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CA 2425271 C 2011/04/19

(11)(21) **2 425 271**

(12) **BREVET CANADIEN**
CANADIAN PATENT

(13) **C**

(86) Date de dépôt PCT/PCT Filing Date: 2001/10/03

(87) Date publication PCT/PCT Publication Date: 2002/04/18

(45) Date de délivrance/Issue Date: 2011/04/19

(85) Entrée phase nationale/National Entry: 2003/04/08

(86) N° demande PCT/PCT Application No.: ES 2001/000367

(87) N° publication PCT/PCT Publication No.: 2002/030397

(30) Priorité/Priority: 2000/10/10 (ES P 200002447)

(51) Cl.Int./Int.Cl. *A61K 9/127* (2006.01), *A61P 35/00* (2006.01)

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(54) Titre : LIPOSOMES D'ENCAPSULATION DE MEDICAMENTS ANTICANCEREUX ET UTILISATION DE CEUX-CI DANS LE TRAITEMENT DES TUMEURS MALIGNES

(54) Title: LIPOSOMES ENCAPSULATING ANTICANCEROUS DRUGS AND USE THEREOF IN THE TREATMENT OF MALIGNANT TUMOURS

(57) Abrégé/Abstract:
Liposomes encapsulating anticancerous drugs and the use thereof in the treatment of malignant tumours. The liposomes are coated with a lipopeptide composed of three substructures: a lipid fragment, an active oligopeptide and an oligopeptide spacer between the other two fragments. Applicable in intravenous administration for treatment of malignant tumours.

ABSTRACT

Liposomes encapsulating anticancerous drugs and the use thereof in the treatment of malignant tumours. The liposomes are coated with a
5 lipopeptide composed of three substructures: a lipid fragment, an active oligopeptide and an oligopeptide spacer between the other two fragments. Applicable in intravenous administration for treatment of malignant tumours.

LIPOSOMES ENCAPSULATING ANTICANCEROUS DRUGS AND USE THEREOF IN THE TREATMENT OF MALIGNANT TUMOURS

FIELD OF THE INVENTION

The present invention is related with procuring a system of anti-
5 cancerous treatment capable of destroying selectively the cancerous cells inside a living being without affecting the remaining cells of the treated organism. More particularly, the invention is related with liposomes containing anti-cancerous drugs of utility in the aforementioned treatment.

STATE OF THE TECHNIQUE

10 Cancer is one of the most widespread illnesses in developed countries and, specifically, tumoral metastases are the main cause of mortality in patients with solid malignant tumours. They consist of the appearance of a new cancerous centre, starting from a primary tumour, in another organ or different tissue. This metastasic process includes a series of sequential stages in which
15 the tumoral cells must interact with the cellular components and the tissues of the host. These stages are as follows: separation of tumoral cells from a primary tumour; invasion of intravascular space; migration through the vascular or lymphatic system to other tissues; adhesion to the vascular endothelium; extravasation and invasion of the new tissue; and formation of the secondary
20 tumour.

Throughout this process, the metastasic tumoral cells interact with the components of the extracellular matrices and, specifically, with the basal membranes, through their adhesion thereto, provoking their deterioration through the action of proteolytic enzymes produced by themselves and/or by the
25 actual host cells, stimulated by the tumoral cells. Thus, the alteration to the cellular adhesion properties is an indispensable element for the appearance of metastasis, since it is a process tied to the liberation of cells from the initial tumour, to their migration and to their implantation in new tissue.

The principal molecules of adhesion that intervene in this interaction
30 are the integrins. The integrins are a family of transmembrane glycoproteins, formed by two chains α and β joined by non-covalent bonds (hydrophobes). Among other functions, the integrins act a receptors of determined proteins of the extracellular matrix, like the Lamanin, the Fibronectin, the Vitronectin and the Collagen **(Ruoslahti, E., Giancotti, F.G., *Cancer Cells* (1989), 1, 4, 119-126).**
35 Recently that a change in the expression of the integrins in the tumoral cells has

been demonstrated whereby their presence in this type of cell is increased **(Dedhar, S., Saulmier, R., *Cell Biol.* (1990), 11, 481-489).** This increase is the responsible factor for the adhesion to the extracellular matrix and for the acquisition of metastasic potential.

5 The main treatment employed for the elimination of tumours is the administration of cytostatics by endovenous route, particularly those belonging to the anthracycline family **(Young, R.C., Ozols, R.F., Myers, C.E., *N. Eng. J. Med.* (1981), 305, 139-153).** But, due to their lack of selectivity with respect to the tumoral cells, to the development of resistance to these drugs by the 10 malignant cells and to the different response of the primary tumour and of the metastasis with respect to their action, this type of therapy usually gives rise to the appearance of serious secondary effects, some of which are of a chronic or irreversible nature.

Consequently, the main objective of current chemotherapy centres on 15 achieving an enhanced antitumoral effectiveness and reducing the toxicity of these drugs. One way of doing this could consist in getting the cytostatic, in a suitable concentration, to reach the target cells, resulting in the selective destruction of the primary tumour or the metastases without any healthy cell being affected. In this manner an increased therapeutic index of the drug would 20 be produced and more effective therapies achieved.

In these systems the drug is incorporated in the liposome aqueous spaces when it is hydrophilic or it is distributed between these and the lipid bilayers when it has a more lipophilic character. Once the drug is encapsulated, it can be administered to the patient under treatment.

25 Various researchers have shown that the use of liposomes for the administration of antineoplastics often enhances the traditional methods of administration, see, for example: **Gabizon et al.: Cancer Res. (1982) 42, 4734-4739 and Van Hossel et al.: Cancer Res. (1984) 44, 3698-3705.**

It has been observed, by means of the employment of various animal 30 models, that the encapsulation of doxorubicin in liposomes reduces significantly the secondary effects of toxicity, both chronic and acute. See, by way of example, **Rahman et al.: Cancer Res. (1980) 40, 1532-1537, Forssen et al.: Proc. Natl. Acad. Sci. USE (1981) 78, 1873-1877, Olson et al.: Eur. J. Cancer Clin. Oncol. (1982) 18 167-176, Rahman et al.: Cancer Res. (1985) 45, 796-** 35 **803 and Gabizon et al.: J. Natl. Cancer Inst.(1986) 77, 459-467.** Additionally,

other toxicity indicators, such as alopecia, weight loss, nausea, vomiting, and also dermal necrosis by extravasation can be reduced in a significant manner with the administration of doxorubicin in liposomes. **Forssen et al.: Cancer Treat. Rep. (1983) 67, 481-484;** see also the references cited above in this
5 paragraph.

It has likewise been established in various tumoral models that this significant reduction in toxicity is not produced at the expense of a diminution in antitumoral effectiveness. As well as the references cited above, see **Rahman et al.: Chemother. Pharmacol. (1986) 16, 22-27, Gabizon et al.: Cancer Res.**
10 **(1983) 43, 4730-4735 and Br. J. cancer (1985) 51, 681-689, Mayhew et al.: J. Natl. Cancer Inst. (1987) 78, 707-713, Forssen et al.: Cancer Res. (1983) 43, 546-550, and Storm et al.: Cancer Res. (1987) 47, 3366-3372.**

Given the incidence and the special characteristics of the cancerous metastases, antimetastasic therapy is one of the fields in which most effort has
15 been applied in the hunt for new alternatives to conventional treatments. For their mechanisms of action, their proven high effectiveness and their high toxicity, the anthracyclines form the family of cytostatics most studied in the field of drug encapsulation in controlled release systems such as the liposomes, which can be appreciated from the growing number of patents that have been
20 appearing under this heading, of which 80% of the total of those existing for liposomes are applied to cytostatics.

The first generation of liposomes containing anthracyclines corresponded to vesicles formed by PC, PG and cholesterol, in the aqueous interior space of which the drug was encapsulated. These liposomes showed a
25 diminution in the toxicity of the drug, though their antitumoral activity was no greater than that of the free drug, they only improved the activity of the drug in the case of tumoral models in which the metastasic cells were spread through the liver, the most accessible organ for the liposomes, but not when the tumoral growth is local **(Mayhew, E., Rustum, Y., *Biol. Cell.* (1983), 47, 81-86).** In
30 addition, due to their rapid capture by the macrophages of the endoplasmatic reticle, their permanence in the organism after the intravenous injection was reduced to a few hours. For this reason, and despite the intensive research carried out, that has been no formulation to satisfy the expectations initially placed on liposomes as transporters of cytostatics. It was during the eighties
35 that the situation changed with the appearance of the first publications in which

liposomes were described that presented glycolipids **(Allen, T.M., Hansen, C., Rutledge, J., *Biochem. Biophys. Acta* (1989), 981, 27-35; Mori, A., Klivanov, A.L., Torchilin, V.P., Huang, L., *FEBS Lett.* (1991), 284, 263-266)** or hydrophilic polymers like the polyethylene glycol (PEG) **(Blume, G., Ceve, C.,**
5 ***Biochem. Biophys. Acta* (1990), 1029, 91-97, Allen, T.M., Hansen, C., Martin, F., Redemann, C., Yau-Young, A., *Biochem. Biophys. Acta* (1991), 1066, 29-36)** on their surface for the purpose of augmenting their time of circulation in the blood stream, obtaining thereby the so-called "second generation liposomes" or "stealth liposomes". It seems this stabilising effect of
10 the PEG and the glycolipids is due to their hydrophilic properties which prevent aggregates being formed on the surface of the liposome and permit it not to be recognised as ligand of any cellular receptor nor of any plasmatic protein. Moreover, their presence on the surface of the liposomes produces a steric effect, as it hinders the action of the opsonins and other blood proteins, and
15 reduces the accessibility of the macrophage receptors to the phosphate groups of the phospholipids, which results in an increased time of circulation in the blood.

Subsequently some authors found that it was possible to improve the stability and effectiveness characteristics of these liposomes through the
20 incorporation of additives which inhibited lipid peroxidation like vitamin E acetate, BHT or those derived from chromans **(EP-0274174, WO-8500968, WO-9202208 and US-5605703).**

Despite the fact that these galenic forms offered a series of benefits over conventional forms, there remains pending the possibility of steering the
25 vesicles to target cells in order to improve the effectiveness with smaller drug doses and of suppressing or reducing secondary effects.

The basic idea is to incorporate on the surface of the liposome any chemical body capable of being recognised selectively by the target cells. The success in steering the liposomes toward the target cells resides in an adequate
30 choice of vector molecule.

The process of selecting any chemical structure capable of steering the liposomes to tumoral cells is by no manner trivial, as specifically in the case of tumoral cells there exists a great diversity of proteins located in the membrane as well as of antigens and surface receptors that vary according to the
35 metastasic potential, the proliferating activity and the tissue in question, in such

a manner that, although they could be used as a base for selecting recognition structures, in practice the choice is not immediate. Furthermore, in many cases, the true situation is that the proliferating and/or tumoral cells have as a differentiating feature the over-expression of determined structures with respect to normal cells.

Knowledge accumulated to date indicates that the adhesion processes and the proteins involved play an essential role in the development of the metastasic process and of the necessary vascularization for cellular proliferation.

From among the proteins most involved in these mechanisms, Laminin has proved to be a good candidate for steering liposomes, since it has been conclusively shown that its receptors are over-expressed in the tumoral cells.

Laminin is the majority component of the basal membrane of cells after collagen. It is a glycoprotein formed by three polypeptide chains: α (440 kDa), β (200 kDa) and γ (220 kDa), which are arranged in the shape of a cross, with two short arms and one long arm. The bonds between the chains are formed by di-sulphide links and by interactions of the non-covalent type, forming an asymmetric molecule in which different structural domains are located.

The cells have different specific membrane receptors that recognise peptide sequences and/or functional domains of the molecule of the Laminin. These receptors can be classified into two groups: integrins and non-integrins.

The integrins are formed by two trans-membrane polypeptide chains, α and β, in non-covalent association. These molecules are the receptors through which the cells adhere to the components of the extracellular matrix. Some of them also intervene in the cell-to-cell recognition. Each one recognises specific peptide sequences which are present in the molecules of the matrix, like for example Laminin.

Among the non-integrin-type receptors, that most studied is the receptor for 67 kDa for Laminin, and it has been isolated and identified starting from various cellular tissues, among which are the carcinomas. Moreover, it has been verified that the metastasic tumoral cells express on their cellular surface more receptors for Laminin than normal cells, for which reason this receptor could be considered to be a marker of tumoral progression and an indicator of the aggressivity of many types of tumour.

The Laminin presents various metastasic activities, such as:

- causing cellular adhesion, growth and extension;
- stimulating the distinction between epithelial and tumoral cells;
- provoking cellular migration;
- facilitating malignancy of tumoral cells by their presence on the surface, which makes their invasivity and metastasic activity increase.

Determined studies have shown the different functions of Laminin are mediatized by specific peptide sequences present in the Laminin molecule, such as: the sequence of five amino-acids SIKVAVS, that is found located in the fragment PA22-2 of the Laminin α chain. Specifically the most active zone of this region is the pentapeptide SIKVAVS.

SUMMARY OF THE INVENTION

At present, studies on liposomes are directed at their steering towards or targeting of tumoral cells through incorporation on the surface of the ligand vesicles, as may be antibodies, peptides and proteins, capable of recognising and linking specifically with this type of cell (Allen T.M., Austin, G.A., Chonn, A., Lin, L., Lee, K.C., *Biochem. Biophys. Acta* (1991), 1061, 56-63).

Thus, an object of an aspect of the present invention consists of a new application for anticancerous drugs encapsulated in liposomes, which present as main characteristic their being covered by lipopeptides proceeding from the structure of the laminins – especially the SIKVAVS sequence - in such a manner that the liposomes so prepared demonstrate a high selectivity regarding tumoral cells and therefore increase the effectiveness of the encapsulated anticancerous drug.

Accordingly, in an aspect of the present invention there is provided liposomes encapsulating anticancerous drugs comprising a coating with a lipopeptide composed of three substructures: a lipid fragment, an active oligopeptide targeting tumoral cells and an oligopeptide spacer between the other two fragments, wherein said oligopeptide spacer is inactive with respect to Laminin and has a length lying between five and ten amino acids.

In accordance with a final aspect of the present invention, there is provided liposomes encapsulating anticancerous drugs comprising a coating with a lipopeptide composed of three substructures: a lipid fragment, an active

oligopeptide derivatized from laminin targeting tumoral cells and an oligopeptide spacer between the other two fragments, wherein said oligopeptide spacer is inactive with respect to Laminin and has a length lying between five and ten amino acids.

5

MEANING OF THE ABBREVIATIONS USED IN THE INVENTION

DXR: Doxorubicin

PC: Phosphatidylcholine 6a

10

PL: Phospholipids of hydrogenated egg

PG: Phosphatidylglycerol

CHOL: Cholesterol

CROM: Chroman-6

A Ala Alanine

C	Cys	Cisteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycin
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

Lam2M:	myristoyl-PEGAD (SEQ. ID. NO: 5)
Lam9:	myristoyl-YESIKVAVS (SEQ. ID. NO: 6)
Lam9Cys:	myristoyl-CYESIKVAVS (SEQ. ID. NO: 7)
Lam9Cys-b-Ala:	myristoyl-AAAAACYESIKVAVS (SEQ. ID. NO: 8)
AG10:	GYSRARKEAASIKVAVSARKE (SEQ. ID. NO: 9)
(E8)-2-4G:	NPWHSIYITRFG (SEQ. ID. NO: 10).
mir:	myristoyl
DOX:	Doxorubicin

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 represents the percentages of cellular adhesion by Laminin-1 and different synthetic peptides.

Figure 2 represents the percentage of inhibition of cellular adhesion to Laminin (complete molecule) by different peptides of the Laminin at different concentrations.

Figure 3 represents the percentage of cellular survival or cytostatic

activity for doxorubicin liposomes with different coating lipopeptides at different concentrations of doxorubicin.

5 Figure 4 represents the concentration of doxorubicin in plasma at different times after the intravenous administration for a sample of liposomes of the invention and for a sample of free doxorubicin.

Figure 5 represents the concentration of doxorubicin in tissue at different times after the intravenous administration for a sample of liposomes of the
10 invention and for a sample of free doxorubicin.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is related with the preparation and use of liposomes containing anticancerous drugs.

The liposomes of the present invention have as their main
15 characteristic that they are coated with fragments of hydrophobically derivatized peptides (overlaid lipopeptides) in such a manner that the liposome so prepared offer a high targeting capability with regard to tumoral cells, thereby increasing the effectiveness of the encapsulated anticancerous drug.

Surprisingly it was shown that the active overlaid lipopeptides in vitro prior to being incorporated in liposomes, would totally lose their targeting capability when incorporated in liposomes, for which reason, according to the invention, peptide spacers were developed which, intercalated between the sequence and the lipophile chain of the overlaid lipopeptide would, strangely, permit the targeting ability of the active peptide sequence to be maintained.

In this way the structure of the overlaid lipopeptide (hydrophobically derivatized peptide) is as follows:

Lipid Fragment Spacer Active Sequence

Consequently, the object of the present invention resides in the preparation and use of liposomes containing anticancerous drugs which on their surface have peptide fragments derivatized from Laminin (overlaid lipopeptides), made up of the following three structural blocks: a lipid fragment, an active oligopeptide and an oligopeptide spacer among other fragments.

The lipid fragments are fatty acids of carbonated chain length between C6 and C20. More specifically decanoyl, myristoyl and stearoyl.

The spacer fragment consists of oligopeptides inactive with respect to Laminin having a length lying between five and ten amino acid residues. More specifically, with a length of seven to nine amino acid residues, and more specifically the sequences AAAAACYE (SEQ. ID. NO:1), SSAAACYE (SEQ. ID. NO:2) and RKERKECYE (SEQ. ID. NO:3.)

The active sequence consists of the oligopeptide SIKVAVS (SEQ. ID. NO:4.)

The liposome-forming lipids are well known. The ratio of total liposome-forming lipids to drug lies between 20:1 and 2:1, and is preferably 10:1. Generally phospholipids are included, with net neutral or negative charge, and a sterol, like cholesterol. The choice of the lipids is performed based on the requirements with respect to the final liposome size, to the drug to be encapsulated and to the desired stability for the preparation. Usually the largest lipid component of the liposomes is the phosphatidyl choline (PC). The PCs differ from each other in the length and

degree of saturation of their acylic chains and can isolated from natural or synthesised sources. The inclusion of a negatively charged phospholipid favours
5 the stability of the liposome solution and prevents the spontaneous aggregation of the liposomes.

The negatively charged phospholipids most employed are the phosphatidyl glycerol (PG), phosphatidyl serine (PS) and the phosphatidyl

inositol (PI), among others. The proportion used, of neutral phospholipid to negatively charged phospholipid ranges from 10:2 to 10:10 respectively. The
5 inclusion of cholesterol generally favours the stability of the liposomes by causing the permeability of the membrane to diminish with respect to ions and small polar molecules and likewise reduces the penetration capacity of a series of proteins between the bilayers that could result in a greater disorder among these. Typically the proportion of cholesterol used runs from 0 to 50% of total
10 lipids.

Optionally, the liposomes object of the present invention, can contain additives that permit enhancement of their stability properties or reduce the toxicity of the encapsulated drug. For example, mention can be made of the lipid oxidation inhibitors such as those described in the **patents US 5605703, EP**
15 **0274174, WO-8500968 and WO 9202208.** Such lipid oxidation inhibitors include, for example, Vitamins and their derivatives like Vitamin E or Vitamin E acetate, an antioxidant authorized for pharmaceutical use like BHT or a chroman or chromen, such as 3,4-dihydride-2,2-dimethyl-6-hydroxy-7-methoxy-2H-1-benzopyrane.

20 The proportion of coating lipopeptide with respect to the total amount of lipids lies between 0.1% and 30%, preferably between 1% and 15%.

The anticancerous drugs that can be encapsulated in the liposomes of the present invention include, but are not limited to:

Nirogenated mustard analogues like Cyclophosphamide; Melphalan;
25 Iphosphamide; or Trophosphamide;

Ethylenimines like Thiotepa;

Nitrosoureas like Carmustine;

Alkylating agents like Temozolomide; or Dacarbazine;

Analogous antimetabolites of Folic acid like Methotrexate or Raltitrexed;

30 Analogues of Purines like Thioguanine, Cladribine or Fludarabine;

Analogues of Pyrimidines like Fluorouracil, Tegafur or Gemcitabine;

Alkaloids of Vinca and analogues like Vinblastine, Vincristine or Vinorelbine;

Derivatives of Podophyllotoxin like Etoposide, Taxanes, Docetaxel or Paclitaxel;

Anthracyclines and similar like Doxorubicin, Epirubicin, Idarubicin and
35 Mitoxantrone;

Other cytotoxic antibiotics like Bleomycin and Mitomycin;
Platinum compounds like Cisplatin, Carboplatin and Oxaliplatin;
5 Monoclonal antibodies like Rituximab;
Other antineoplastic agents like Pentostatin, Miltefosine, Estramustine, Topotecan, Irinotecan and Bicalutamide.

In accordance with that described above, the liposomes of the present invention present the following characteristics:

10 a) A lipid concentration of 1 and 100 mg/ml, and preferably

around 10 mg/ml.

b) The component lipids are phospholipids, of both natural and synthetic origin, and cholesterol.

5 c) The proportion of cholesterol, with respect to the quantity of total lipids, is between 0 and 50%, preferably between 35 and 50%.

d) The phospholipids present are phosphatidyl choline, which has no net charge, and optionally another, negatively-charged phospholipid, preferentially phosphatidyl glycerol.

10 e) The ratio of the neutral phospholipid to that negatively charged lies between 10:2 and 10:10 and preferably between 10:7 and 10:10 respectively.

f) Optionally the liposomes can contain other additives like for example lipid oxidation inhibitors like those described in the

15 **patents US 5605703, EP 0274174, WO-8500968 and WO-9202208.**

g) The concentration of peptide would oscillate between 0.1 and 1 mg/ml, and preferably around 0.5 mg/ml.

h) The liposomes are formed in an aqueous solution, tamponed or

20 not, physiologically isotonic. For example, 0.9% NaCl.

i) The size of the liposomes shall be in any case less than 500 nm, and preferably less than 300 nm and more specifically between 50 nm and 250 nm.

Preparation of the liposomes and incorporation of the drug:

25 A preferred method is that presented by **Bangham et al.** ("Diffusion of univalent ions across the lamellae of swollen phospholipids" Bangham AD, Standish MM, Watkins JC, J. Mol. Biol.1965, 13, 238-252). in which multilamellar liposomes (MLVs) are obtained which heterogeneous in size. In this method the forming lipids are dissolved in a suitable organic solvent that is subsequently removed by rotary

30 evaporation under vacuum. The lipid film formed is subjected to hydration with an adequate aqueous medium containing the drug, by means of manual or mechanical agitation. The heterogeneous suspension of MLVs is subjected to whatever of the known procedures for reduction and homogenisation of sizes. For example, two preferred procedures are that of sonication with Titanium probe to obtain SUV

35 liposomes and the extrusion through polycarbonate filters of the MLV solution to obtain VET liposomes.

Preparation of the peptide fragments:

The sysnthesis of the peptides is carried out using the solid phase
5 method of **Merrifield (1962)**) ("Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide" Merrifield et al., meeting of the Federation of the American Societies for Experimental Biology (FASEB), 1962, Merrifield R.B., J. Am. Chem. Soc. 1963, 85, 2149-2154) with Fmoc/tBu approach.

Incorporation of lipopeptide:

10 The lipopeptides employed were acyl-oligopeptides, being of preference the acyl group with linear saturated hydrocarbon chains of length C6 to C20 – preferentially the decanoyl, myristoyl or stearoyl. The lipopeptides were mixed with the rest of the components that were to constitute the liposomes, or else they were incorporated in the liposomes by incubation at 60° C of these
15 lipopeptides and the vesicles, since, as the bilayers were in a gel state, permitted the incorporation of the hydrophobic part of these derivatives in their interior. In both cases the hydrophobic zone of the derivatives ought to remain forming the bilayer, whilst the peptide sequence would remain on the hydrophilic exterior.

20 By way of illustration, but not restrictively, the procedure detailed in the present patent is described hereunder by means of several practical examples.

EXAMPLE 1: Synthesis of active peptides with carboxylic end:

The synthesis of peptides derived from Laminin is carried out following the solid phase method of **Merrifield (1962)** ("Solid Phase Peptide Synthesis. I.
25 The Synthesis of a Tetrapeptide" Merrifield et al., meeting of the Federation of the American Societies for Experimental Biology (FASEB), 1962, Merrifield R.B., J. Am. Chem. Soc. 1963, 85, 2149-2154) with Fmoc/tBu approach.

In order to obtain a sequence with carboxylic end, as the solid synthesis support, a **Wang** resin is employed with a degree of functionalisation of 0.72
30 meq/g of resin which was submitted to the treatment outlined in the table below:

35

Step	Reagent	Repetitions	Time
1	DMF	3 times	1 minute
2	Dichloromethane	3 times	1 minute
3	Tertiary amyl alcohol	3 times	1 minute
4	Ether	Until dry	-

Table 1: Washing protocol for the peptidyl resin

In general, the starting point was 1 gram of Wang resin in a syringe with a
5 filter coupled to a vacuum system, and it was dimethylformamide (DMF) was blown in for 30 minutes. In parallel, in a filter weight scale, the necessary amount was weighed of the first Fmoc-amino acid and it was dissolved in DMF,

adding to this solution the coupling agents 4-dimethyl amino pyridine (4-DMAP) and diisopropylcarbodiimide(DIPCDI)(0.3:1, molar). All the reagents were used in an excess of 5 times with respect to the quantity required to complete the reaction. Thereafter, this mixture was added to the previously drained resin and 5 left to react for 2 hours at room temperature with occasional stirring. After this time the resin was washed with different solvents until completely dry.

Finally the joint quantity of amino acid was evaluated. In the cases where the reaction was incomplete, more reagents were added, in a quantity corresponding to one half the initial quantity employed, and left to react for a 10 further two hours, repeating thereafter the same process of resin-drying and quantifying of the amino acid incorporated.

The union of the remaining Fmoc-amino acids was carried out through successive stages of deprotection of the amino group and formation of the amide bond.

15 Thus, for the suppression of the Fmoc amino protector group, the peptidyl-resin was treated once with DMF/piperidine 20% for a minute, the treatment being repeated a second time for 5 minutes. Afterwards, the piperidine was removed with various washings with DMF and the ninhydrin test was carried out to check for the complete elimination of the Fmoc group (blue 20 colouring). In some cases the deprotection was performed with the reagent 1.8-diazabicyclo [5.4.0.]-undec7-eno (DBU) used in the mixture of DMF/piperidine/DBU (48:2:2, v/v/v) by means of a single treatment of the resin for 7 minutes. At the end of this time the resin was washed various times with DMF and the ninhydrin test performed again just as in the previous case.

25 Once the peptidyl-resin was unprotected, the pertinent Fmoc-amino acid was added to it and the coupling reagents. Depending on the difficulty the synthesised sequence presented, two different combination of reagents were used:

HOBt and DIPCDI, in the molar proportion 1:1, with the Fmoc-amino acid.

30 HOBt, DIEA and 2-(1H-benzotriazol-1-il)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU), in the molar proporation 1:2:1.

All the reagents were used in an excess of 2.5 times with respect to the quantity necessary.

In both cases the reaction was left for 1 hour, the conclusion being 35 controlled by means of the ninhydrin test for the disappearance of the free

amino groups (yellow colouring). When the reaction was not complete, the mixture was left in contact with the resin for 1 more hour, after which the ninhydrin test was repeated. In the event that there were still free amino groups in the resin, the latter was washed several times with DMF and the reagents
5 were added again in half the quantity initially employed. On some occasions, and despite the reaction being repeated, incomplete couplings were produced. In order to be able to continue with the synthesis without anomalous chains being formed, it was necessary to block the incomplete chains by acetylation of the amino groups that still remained free. To this end, the resin was treated with
10 2 mequivalents of acetic anhydride and 1 mequivalent of 4-DMAP for each mequivalent of peptidyl-resin during 30 minutes. Next it was washed with DMF and a ninhydrin test was run to check the total disappearance of the amino groups (yellow colouring).

The ninhydrin test was substituted by the chloranil test in the event of
15 detecting secondary amino groups of amino acids like proline, since the ninhydrin does not react with said groups.

EXAMPLE: Sysnthsis of the peptides with terminal amino end:

The peptides with carboxamide end are obtained from the resin p-methylbenzhydrylamine (MBHA). This resin needs a special initial treatment
20 which comprises various washings with an acidic mixture of DCM/TFA at 40%, being left finally in contact with the resin for 20 minutes. Afterwards, to remove the acid, it was washed 5 times with DCM for 1 minute each time, and to neutralise it, the resin was treated with the base mixture DCM/diisopropylethylamine (DIEA) at 5%, until it was found that the resin pH
25 was base. Finally, to remove the DIEA, it was washed various times with DCM.

Next, the coupling was carried out of the acidic spacer p-[(R,S)-alpha[1-9H-fluorene-9-e)-methoxy formamide]-2,4-dimethoxybenzyl]-phenoxyacetic (AM), protected with the Fmoc group, which is what provides the sequence with its amide end. For this, the Fmoc-AM was weighed in an excess of 1.5 times the
30 quantity required, and it was added to the resin together with the reagents hydroxybenzotriazole (HOBt) and DIPCDI (1:1, molar), also in excess, leaving the reaction to take place for 90 minutes. The conclusion of the reaction was determined by means of the Kaiser test or ninhydrin test, checking for the disappearance of free amino groups from the resin. In the event that all the
35 spacer had not linked, the reaction was repeated once more, using half of the

initial quantity of reagents employed. Once all the spacer had linked, the resin was washed various times with DMF in order to remove the reagents in excess.

The coupling of the remaining Fmoc-amino acids was performed through successive stages of deprotection of the amino group and of amide link formation, just as described in Example 1.

EXAMPLE 3: Deprotection and de-anchoring of the peptide

For the deprotection of the free peptide sequence, the Fmoc: group is first removed from the terminal-amino end following the protocol given in the table below:

Step	Reagent	Repetitions	Time
1	DMF	3 times	1 minute
2	DMF/piperidine 20%	1 time	1 minute
3	DMF/piperidine 20%	3 times	5 minutes
4	DMF	3 times	1 minute
5	DCM	3 times	1 minute
6	Tertiary amyl alcohol	3 times	1 minute
7	Ether	Until dry	

Table 2. Deprotection and de-anchoring protocol of the peptide

In some syntheses the DMF/piperidine at 20% is replaced with the mixture DMF/piperidine/DBU (48:2:2, v/v/v), which is left in contact with the peptidyl-resin for 7 minutes.

Next the peptide was de-anchored from the resin and the protector groups removed from the amino acid functional chains, in a single step. To achieve this, various TFA mixtures were prepared with different scavengers like anisol, thioanisole, phenol, mercaptoethanol, and water, according to the protector groups present in the peptide chains. An aliquot was weighed of the peptidyl-resin in a syringe with filter coupled to a vacuum system and the acidic mixture of scavengers added to it, being left in contact with the resin for 2 to 3 hours, at room temperature with occasional stirring. After this time elapsed, the resin was filtered and washed 3 times with TFA, the filtrates and the washing products being collected in a tube. First the TFA was evaporated

off with Nitrogen and afterwards cold diethyl ether was added, obtaining a white precipitate (free peptide). The precipitate was centrifuged at 3000 rpm for 15 minutes, the supernatant being drained off and the process repeated 5 more times. Finally the traces of ether were removed from the solid with Nitrogen, it was re-dissolved in water or acetic at 10%, depending on the peptide solubility, and lyophilised to obtain the raw free peptide product completely dry.

EXAMPLE 4: Hydrophobic derivatization of the peptide sequences in solid phase:

The fatty acids were coupled to the sequences in the same form as the Fmoc-amino acids, by means of the formation of an amide bond with the carboxylic group of the fatty acid.

Thus, an aliquot was weighed of the peptidyl-resin in a syringe with filter attached to a vacuum pump and was swollen with DMF. Next the deprotection of the Fmoc group was carried out. Once deprotected, the fatty acid employed in each case was added, in an excess of 2.5 times, together with the systhesis reagents DPCDI/HOBt or, TBTU/DIEA/HOBt, depending on the peptide sequence in question. The conclusion of the reaction was determined, just as during the synthesis, by the ninhydrin test for disappearance of free amine groups.

To obtain the free hydrophobic derivative, the peptidyl-resin was treated with the same acidic mixture of TFA and scavengers, and under identical conditions to those employed in the de-anchoring of the initial peptide sequence.

EXAMPLE 5: Obtaining liposomes containing Doxorubicin and a lipopeptide covering the surface of the liposome:

Initially, and in all cases, large multilamellar liposomes (MLV) were prepared following the method described by Bangham ("Diffusion of univalent ions across the lamellae of swollen phospholipids" Bangham AD, Standish MM, Watkins JC, J. Mol. Biol.1965, 13, 238-252). From these, and by sonication, the small unilamellar liposomes (SUV) were obtained.

All the material and the solutions employed were sterile and, during the whole process, the work was carried out under a laminar flow hood to maintain sterility.

The liposomes prepared with the hydrophobic derivatives of the two active sequences had in their composition: phosphatidyl choline (PC), phosphatidyl glycerol (PG), cholesterol and Chroman-6. To obtain them the

following procedure was adopted:

Thus, in the first place, SUV liposomes were prepared. The PC, PG, cholesterol and the Chroman-6 were weighed, and dissolved in Chloroform, the solvent being evaporated off in the rotary evaporator in order to form a lipid film. Any traces of solvent that might remain were removed by lyophilisation lasting 1 hour.

After this period had elapsed, the film was hydrated with 1 mL of NaCl at 0.9%, maintaining the ball in a bath at 60 degrees Celsius for 1 hour. To the MLV liposomes obtained, 1.2 mL of a Doxorubicin solution was added having a concentration equal to 2 mg/mL (2.4 mg). The preparation was left in repose for 15 minutes in a bath at 60 degrees Celsius and afterwards the ball was kept in a vacuum-free rotary evaporator which turned slowly for a period of 20 minutes.

In order to obtain SUV liposomes, the MLV were subjected to sonication in an ultrasonic bath for 8 cycles each lasting 2 minutes, separated by 5-minute intervals of repose in a bath at 60 degrees Celsius.

The incorporation of the lipopeptides was carried out by mixing an aliquot of 200 µL of liposomes, 200 µL of NaCl at 0.9% and 12 µL of a solution of lipopeptide in DMSO (c=10 mg/mL). The mixture was left in repose at 60 degrees Celsius for one hour and afterwards at room temperature for a further 30 minutes.

Alternatively, the liposomes were prepared incorporating the lipopeptide from the beginning. Thus, the PC, PG, Cholesterol and Chroman-6 lipids were mixed with an aliquot of the lipopeptide dissolved in chloroform/methanol, in the same molar ratio as in the previous case. The rest of the procedure is identical to the previous case.

Finally, to remove the Doxorubicin not encalsulated and the lipopeptide not incorporated, the sample was placed in a PD-10 column (Sephadex G-25). For this, the column was first balanced with NaCl at 0.9%. Once balanced, the sample was added, which was also eluted with NaCl at 0.9%, until it overflowed from the column. The volume of liposomes obtained was made up to 2 mL.

Following this process, the following types of liposomes were prepared incorporating Doxorubicin:

Lipid composition	Conc. of Lipids	Conc. of drug	Coating lipopeptide	Conc. of peptide	Liposome size
PC/PG/Chol./Chr.	9.91 mg/mL	1.04 mg/mL	Myristic-$(A)_5$-CYESIKVAVS (SEQ. ID. NO:7)	0.42 mg/mL	160 nm
PC/PG/Chol./Chr.	14.05 mg/mL	1.5 mg/mL	Myristic-PEAGD (SEQ. ID. NO:5)	1.1 mg/mL	115 nm

5 **EXAMPLE 6:** Obtaining liposomes containing Paclitaxel and a lipopeptide coating the the surface of the liposome:

Initially, and in all cases, large multilamellar liposomes (MLV) were prepared following the method described by Bangham ("Diffusion of univalent ions across the lamellae of swollen phospholipids" Bangham AD, Standish MM,
10 Watkins JC, J. Mol. Biol.1965, 13, 238-252). From these, and by sonication, small unilamellar liposomes were obtained.

All the material and the solutions employed were sterile and, during the whole process, the work was carried out under a laminar flow hood to maintain sterility.

15 The liposomes prepared with the hydrophobic derivatives of the active sequences had in their composition: phosphatidyl choline (PC), phosphatidyl glycerol (PG) and cholesterol. To obtain them the following procedure was adopted:

Thus, in the first place, SUV liposomes were prepared. The PC, and
20 cholesterol were weighed, and dissolved in Chloroform, the solvent being evaporated off in the rotary evaporator in order to form a lipid film. Any traces of solvent that might remain were removed by lyophilisation lasting 1 hour.

After this period had elapsed, the film was hydrated with 1 mL of NaCl at 0.9%, maintaining the ball in a bath at 60 degrees Celsius for 1 hour. To the
25 MLV liposomes obtained, 1.2 mL of a Paclitaxel solution was added having a concentration equal to 0.5 mg/mL (0.6 mg). The preparation was left in repose

for 15 minutes in a bath at 60 degrees Celsius and afterwards the ball was

5 kept in a vacuum-free rotary evaporator which turned slowly for a period of 20 minutes.

In order to obtain SUV liposomes, the MLV were subjected to sonication in an ultrasonic bath for 8 cycles each lasting 2 minutes, separated by 5-minute intervals of repose in a bath at 60 degrees Celsius.

10

The incorporation of the lipopeptides was carried out by mixing an aliquot of 200 μL of liposomes, 200 μL of NaCl at 0.9% and 12 μL of a solution of lipopeptide in DMSO (c=10 mg/mL). The mixture was left in repose at 60 degrees Celsius for one hour and afterwards at room temperature for a further
5 30 minutes.

Alternatively, the liposomes were prepared incorporating the lipopeptide from the beginning. Thus, the PC, PG and Cholesterol lipids were mixed with an aliquot of the lipopeptide dissolved in chloroform/methanol, in the same molar ratio as in the previous case. The rest of the procedure is identical to the
10 previous case.

Finally, to remove the Paclitaxel not encapsulated and the lipopeptide not incorporated, the sample was placed in a PD-10 column (Sephadex G-25). For this, the column was first balanced with NaCl at 0.9%. Once balanced, the sample was added, which was also eluted with NaCl at 0.9%, until it
15 overflowed from the column. The volume of liposomes obtained was made up to 2 mL.

Following this process, the following types of liposomes were prepared incorporating Paclitaxel:

Lipid composition	Conc. of Lipids	Conc. of drug	Coating lipopeptide	Conc. of peptide	Liposome size
PC/PG/Chol.	8.93 mg/mL	0.26 mg/mL	Myristic-$(A)_5$-CYESIKVAVS (SEQ. ID. NO:7)	0.42 mg/mL	140 nm
PC/PG/Chol.	13.5 mg/mL	0.4 mg/mL	Myristic-PEAGD (SEQ. ID. NO:5)	1.1 mg/mL	105 nm

EXAMPLE 7: Cellular adhesion tests

Solutions of Laminin-1 and synthetic peptides (50 mg/well) were fixed in wells of the 96-well tissue culture plate of TPP (Switzerland). The wells were dried at room temperature during the night. Before using them, the wells were washed with tamponed saline solution free from Calcium and Magnesium ions. The remaining free radicals of the polystyrene were blocked by using a 1% BSA solution.

They were cultivated and marked with ^{51}Cr cells of human fibrosarcoma HT1080. The marked cells were placed (1cpm/well) in the wells which contained the Laminin and the synthetic peptides.

After 30 minutes of incubation at 37 degrees Celsius, the unadhered cells were removed by washing. The adhered cells were smoothed and the radioactivity measured. The specific percentages of adhesion encountered are shown in the attached Figure 1.

EXAMPLE 8: Inhibition of cellular adhesion to Laminin (complete molecule) in vitro by peptides of the Laminin.

Following the procedure described under Example 1 HT-1080 cells marked with ^{51}Cr were adhered, in wells($0.32 cm^2$) coated with 1μg of Laminin. The adhered cells were incubated with different concentrations of of synthetic peptide fragments of Laminins. The results obtained are shown in the attached figure 2.

EXAMPLE 9: Anti-proliferative effect of Doxorubicin liposomes directed against specific receptors of laminin peptides in tumoral cells.

The anti-proliferative effect of Doxorubicin was analysed by following the MTT method. HT1080 cells obtained from exponential cultures were sown in $0.36 cm^2$ wells (96-well tissue culture plates of TPP, Switzerland) with a

density of 5000 cells per well. One day later, the cells were washed and incubated for two hours with liposomes containing Doxorubicin. The different liposome formulations were adapted to the same drug concentration and the test was carried out in parallel wells (increasing the concentration of
5 Doxorubicin from 0.01 µg/ml to 10 µg/ml. After the incubation, the cells were washed five times with PBS and incubated for three days in a complete medium. After this period, to each well was added 50 µL of PBS containing 1mg/ml of MTT (tetrazolium salt, Sigma) and they were incubated for a further four hours. The intracellular crystals of Fromazan resulting from the reduction
10 of the tetrazolium salt, only present in the active cells, were dissolved in DMSO. The number of metabolically active cells was estimated by measuring the absorbance of this solution of DMSO at 540nm.

The percentage of cytostatic activity was calculated according to the formula (A-B)/Ax100, where A is the absorbance in tumoral cells incubated in
15 a control medium and B is the absorbance in tumoral cells incubated with the liposome preparations.

The results of the resulting cytostasis are shown in the attached Figure 3, in which:

- the results present the mean +/- the standard desiccation of three
20 independent experiments performed in triplicate;
- The IC_{50} is defined as the drug concentration at which 50% of the cells survive in comparison with the control lot; and
- $P > 0.05$; Student test t.

EXAMPLE 10: Biodistribution of Doxorubicin administered as free drug or
25 liposome preparation (PC/PG/Chol/myristoyl-AAAAACYESIKVAVS)/Doxorubicin) in tumour-bearing animals.

Animals: The tests were performed on naked and immuno-suppressed BALB/c mice obtained for the animal production area of IFFA CREDO Inc. (Lyons, France). The animals were kept in laminar flow cabins in pathogen-free
30 conditions and were used when they reached an age of 8 weeks.

Cellular culture conditions: Cells of HT1080 human fibrosarcoma were made to grow in Ham's F-12 medium (GIBCO, Grand Island, NY) supplemented with 10% of bovine fetal serum, Sodium pyruvate, non-essential amino acids, L-glutamine, and vitamin solution (GIBCO, Grand Island, NY). The cultures were
35 kept in plastic and incubated in 5% CO2-95% air at 37 degrees Celsius in

humidified incubators. The cellular line was examined to certify the absence of Mycoplasma.

The tumoral cells were harvested from the sub-confluent cultures (50-70% confluence) by treating with trypsin (0.25%) and EDITA (0.02%). The cells
5 were washed in a supplemented medium and afterwards were re-suspended in a Hank Balanced Saline Solution (HBSS) for their subsequent injection. Only monocellular suspensions with a viability of more than 90% (determined by colouring with Trypan blue) were used for the *in vivo* studies.

Biodistribution test: HT-1080 cells at a concentration of 1x107 cells/mL of
10 HBSS were pre-mixed with an equal volume of liquid Matrigel (Collaborative Biomedical Products, Bedford, MA) 10 mg/mL. Of the resulting suspension, 0.02 mL were inoculated subcutaneously into the left-hand flank of the mice. Tumour growth was monitored twice weekly. When the tumours attained a volume of 1 cm^3 (day 25 after injection of the cells), the mice received a single intravenous
15 dose of Doxorubicin (5mg/kg) in liposome preparation or free drug form. At times of 30 minutes, 5 hours and 24 hours from the administration of the drug, the mice were sacrificed and samples were taken of tumoral tissue and plasma. The results obtained are shown in Figures 4 and 5 attached.

SEQUENCE LISTING

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<120> LIPOSOMES ENCAPSULATING ANTICANCEROUS DRUGS AND USE THEREOF IN THE TREATMENT OF MALIGNANT TUMOURS

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<141> 2001-10-03

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CLAIMS

1. Liposomes encapsulating anticancerous drugs comprising a coating with a lipopeptide composed of three substructures: a lipid fragment, an active oligopeptide derivatized from laminin targeting tumoral cells and an oligopeptide spacer between the other two fragments, wherein said oligopeptide spacer is inactive with respect to Laminin and has a length lying between five and ten amino acids.

2. Liposomes encapsulating anticancerous drugs in accordance with claim 1, wherein the lipid fragment of the coating lipopeptide are fatty acids of carbon chain length between C6 and C20.

3. Liposomes encapsulating anticancerous drugs in accordance with any one of claims 1 and 2, wherein said lipid fragment of the coating lipopeptide is selected from the group consisting of decanoyl, myristoyl and stearoyl.

4. Liposomes encapsulating anticancerous drugs in accordance with any one of claims 1 to 3, wherein said active oligopeptide of the coating lipopeptide is SEQ. ID. NO:4.

5. Liposomes encapsulating anticancerous drugs in accordance with any one of claims 1 to 4, wherein said oligopeptide spacer between the active oligopeptide and the lipid fragment of the coating lipopeptide is one of the following sequences: SEQ. ID. NO:1, SEQ. ID. NO:2 or SEQ. ID. NO:3.

6. Liposomes encapsulating anticancerous drugs in accordance with any one of claims 1 to 5, wherein the ratio of total liposome-forming lipids to drug lies between 20:1 and 2:1.

7. Liposomes encapsulating anticancerous drugs in accordance with any one of claims 1 to 6, wherein said liposome-forming lipids are phospholipids, of both natural and synthetic origin, and cholesterol.

8. Liposomes encapsulating anticancerous drugs in accordance with claim 7, wherein said phospholipids are a neutral phospholipid, and another phospholipid charged negatively.

9. Liposomes encapsulating anticancerous drugs in accordance with claim 8, wherein said neutral phospholipid is phosphatidylcholine.

10. Liposomes encapsulating anticancerous drugs in accordance with claim 8, wherein said negatively charged phospholipid is phosphatidylglycerol.

11. Liposomes encapsulating anticancerous drugs in accordance with any one of claims 8 to 10, wherein the proportion of said neutral phospholipid to said negatively charged phospholipid ranges from 10:2 to 10:10.

12. Liposomes encapsulating anticancerous drugs in accordance with any one of claims 7 to 11, wherein the proportion of cholesterol, with respect to the total amount of lipids, lies between 0 and 50%.

13. Liposomes encapsulating anticancerous drugs in accordance with any one of claims 1 to 12, wherein said liposomes contain a lipid oxidation inhibitor.

14. Liposomes encapsulating anticancerous drugs in accordance with claim 13, wherein said lipid oxidation inhibitor is Vitamin E acetate, butylhydroxytoluene (BHT) or a chroman.

15. Liposomes encapsulating anticancerous drugs in accordance with any one of claims 1 to 14, wherein the proportion of coating lipopeptide with respect to the total amount of lipids lies between 0.1% by weight and 30% by weight.

16. Liposomes encapsulating anticancerous drugs in accordance with any one of claims 1 to 15, wherein said liposomes have an average size lying between 50 nm and 250 nm.

17. Liposomes encapsulating anticancerous drugs in accordance with any one of claims 1 to 16, wherein said anticancerous drugs are selected from the group of:
nitrogenated mustard analogues, ethylenimines, nitrosoureas, alkylating agents, analogous antimetabolites of Folic acid, analogues of purines, analogues of Pyrimidines, alkaloids of Vinca and analogues, derivatives of Podophyllotoxin, Taxanes, anthracyclines, cytotoxic antibiotics, platinum compounds, monoclonal antibodies, Pentostatin, Miltefosine, Estramustine, Topotecan, Irinotecan and Bicalutamide.

18. Intravenous use of the liposomes encapsulating anticancerous drugs in accordance with any one of claims 1 to 17, for the treatment of malignant tumours in humans.

19. Use of the liposomes encapsulating anticancerous drugs in accordance with any one of claims 1 to 17, for the preparation of an intravenous medicament for treatment of malignant tumours in humans.

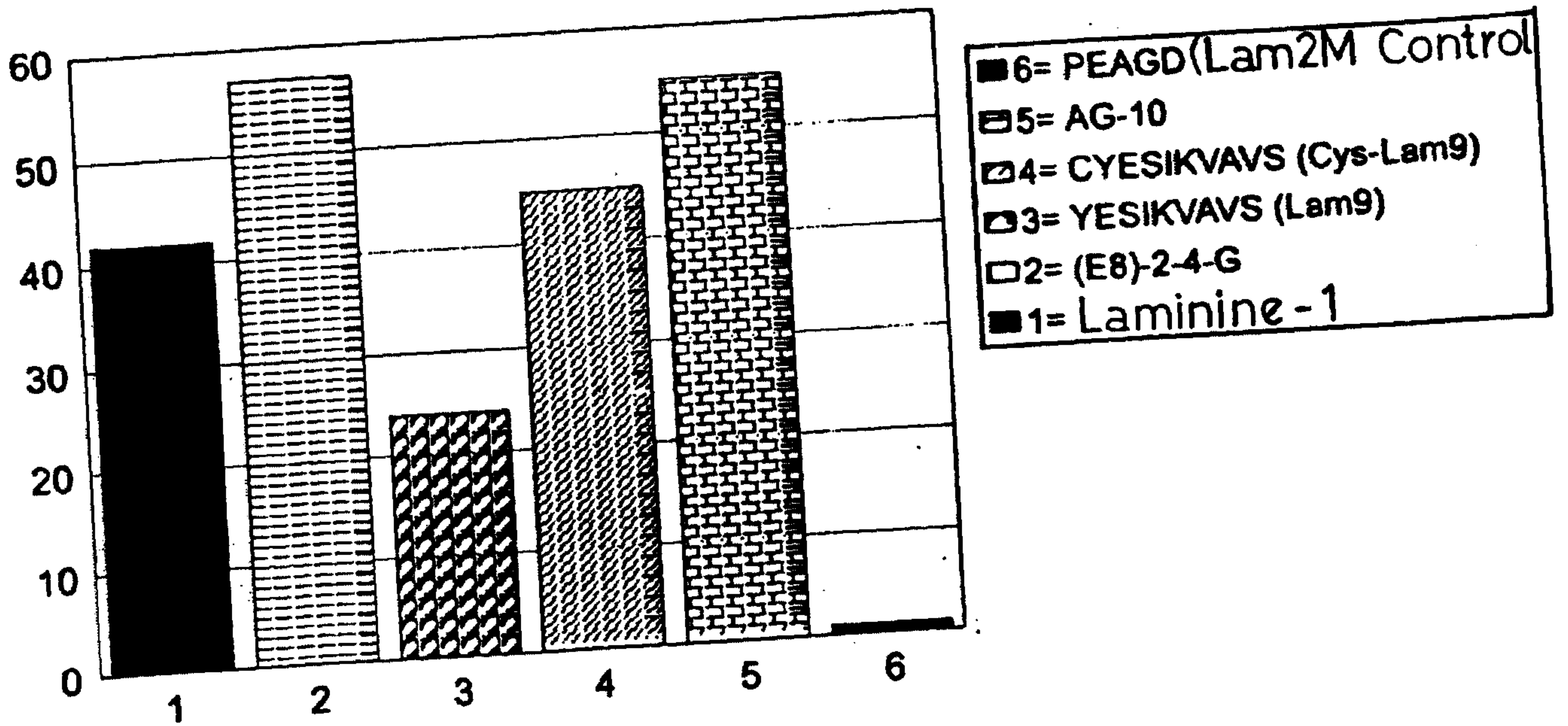

FIG. 1

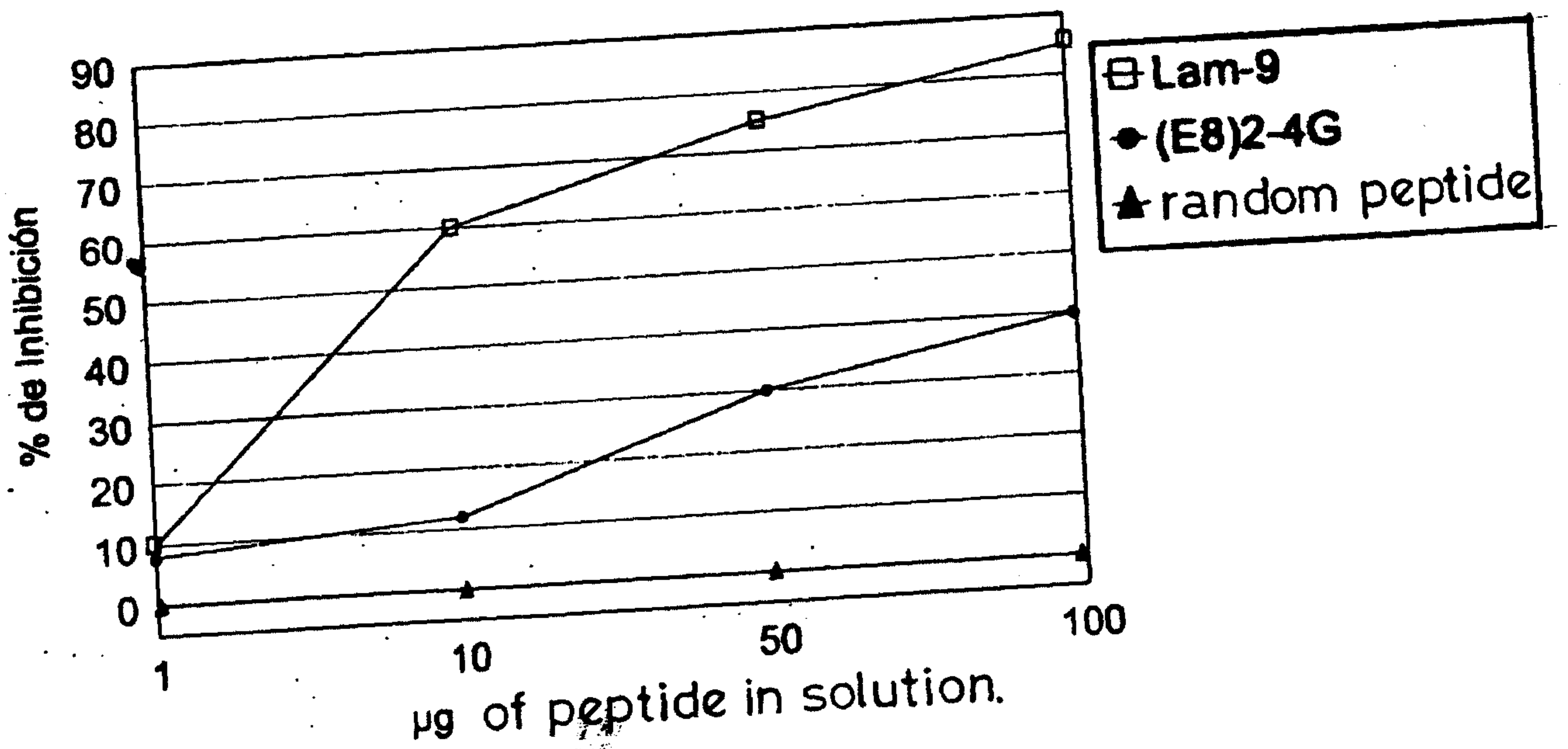

FIG. 2

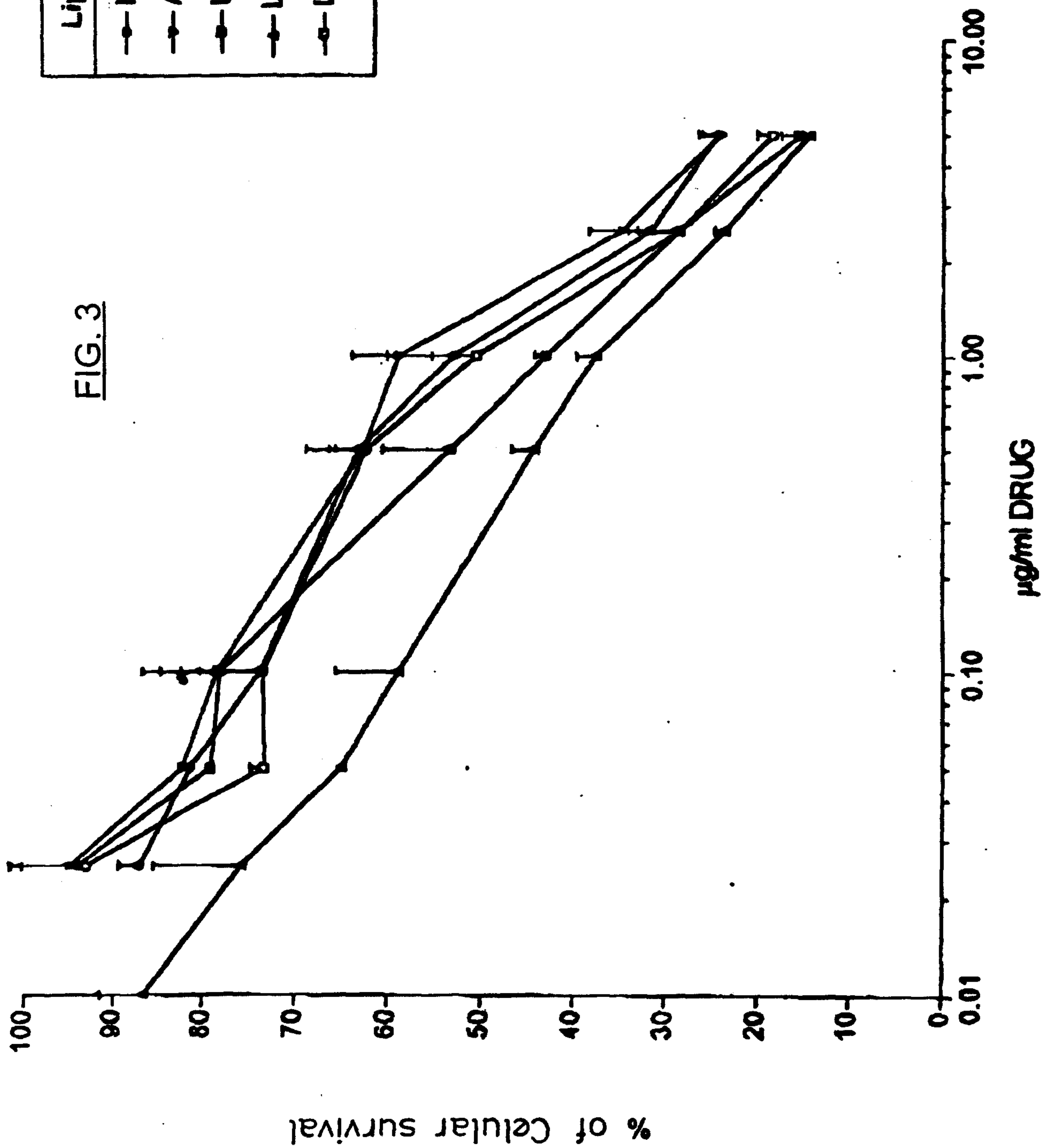

FIG. 3

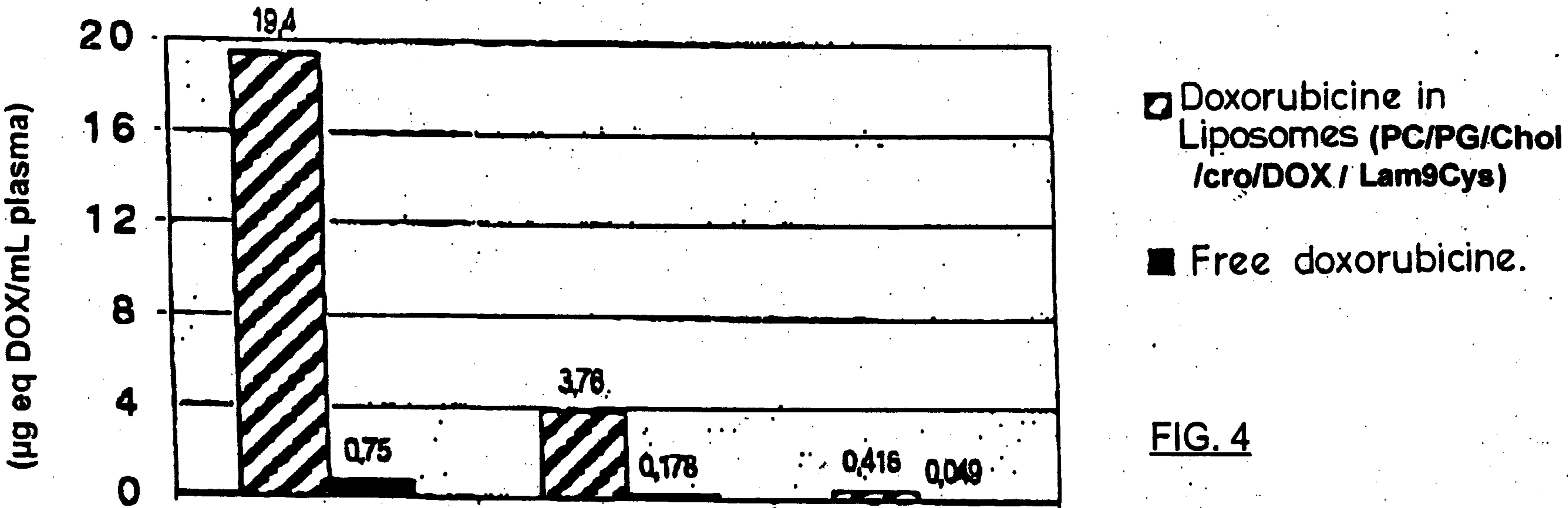

FIG. 4

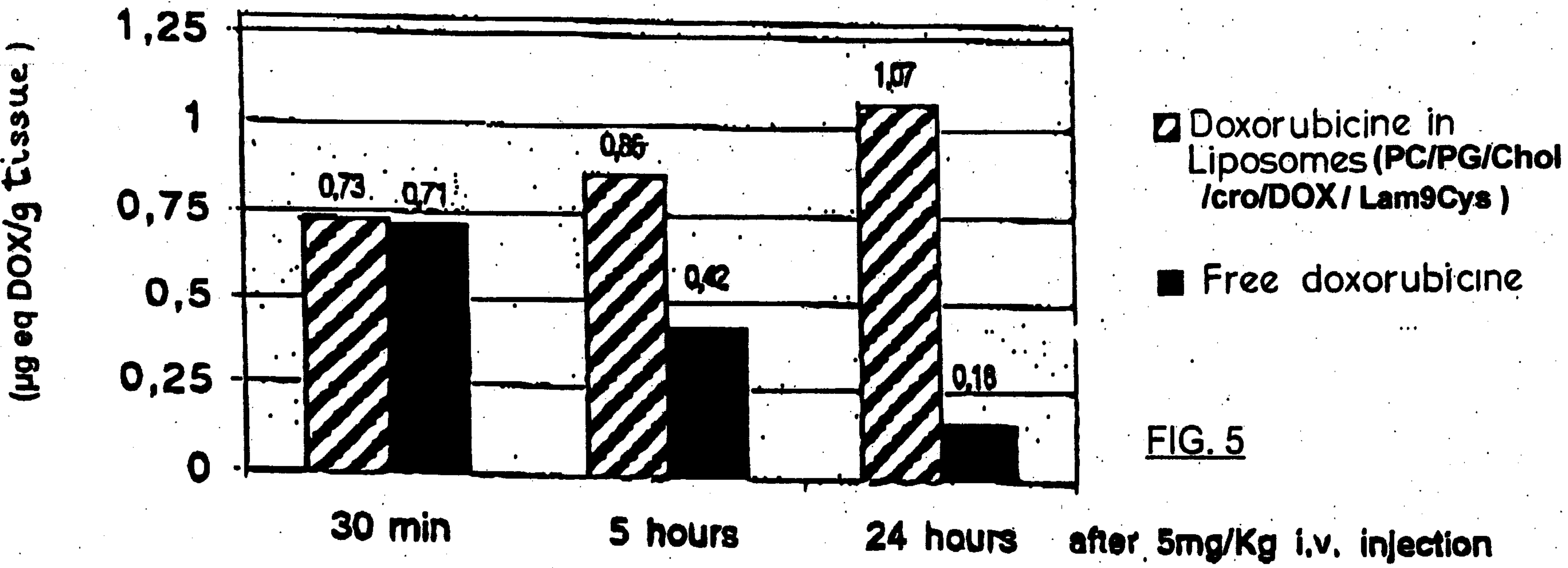

FIG. 5