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(54) ANTITUMOR AGENTS AND PROCESS FOR PRODUCING THE SAME

(57) Anticancer agents which contain as the active ingredient heme oxygenase inhibitory metalloporphyrin derivatives which are conjugated with amphipathic or water-soluble polymers (in particular, Zn-protoporphyrin (ZnPP) conjugated with polyethylene glycol). Because of being conjugated to amphipathic or water-soluble polymers, such as polyethylene glycol, the active ingredient can be administered by intravenous injection and can exert a remarkable anticancer effect owing to tumor-selective delivery.

EP 1 421 938 A1

Description

Field of the invention:

[0001] The present invention relates to anticancer agents with little side effect and excellent tumor accumulation thereby exhibiting very potent anticancer effect, and the preparation method of the same. More precisely, it relates to anticancer agents containing as the active ingredient heme oxygenase inhibitory metalloporphyrin derivatives that are conjugated with amphipathic or water-soluble polymers. And it relates to also a preparation method of the same with high efficiency.

Background technology:

[0002] The inventors of the present invention have investigated the relationship between cancer growth or its suppression and the activity of heme oxygenase, and found that heme oxygenase is highly expressed in tumor tissues. The heme oxygenase degrades heme and produces bilverdin, carbon monoxide and free iron in tumor or normal tissues.

[0003] Bilverdin is readily converted into bilirubin in the cells, and this bilirubin is a very potent antioxidant. Thereby, bilirubin can be a defense molecule against active oxygen such as peroxide, H_2O_2, or nitric oxide etc. that are generated by leukocytes of the hosts (cancer patients). Namely, bilirubin, thus generated will nullify the toxic oxidative defense power against cancer cells or infecting microbes of the host. Therefore, if one blocks heme oxygenase, no bilirubin will be available and tumor cells will be killed by the oxidative molecules generated by leukocytes as a result of an innate defense state.

[0004] The inventors had tried to see the antitumor effect of zinc protoporphyrin (ZnPP), an inhibitor of heme oxygenase, administered into the tumor feeding artery of tumor bearing rats thereby targeting the inhibitor into the tumor loci selectively, and they indeed confirmed antitumor effect in rats (K. Doi et al.: Br. J. Cancer 80, 1945-54, 1999).

[0005] However, there are several problems to use ZnPP per se as an antitumor agent. First, it is almost insoluble in water per se, thus, one must use an oily formulation to solubilize ZnPP, and such oily formulated agent may be only injectable via the tumor-feeding artery, and this is rather too elaborate and far advanced skill is required for this procedure compared with ordinary intravenous or subcutaneous injection. Second, native or original ZnPP has no guarantee for selective accumulation of ZnPP in cancer tissues, and to exert tumor selective anticancer effect, whereas the drug will be widely distributed to the whole body besides the tumor. Therefore, unexpected side effects are concerned.

On the contrary, the inventors are experts in tumor biology, particularly studied the vascular permeability of solid tumor tissues, and know that macromolecular therapeutics would permeate more selectively at the tumor tissue by virtue of the unique anatomical character and by the effect of multiple vascular permeability factors; and further, those macromolecules are retained in the tumor tissues for long periods.

Thus, this phenomenon was coined "enhanced permeability and retention (EPR)-effect" (Y. Matsumura, H. Maeda: Cancer Res. 47: 6387-92, 1986; H. Maeda: In Advances in Enzyme Regulation (by G. Weber ed), Elsevier Scientific Ltd., Amsterdam, 41, 189-207, 2001).

[0006] According to the EPR-effect, drugs with molecular size larger than 40,000 exhibit a high concentration in blood plasma for a prolonged time, and several hours to days after intravenous injection, whereas an intratumoral concentration will result in a multiple time, more precisely in 24-48 hr. This means, making the apparent drug size greater than 40,000 would make possible a selective tumor targeting of such macromolecular drugs.

[0007] Meanwhile, various metal porphyrin derivatives having inhibitory activity against heme oxygenase, and improved methods of their administrations as a whole were studied. The result is that an amphipathic or water soluble polymer conjugation to the metal protporphyrins made it possible to yield water soluble metal porphyrin derivatives and they can be administered not only arterially but also intravenously which has more versatile and easy clinical uses. They exhibited EPR-effect by polymer conjugation yielding a highly efficient accumulation in tumor, and enzyme inhibitory activity against heme oxygenase is retained for long periods. As a result, only 2 to 3 times of injections made it possible to suppress tumor growth completely in mice, which was a remarkable result.

[0008] Previously, metal porphyrin derivatives possessing heme oxygenase inhibitory activity with amphipathic or water soluble polymer conjugation were never reported, nor was the method of their preparation before our own. The inventors have developed the method for synthesis of amphipathic or water-soluble polymer conjugation of metalloporphyrin via an amide linkage. Resulting polymer conjugated metalloporphyrin derivatives are novel series of compounds not reported previously.

Disclosure of the Invention

[0009] The present invention are anticancer agents containing as the active ingredient metalloporphyrin derivatives having inhibitory activity against heme oxygenase, especially Zn-protoporphyrin (ZnPP) conjugated with amphipathic polymers which are both water and lipid soluble or water soluble polymers.

[0010] The present invention is also a series of novel useful compounds for an ingredient of anticancer agents where amphipathic or water-soluble polymers and heme oxygenase inhibitory metalloporphyrin derivatives are conjugated via amide bonds, and a preparation method of the compounds.

Brief Explanations of drawings

[0011]

Figure 1 shows the gel filtration chromatography of diaminoethane coupled protoporphyrin and PEG-conjugated protoporphyrin.

Figure 2 shows a Lineweaver-Burk plot of PEG-ZnPP inhibitory profile against heme oxygenase.

Figure 3 shows flow-cytometric analysis data where PEG-ZnPP treated cultured cancer cells exhibit a more oxidant exposured profile.

Figure 4 shows an antitumor effect of PEG-ZnPP in a mouse model with a solid tumor.

Figure 5 shows the profile of body weight change during or after intravenous administration of PEG-ZnPP.

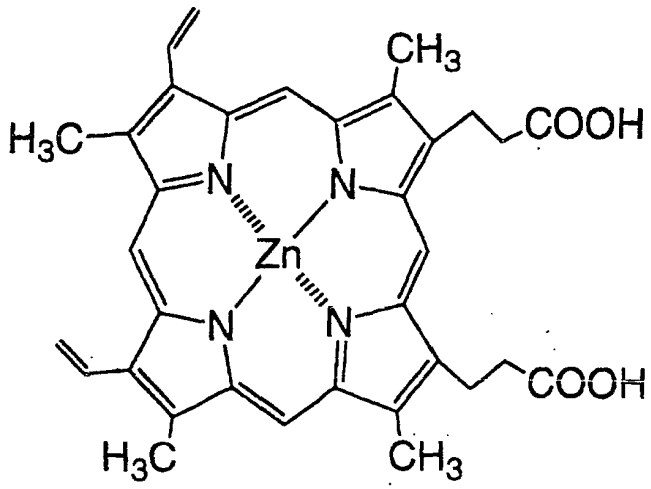
Most preferable embodiment for carrying out the invention

[0012] Amphipathic or water soluble polymers to be conjugated include polyethylene glycol (PEG), polypropylene glycol (PPG), polyvinyl pyrrolidone (PVP), polyvinyl alcohol (PVA), various gelatins, and their derivatives such as succinylated forms, polyamino acids (eg. polymerized aspartic acid, glutamic acid, lysine, alanine, glycine, proline, tyrosine, etc.), hydroxypropyl and other alkyl acrylate polymers, styrene maleic acid copolymers(SMA), and their derivatives. Among these polymers with amphipathic and water-soluble characters, PEG and SMA are more preferable. PEG with molecular weight of 2000-5000 is preferably used.

[0013] SMA is a copolymer of styrene and maleic acid in the alternative order where the carboxyl group of maleic acid can be utilized to conjugate with metalloporphyrin directly or indirectly. SMA can be used as such or as its derivatives where maleic acid is partially esterified.

[0014] Metal porphyrin derivative is a complex porphyrin compound, where the metal is chelated in stable coordination to the porphyrin ring, and protoporphyrin is preferably used because of its easy availability among porphyrin compounds.

[0015] Among the metals to be coordinated, iron that give no heme oxygenase inhibitory action, mercury with poisonous nature, monovalent metals which do not form coordinated chelation, can not be used. Although, various metals other than above such as zinc, tin, cobalt, and copper can be used. Among them, tin and zinc complexes are more preferred. However, tin is also known to be poisonous. Thus, ZnPP is most preferable and its chemical structure is shown in formula B.

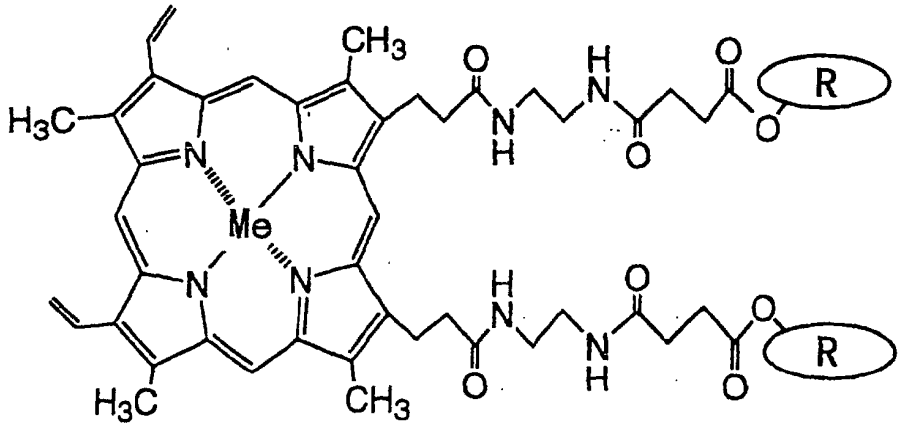


(B)

[0016] The anticancer agents of the present invention are any macromolecular compounds obtained by conjugation of a metal porphyrin with amphipathic or water-soluble polymers. However we found it difficult to carry out the conjugation of the polymers to the metal porphyrin directly, because a metal porphyrin derivative is water-insoluble. To undertake this chemical conjugation, it is preferable to conjugate the polymers to porphyrin before the coordination of metal, then to coordinate the metal.

[0017] The conjugation of porphyrin with the polymer can be facilitated directly as well as by introducing a desired functional spacer group.

[0018] For example, in the synthesis of ZnPP, the polymer can be directly conjugated to the two carboxyl groups in protoporphyrin, but this direct conjugation method is not advantageous because of poor activity of said carboxyl groups for this reaction. The inventors of the present invention studied effective synthesis methods of PEG conjugated ZnPP, and succeeded to synthesize ZnPP conjugated with PEG via an amide bond (formula A).



(A)

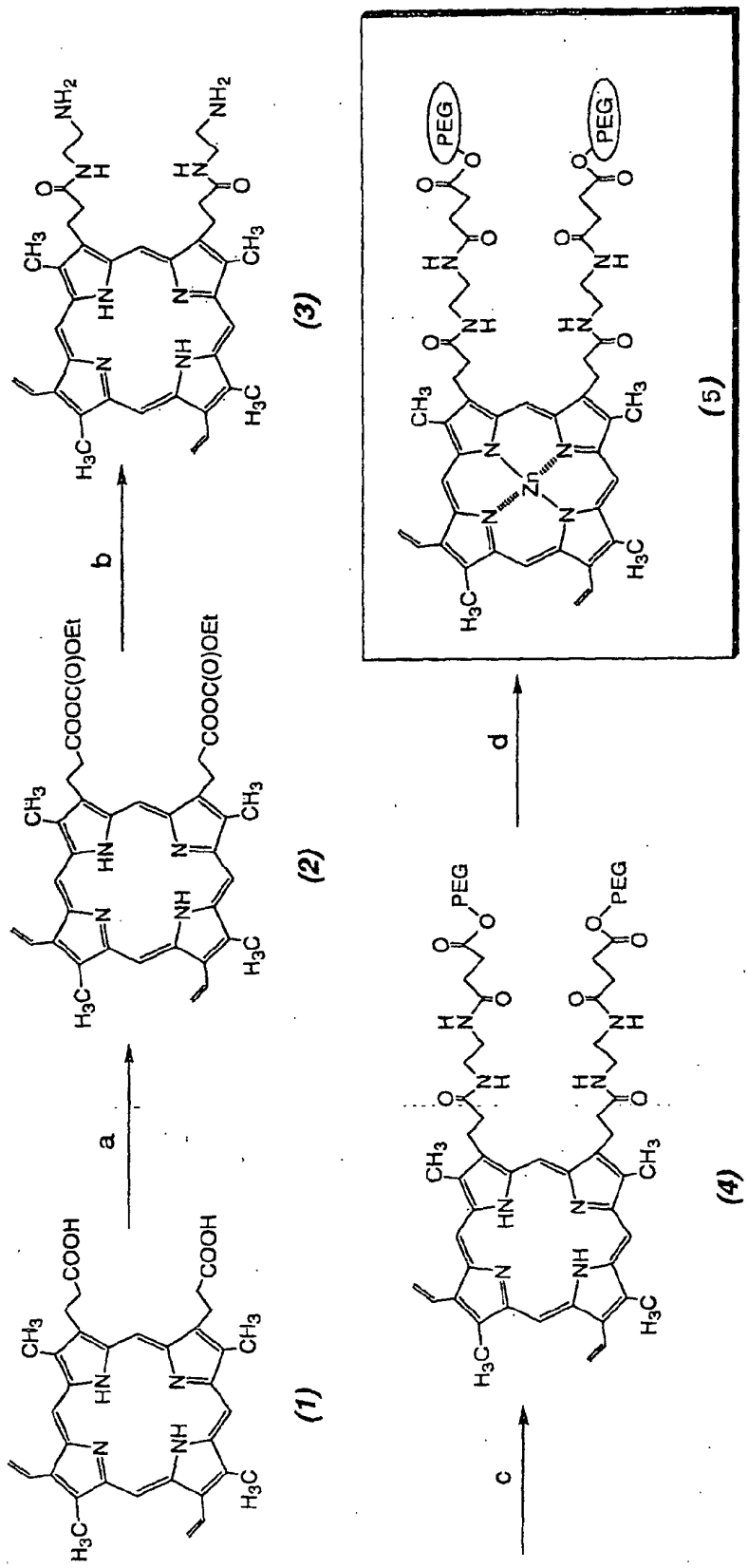
Therein, R means an amphipathic or water-soluble polymer, and Me is a metal.

[0019] The polymer conjugated ZnPP B may be synthesized by a successive reaction as follows:

(1)Introduction of amino group to protoporphyrin IX;
(2)Conjugation of the polymer, and lastly
(3)Coordination of Zn into porphyrin ring.

[0020] For example, the scheme of synthesis of PEG conjugated ZnPP is shown diagrametrically by stepwise reactions as follows.

[Reaction (a)] Protoporphyrin IX (compd.(1)) is activated with ethyl chloroformate in tetrahydrofuran (compd.(2)).
[Reaction (b)] Protoporphyrin with diamino group (compd.(3)) can be obtained by addition of ethylene diamine.
[Reaction (c)] PEG is introduced into protoporphyrin ring by addition of activated PEG (compd.4).
[Reaction (d)]. Lastly, PEG-ZnPP (compd.5) is obtained by addition of zinc acetate to the reaction product of the Reaction (d). One can replace Zn for tin (Sn) and obtain PEG-Sn-PP by addition of tin acetate


(1)
(2)
(3)
(4)
(5)
a
b
c
d
COOH
COOC(O)OEt
PEG
Zn

[0021] Other compounds than PEG, with amphipathic or water soluble polymers, such as SMA, can be attached to protoporphyrin similarly by condensation reaction of compd.(3) and SMA.

[0022] Heme oxygenase inhibitory methalloprotoporphyrin, that is conjugated with amphipathic or water-soluble polymers shown in formula A, is selectively accumulated in solid tumors and exhibit excellent antitumor activity. Thus it is a novel and useful antitumor substance. The compd.(5), which is a typical example of the compound A, wherein metal is zinc and R is PEG, was synthesized by the scheme (a) to (d). The chemical structure of the reaction product was confirmed by following analysis.

[0023] Firstly; the evidence of the amino group of ethylene diamine that was introduced into protoporphyrin (compd. 3) is confirmed by (1)Infrared spectra with absorbance at 1641cm^{-1}, 1552cm^{-1} showed a new formation of the amide bond in the compound (structure of compd.3). (2)Determination of molecular weight of the compound by mass spectroscopy (MS) showed 646, identical to the value calculated by formula based on the compd.3.

[0024] Then, PEG (mw about 5000) was coupled to amino group introduced into the protoporphyrin (compd.3), and zinc is chelated. The structure of thus obtained ZnPP was identified by determination of the molecular weight and absorption spectra (UV/Vis).

[0025] Determination of the molecular weight showed mass of near 11,000 Da by TOF/MS (time of flight-mass spectroscopy). UV absorption showed a max. peak at 425, 543, and 583 indicating formula (5) to be PEG-ZnPP.

[0026] The scheme of PEG-ZnPP synthesis using protoporphyrin IX as starting material via reaction steps [a] - [d] is a novel manufacturing method. The obtained polymer conjugated metalloporphyrin is readily water-soluble and it may be used as injection solution either intravenously or arterially.

[Examples]

[0027] The process for preparing the PEG-ZnPP, inhibitory activity of the PEG-Znpp towards heme oxygenase and anticancer effect of PEG-ZnPP by intravenous injection according to the present invention shall be explained in detail with the following examples. However it should be understood, that the present invention shall not limited to these examples.

[Example of Manufacturing]: Synthesis of polyethylene glycol conjugated ZnPP (PEG-ZnPP)

[0028] 100mg of protoporphyrin IX was dissolved in 20 ml of tetrahydrofuran, and 2.45 ml of triethylamine was added to this solution. This solution was kept at about 0°C on ice, then 1.7 ml of ethyl chloroformate was added to this dropwise under stirring, and allowed to react further for two hrs. Subsequently, triethylamine HCl salt being formed was removed by filtration, and 1.2 ml ethylene diamine was added, and the reaction was continued at room temperature for 24 hrs. The reaction mixture was then subjected to vacuum evaporation to remove tetrahydrofuran, and the solid material obtained was washed 7 times with 50ml of distilled water yielding 60 mg of porphyrin derivative having two amino groups per molecule (reaction a and b).

[0029] Five mg of compound (3) was dissolved in 25 ml of chloroform, and 800 mg of succinimidoester of polyethylene glycol (Shearwater; PEG, MW5000) was added to this solution, and reacted for 24 hrs under stirring at room temperature [reaction c].

[0030] PEG-conjugated protoporpyrin thus obtained was subjected to gel filtration chromatography on Sephadex LH60 using chloroform as eluent. The result of the gel filtration chromatography showed that unreacted aminated compound (3) did not exist in the preparation of PEG-conjugated protoporpyrin at all. It showed all aminated protoporphyrin reacted with PEG to form polymeric form of protoporphyrin. Unmodified protoporphyrin, if any, was eluted at fraction No. 20, where elution volume was similar to aminated protoporphyrin.

[0031] 40mg of zinc acetate was added to the PEG-PP solution and allowed for two hrs at room temperature yielding PEG-conjugated zinc protoporphyrin (PEG-Zn-PP) (reaction d).

[Experimental Example 1]: Inhibitory activity of PEG-ZnPP against heme oxidase.

[0032] This was examined using purified heme oxygenase fraction derived from rat spleen. It was assayed at 37 °C in the presence of hemin, the substrate of heme oxygenase, cofactor (NADPH, nicotine adenine dinucleotide), and cytosolic fraction containing bilirubin reductase, in which biliverdin formed by the oxygenase is converted to bilirubin.

[0033] Bilirubin was extracted with chloroform and quantified by absorption at 465 nm. By the addition of either PEG-ZnPP, or unmodified ZnPP, or no inhibitor, their effect on heme oxidase was examined, and the Lineweaver-Burk plot of heme oxygenase activity was plotted during the inhibition by PEG-ZnPP. The result is shown in Figure 2, indicating that PEG-ZnPP inhibits the heme oxygenase in a dose dependent manner, and inhibitory constant (Ki) was 0.13 μM. Mode of inhibition was competitive, and the value was equivalent to that of unmodified ZnPP. (Ki = 0.12 μM)

[Experimental Example 2]: Effect of PEG-ZnPP on cultured tumor cells.

[0034] Lung adenocarcinoma cell line A549 cells were plated in a plastic dish and after overnight culture, 5 μM and 10 μM of PEG-ZnPP dissolved in distilled water were added to the culture dishes. Then, 8 hrs after cultivation at 37 °C, a reagent that quantifies oxidative stress, called dichlorodihydrofluorescein diacetyl ester (DCDHF), was added and followed by cell culture for 30 minutes. Under oxidative stress, this DCDHF will become oxidized and will fluoresce due to oxystress generated by formation of fluorescein in cells.

[0035] Quantification of fluorescence intensity . represents the extent of oxidative stress induced in the cells. Then, cultured cells were trypsinized and recovered cells were subjected to the flow cytometrory analysis and fluorescence cell population was quantified. The results are shown in Figure 3, where the effect of PEG-ZnPP at 5 μM, 10 μM, is compared with that of no drug. It is clear from these data in the Fig. 3 that PEG-ZnPP brought about a higher intracellular oxidative state in the dose dependent manner of PEG-ZnPP.

[Experimental Example 3]: Inhibition of heme oxygenase in solid tumor model in mouse.

[0036] In male ddY mice with mean body weight of 35 g, S180 sarcoma cells were implanted in the dorsal skin, and when solid tumor size becomes 5 mm in cross diameter after about one week, PEG-ZnPP dissolved in distilled water was injected via the tail vein (i.v.) at 0.5 mg ZnPP equivalent per Kg body weight. The solid tumors were removed after 24 hr, and heme oxygenase activity was quantified similarly as described in Example 1. A control mouse received distilled water without PEG-ZnPP. The tumor specimens were obtained and treated similarly. As shown in Table 1, PEG-ZnPP given i.v. (tail) showed significant reduction of the heme oxygenase activity. Unmodified ZnPP could not be administered i.v. because of its difficulty in solubility.

[Table 1]

Inhibition of intratumor heme oxygenase by PEG-ZnPP given via the tail vein.		
Drug	Activity of heme oxygenase in tumor tissue. (n mol bilirubin/mg protein/hr)	
Control, none	4.17 ±1.07	(P < 0.02)
Group of PEG-ZnPP administered	2.30 ±0.54	
Unmodified ZnPP administered	Impossible to solubilize in water (can not be injected)	

[Experiment 4]: Antitumor effect of PEG-ZnPP and change of body weight in mice bearing solid tumor.

[0037] Similar to Experiment 3 above, with sarcoma S 180 of mice implanted under the dosal skin of ddY mice, and after 10, 13 and 15 days after tumor implantation, PEG-ZnPP at 30 n mole, 30 n mole and 50 n mole (3 times only), respectively, was injected into the tail vein respectively (see also arrow marks in Figure 4). Control mice received distilled water instead of PEG-ZnPP. The sizes of the tumors were measured every week day as shown in Figure 4. It is clear that the PEG-ZnPP group showed remarkable suppression of tumor growth compared with control group.

[0038] The body weight of both treated and non-treated mice was measured simultaneously as seen in Figure 5. There was no remarkable body weight loss in the group treated with PEG-ZnPP.

Applicability of the Invention in the Industrial Sense

[0039] According to the present invention, metal porphyrin derivatives which are inhibitory against heme oxygenase, can be made both water-soluble and lipid soluble. They make the derivatives to become an intravenously injectable medicament by conjugation with amphipathic or water-soluble polymers. This is a novel medicament having an excellent tumor selective accumulation. An effective preparation method of this compound was also found.

[0040] The anticancer agents according to the present invention have an excellent anticancer effect without generating any appreciable side effect or toxicity.

[0041] Thus the polymer conjugated anticancer agents according to the present invention are highly useful drugs having an excellent tumor selective targeting property with a new mode of action different from many of the known low molecular weight anticancer drugs.

Claims

1. Anticancer agents which contain as the active ingredient heme oxygenase inhibitory metalloporphyrin derivatives which are conjugated with amphipathic or water-soluble polymers.

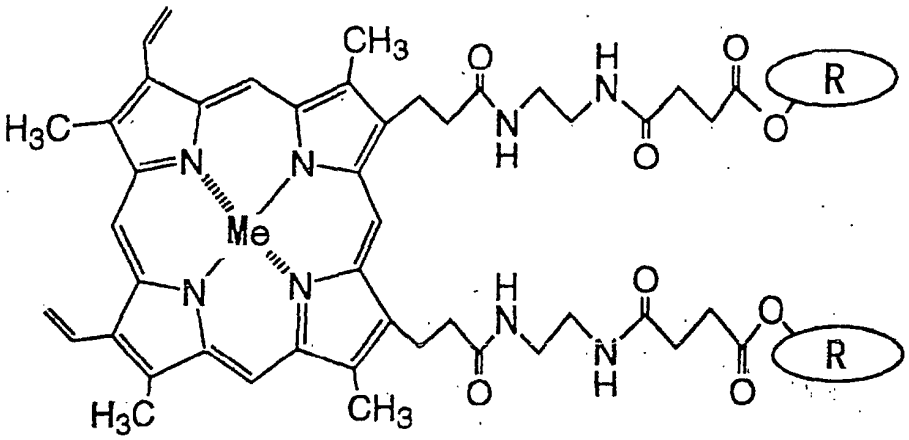
2. The anticancer agents according to Claim 1, wherein the heme oxygenase inhibitory metalloporphyrin derivatives are complex compounds in which a metal atom is chelated to protoporphyrin in stable coordination.

3. The anticancer agents according to Claims 1 or 2, wherein the amphipathic or water-soluble polymers are polyethylene glycol (PEG) or a styrene-maleic acid copolymer (SMA).

4. The anticancer agents according to Claims 1-3, wherein the heme oxygenase inhibitory metalloporphyrin derivatives are conjugated with amphipathic or water-soluble polymers via amide bonds.

5. The anticancer agents according to Claims 1-4, wherein the metal is zinc.

6. Heme oxygenase inhibitory metalloprotoporphyrin derivatives which are conjugated with amphipathic or water soluble polymers represented by the general formula A



(A)

wherein "R" means amphipathic or water soluble polymers and "Me" is a metal.

7. A preparation method of heme oxygenase inhibitory metalloporphyrin derivatives, which are conjugated with amphipathic or water soluble polymers according to Claim 6, which comprises introducing ethylene diamine to protoporphyrin IX, introducing the polyethylene glycol chain into protoporphyrin by adding activated polyethylene glycol, and finally adding a metal salt to introduce the metal atom into the porphyrin ring.

Figure 1

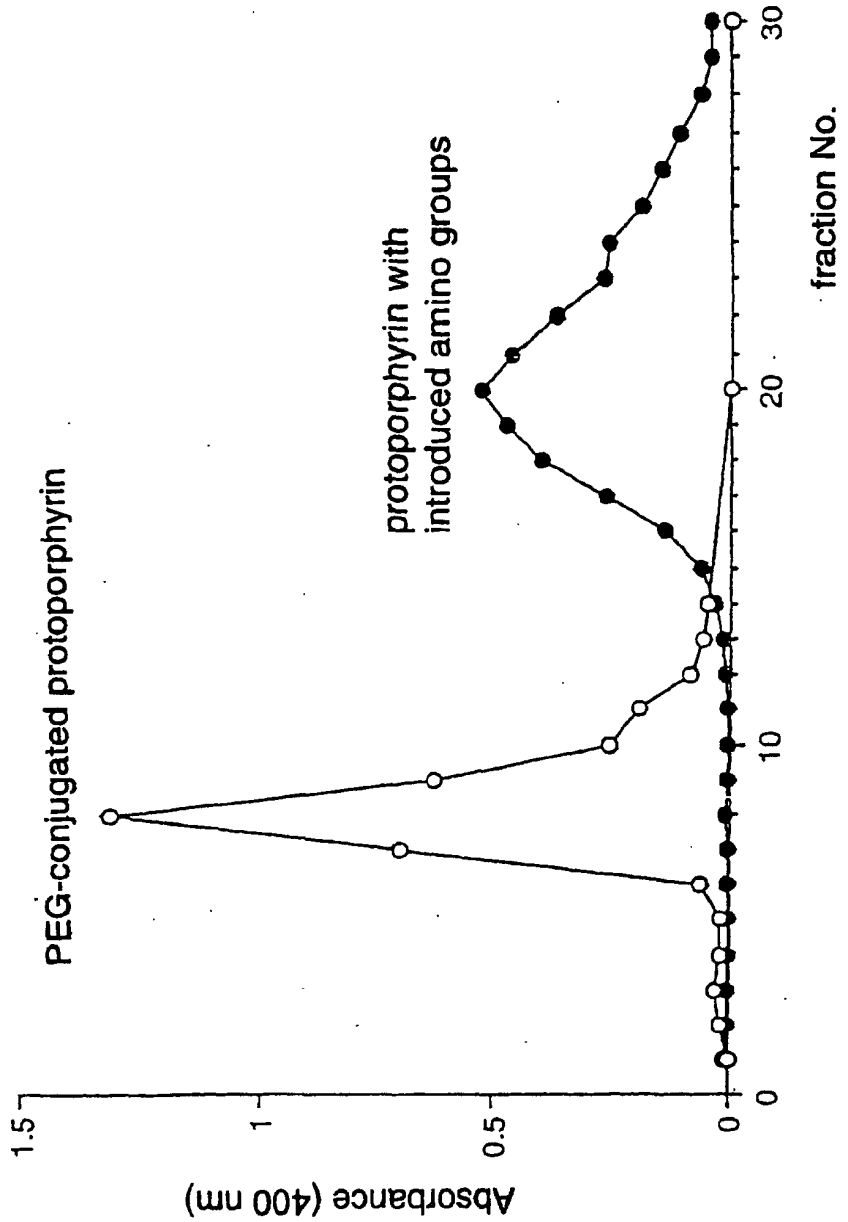

PEG-conjugated protoporphyrin
protoporphyrin with introduced amino groups
Absorbance (400 nm)
fraction No.
0
0.5
1
1.5
0
10
20
30

Figure 2

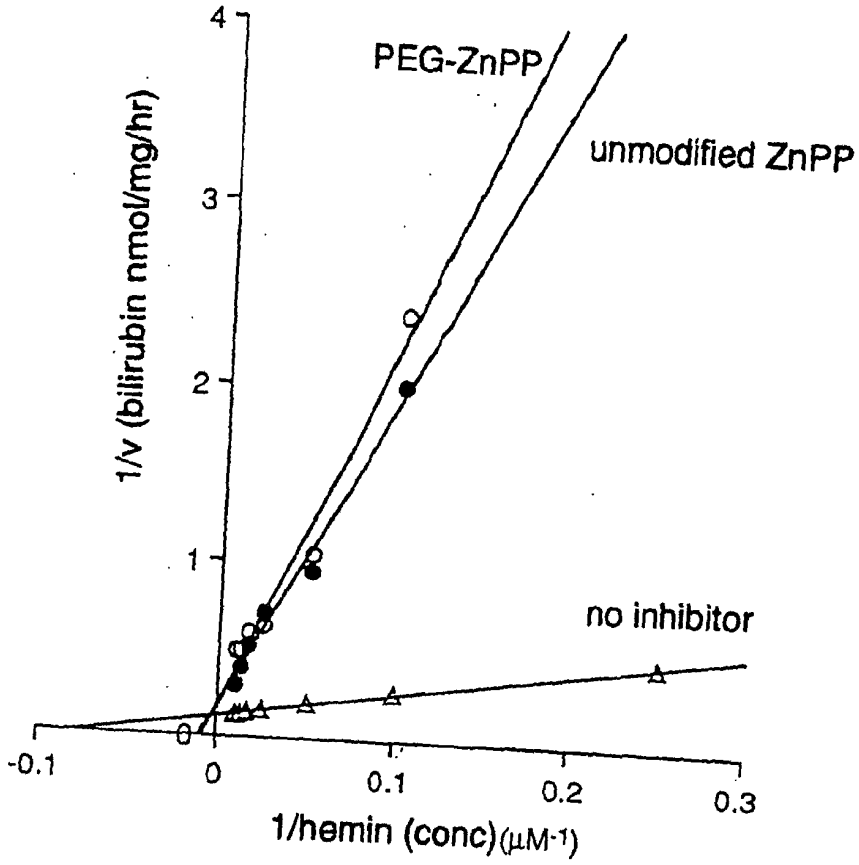

PEG-ZnPP
unmodified ZnPP
no inhibitor
1/v (bilirubin nmol/mg/hr)
1/hemin (conc)(μM-1)
4
3
2
1
0
-0.1
0
0.1
0.2
0.3

Figure 3

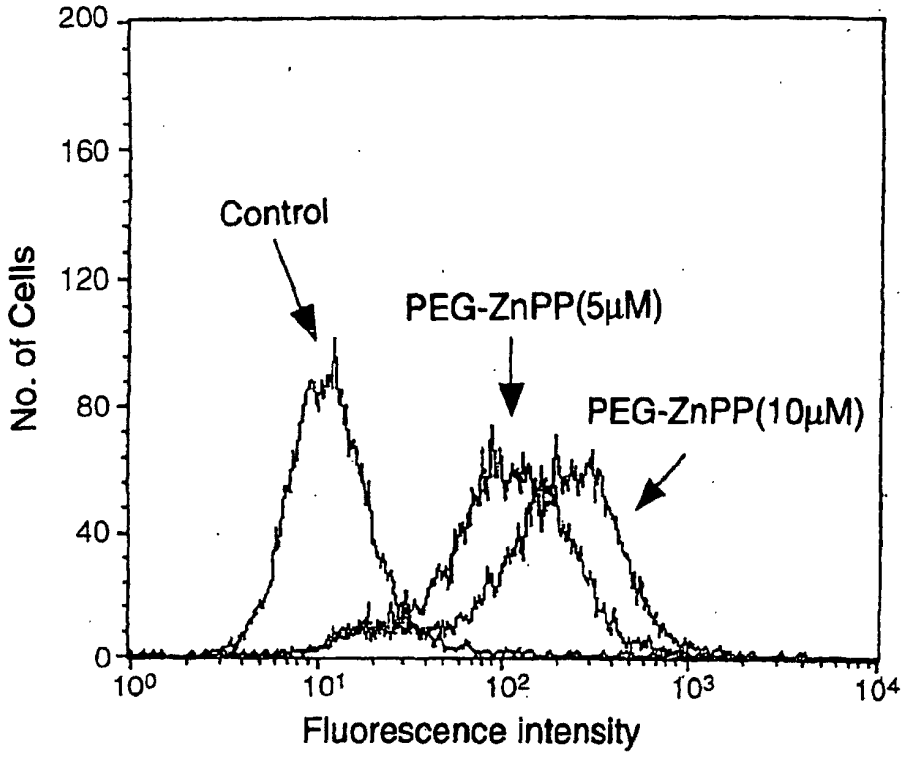

200
160
120
80
40
0
No. of Cells
Control
PEG-ZnPP(5μM)
PEG-ZnPP(10μM)
10⁰
10¹
10²
10³
10⁴
Fluorescence intensity

Figure 4

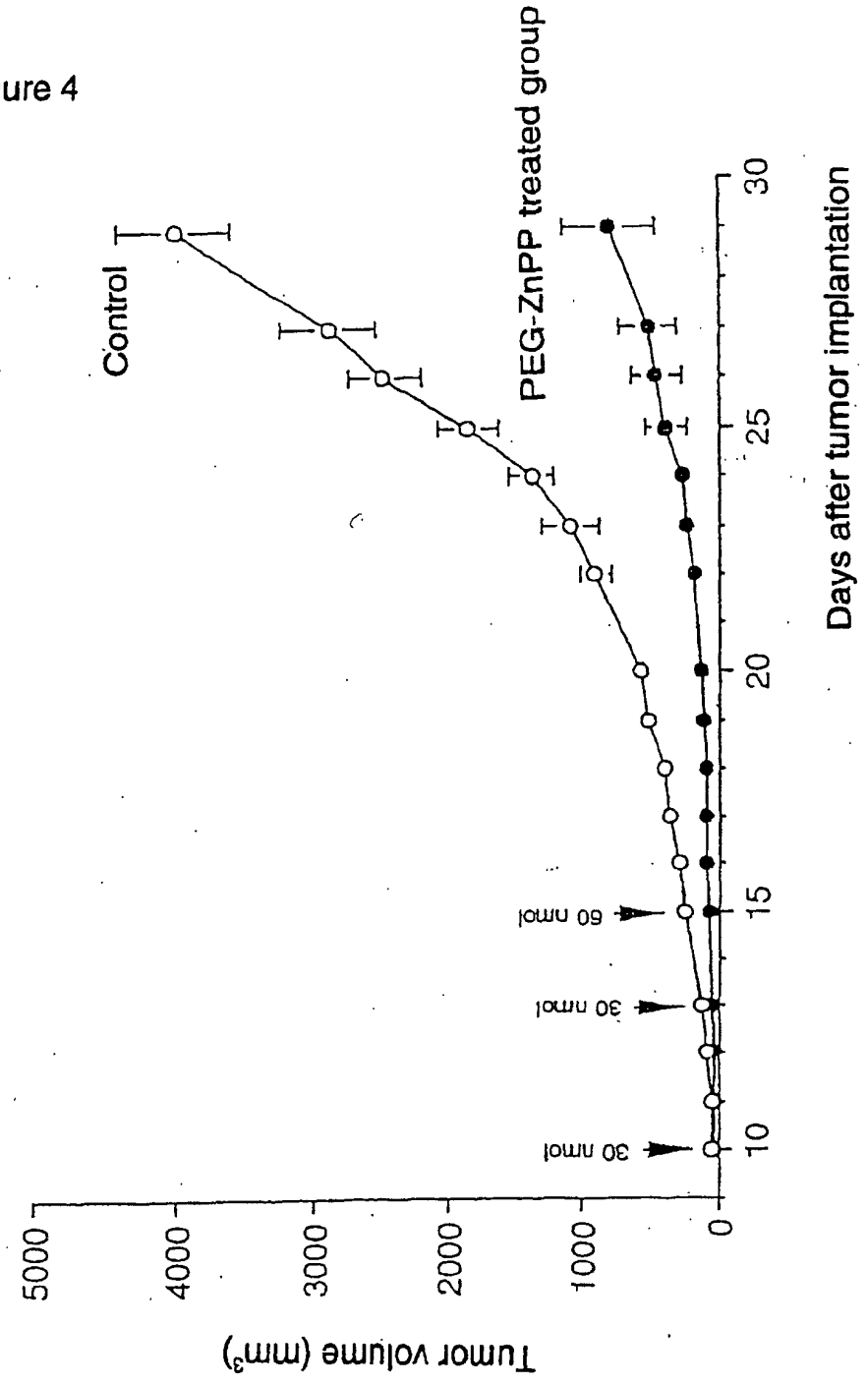
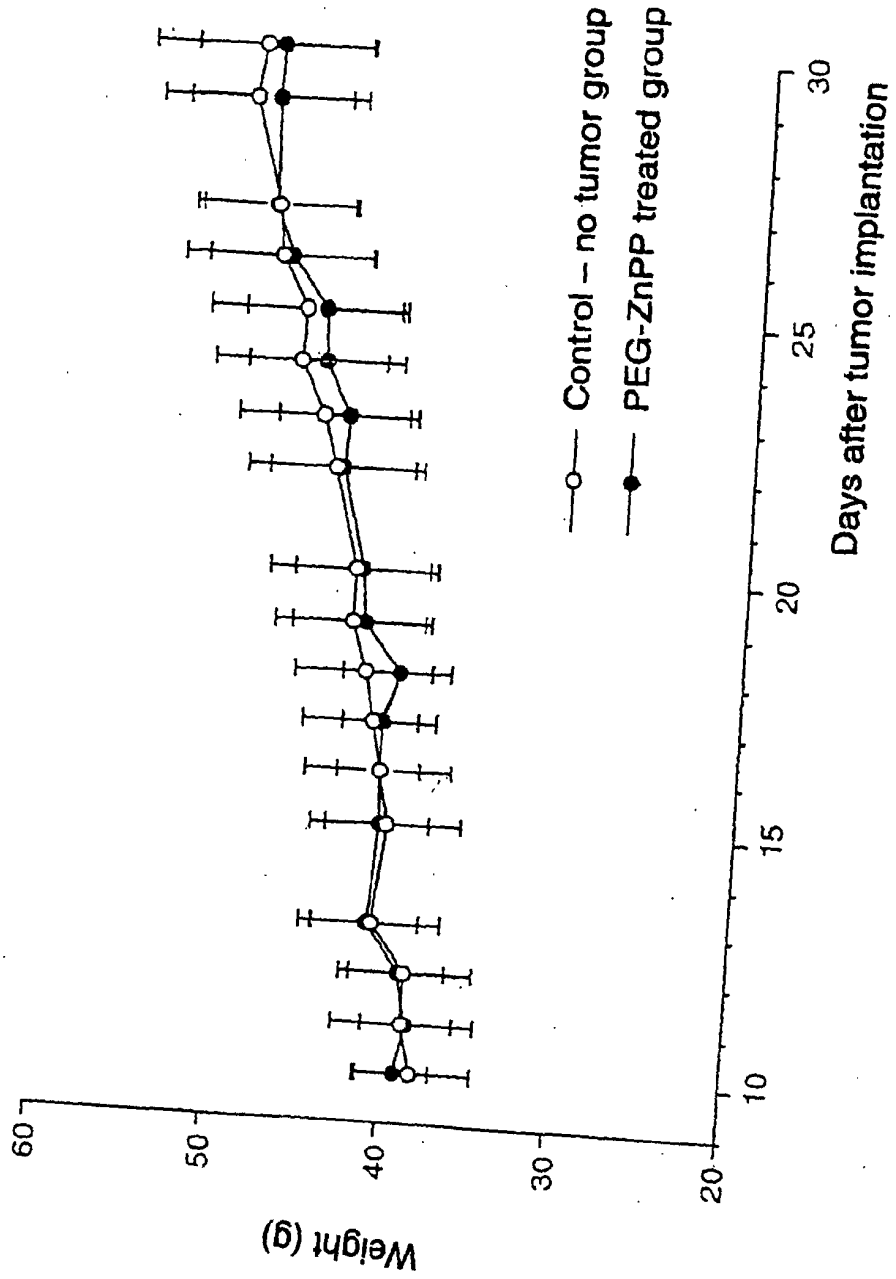

Control
PEG-ZnPP treated group
30 nmol
30 nmol
60 nmol
Tumor volume (mm³)
0
1000
2000
3000
4000
5000
10
15
20
25
30
Days after tumor implantation

Figure 5


Weight (g)
60
50
40
30
20
10
15
20
25
30
Days after tumor implantation
Control – no tumor group
PEG-ZnPP treated group

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP02/08707

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[7] A61K31/409, 47/48, A61P35/00, 43/00, C07D487/22

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[7] A61K31/409, 47/48, A61P35/00, 43/00, C07D487/22

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CAPLUS(STN), CAOLD(STN), REGISTRY(STN), WPI/L(DIALOG)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 91/18006 A (DIATRON CORP.), 28 November, 1991 (28.11.91), Concerning the compound in which PEG is bonded to porphyrin, see the claims, Fig. 1, lines 16 to 31 of paragraph 5, lines 14 to 23 of paragraph 6, and example 1 & EP 529002 A1 & US 5403928 A & JP 5-508015 A	1-7
Y	JP 55-144028 A (Hidetoshi TSUCHIDA), 10 November, 1980 (10.11.80), Concerning the compound in which PEG is bonded to protoporphyrin, see the whole specification. (Family: none)	1-7

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 November, 2002 (20.11.02)	17 December, 2002 (17.12.02)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP02/08707

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DE 2645079 A (BAYER ERNST), 13 April, 1978 (13.04.78), Concernig the compound in which PEG is bonded to porphyrin, see p.4. (Family: none)	1-7
Y	K. DOI, et al., "Induction of heme oxygenase-1 by nitric oxide and ischaemia in experimental solid tumors and implications for tumor growth", Br. J. Cancer, 1999, Vol.80, No.12, pages 1945 to 1954 Concerning the haem oxygenase inhibiting action and tumor suppression action of a zinc complex of protoporphyrin, see the whole article.	1-7
Y	US 5849259 A (INSTITUT FUR DIAGNOSTIKFORSCHUNG GMBH.), 15 December, 1998 (15.12.98), Concerning derivatives in which a side chain is introduced into protoporphyrin through ethylene -diamine and their preparing methods, see the compounds of working examples 2, 10-12 and the Claims. & WO 94/7894 A1 & JP 8-504399 A	6-7
P,X	S. K. SAHOO, et al., Pegylated zinc protoporphyrin : A water-soluble heme oxygenase inhibitor with tumor -targeting capacity, Bioconjugate Chem., 2002, Vol.13, No.5, pages 1031 to 1038	1-7
X		1-3
Y	Chemical Abstracts, 1995, Vol.124, abstract No. 219685 & Su BINGYIN, et al., Synthesis of water soluble derivatives of porphyrin metal complex and its effect on tumor cell growth, Zhongguo Yaoxue Zazhi (Beijing), 1995, Vol.30, No.12, pages 746 to 748	4-7

Form PCT/ISA/210 (continuation of second sheet) (July 1998)