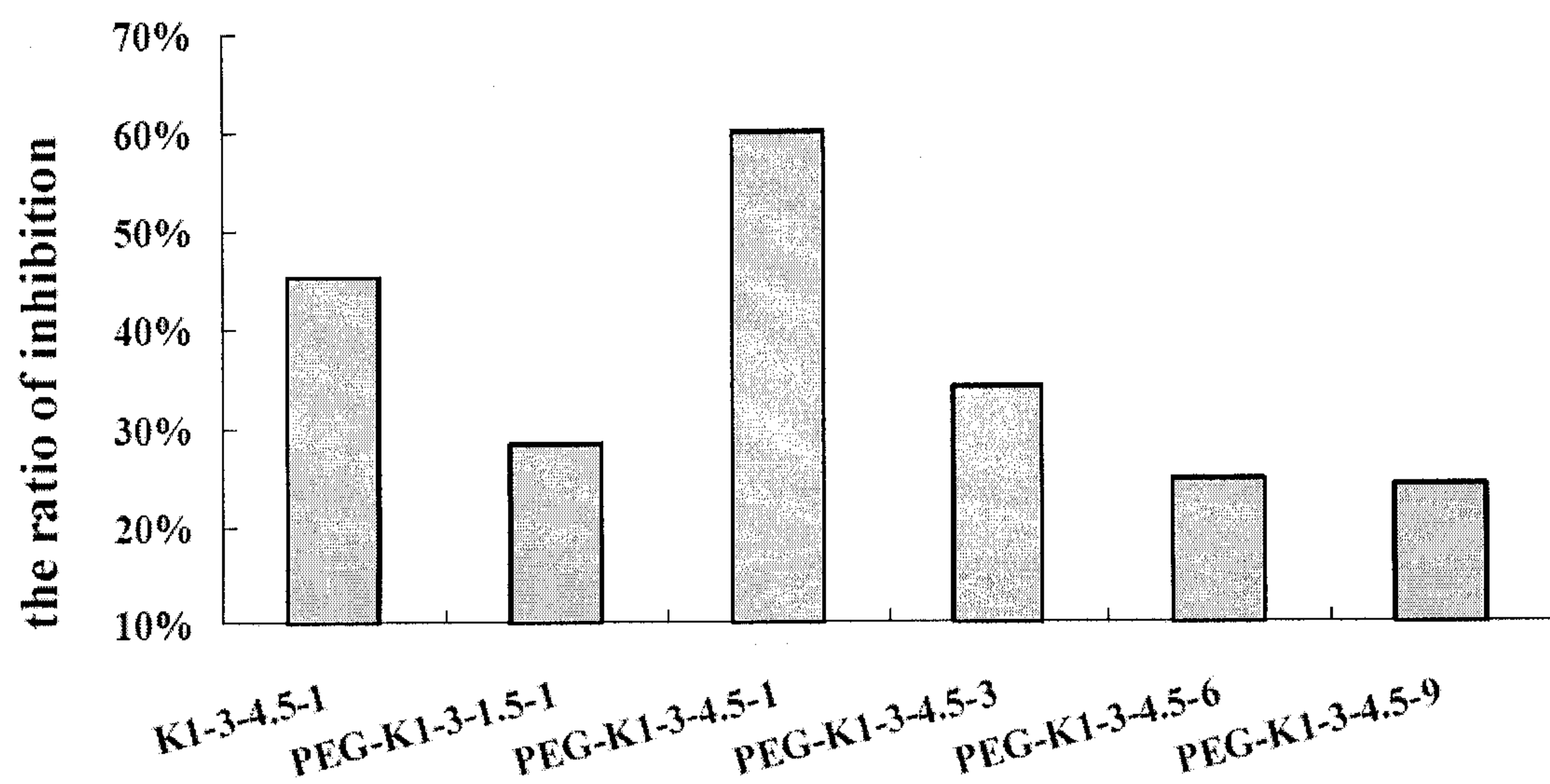


FIG. 17

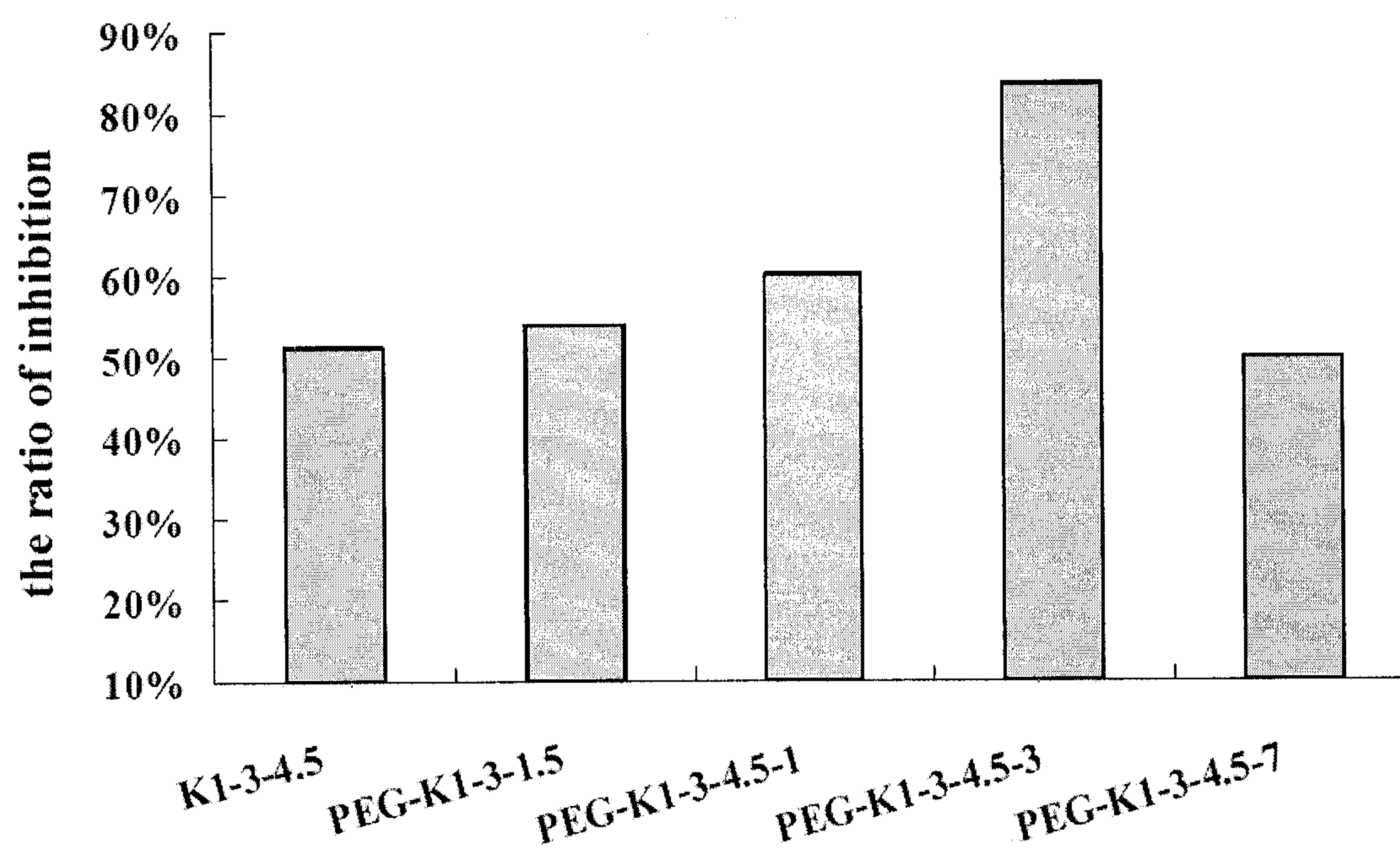


FIG. 18

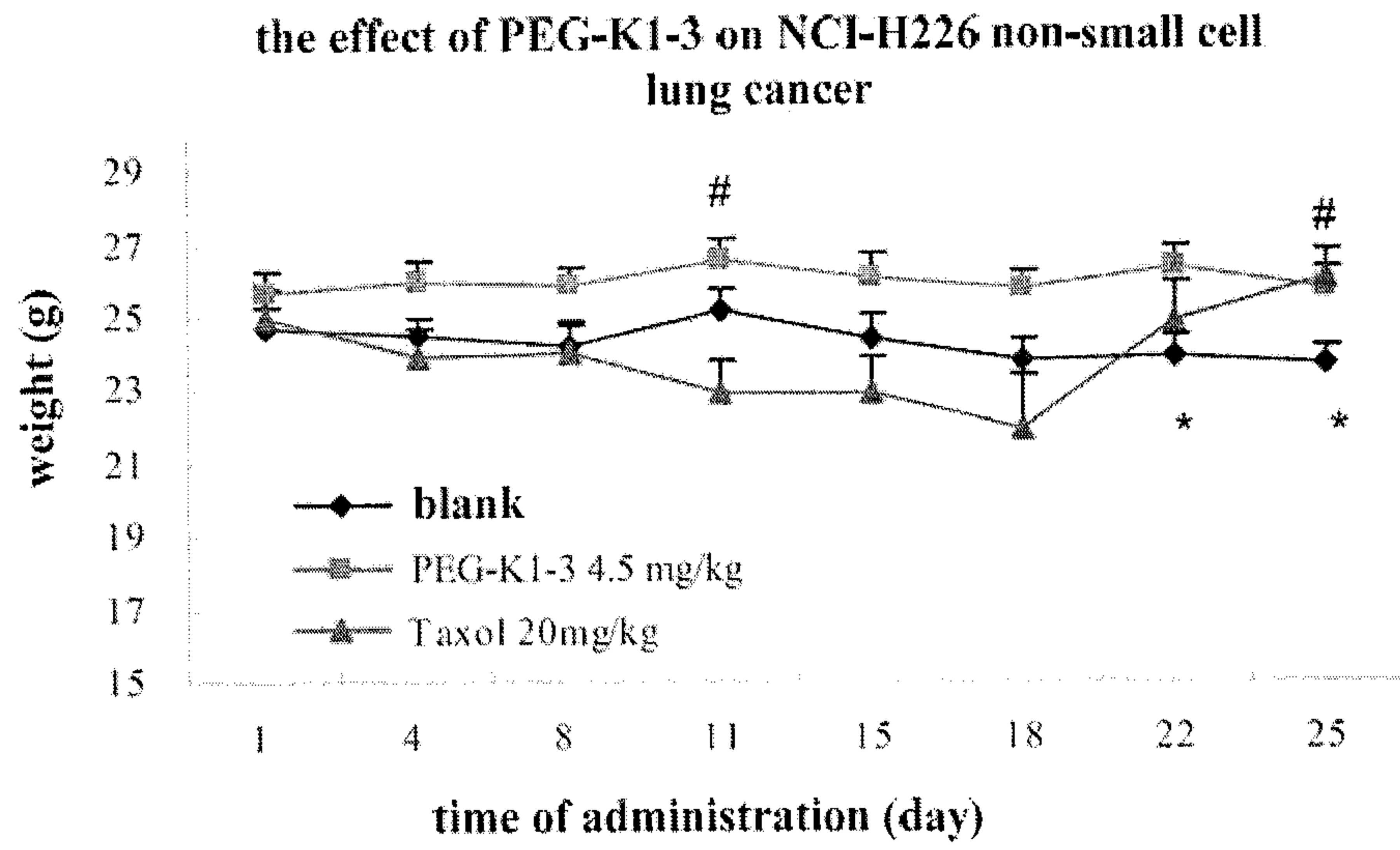


FIG. 19A

inhibition of PEG-K1-3 on NCI-H226 non-small cell lung cancer
(tumor volume)

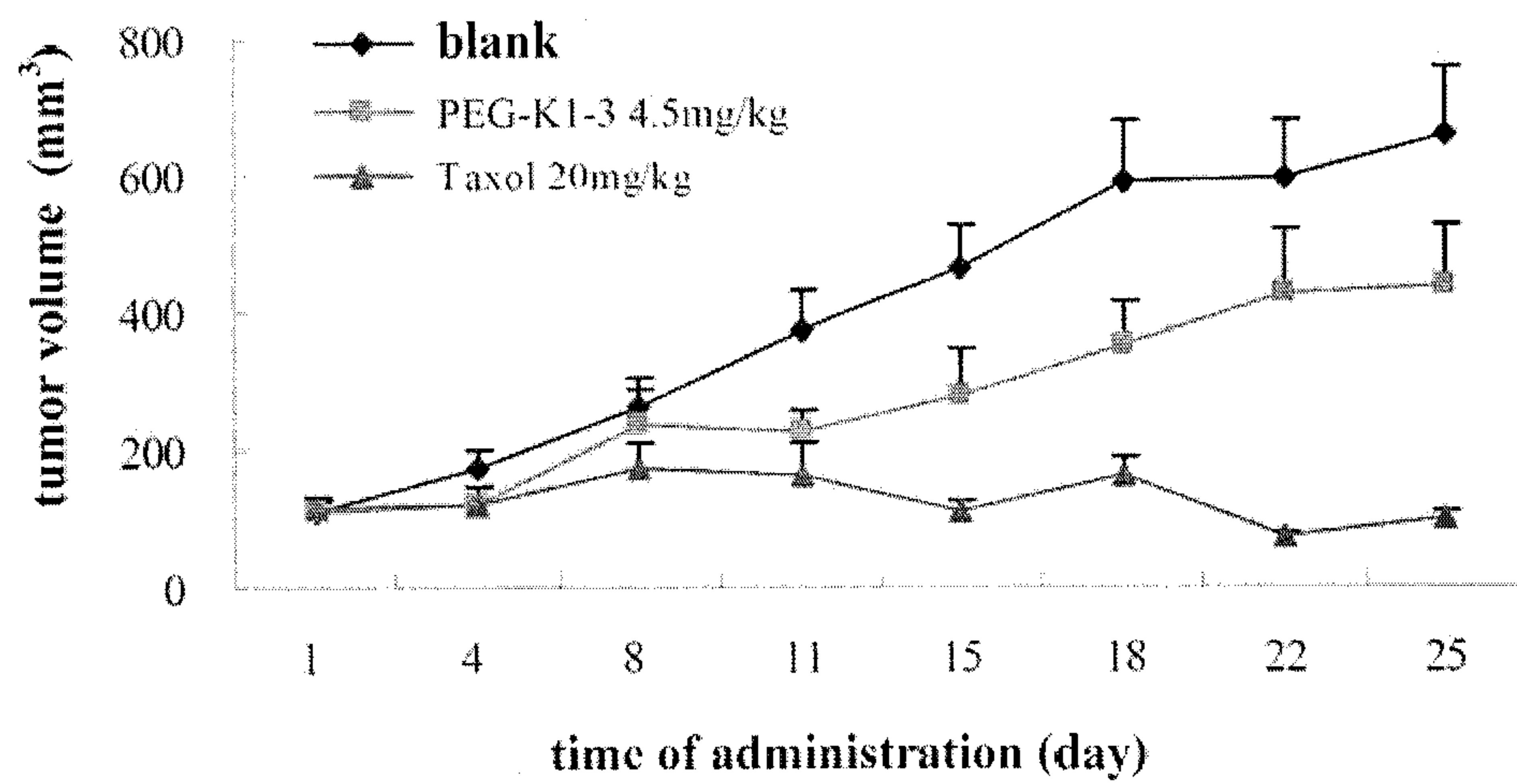


FIG. 19B

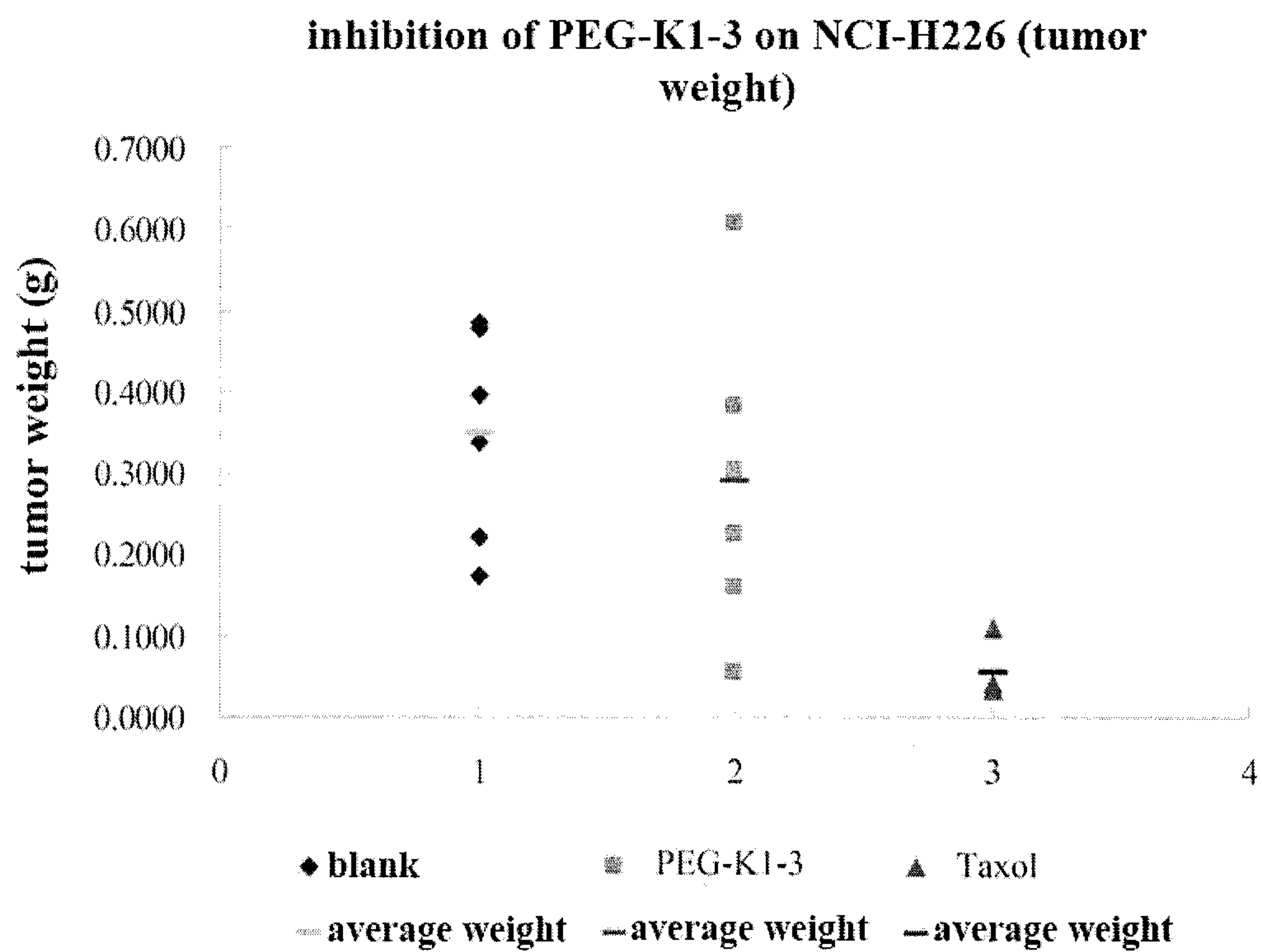


FIG. 19C