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[54] USE OF ETHYLENE GLYCOL ESTERS OF MONOHYDROBENZOPORPHYRIN DERIVATIVES AS PHOTOACTIVE AGENTS 一氫苯並卟啉衍生物的乙二醇酯作為光活性試劑的用途

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(54) **Use of ethylene glycol esters of monohydrobenzoporphyrin derivatives as photoactive agents**

Verwendung von Ethylenglycolestern von Monohydrobenzoporphyrinderivativen als photoaktive Mittel

Utilisation d'esters éthylèneglycol des dérivés de la monohydrobenzoporphyrine comme agents photoactifs

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Description

Technical Field

[0001] The invention relates to compounds useful in photodynamic therapy (PDT) and related applications. In particular, it concerns ethylene glycol esters of monohydrobenzoporphyrins.

Background Art

[0002] Photodynamic therapy (PDT) generally involves the administration of compounds that are capable of absorbing light, typically in the visible range, but also in the near ultraviolet, followed by irradiation of locations in the subject for which a toxic or inhibitory effect is desired. PDT was initially developed using hematoporphyrin and related compounds in the treatment of tumors, as it appeared that these compounds would "home" to locations containing rapidly dividing cells. The tumor could then be irradiated with light absorbed by the hematoporphyrin and destruction of the surrounding tissue resulted. PDT has since been shown to be useful for treatment of atherosclerotic plaques, restenosis, infections in the blood stream, rheumatoid arthritis, psoriasis and in the treatment of ocular conditions not necessarily limited to tumors.

[0003] U.S. Patent No. 5.171.749 and patents issuing on related applications, U.S. Patents Nos. 5.283.255; 5.399.583; 4,883,790; 4,920,143; and 5,095,030, describe and claim a class of photoactive compounds useful in PDT designated the monohydrobenzoporphyrins, or "BPDs." This class is obtained by Diels-Alder reaction of a mono- or disubstituted alkyne with protoporphyrin-IX and the resultant compounds can further be isomerized, reduced, and/or derivatized to obtain a large class of BPDs. As disclosed in these patents, a particularly useful subclass of this group results from hydrolysis or partial hydrolysis of the ester groups of the 2-carboxyethyl side-chains on rings C and D. Esterification as protection of these groups during the Diels-Alder reaction results in initial products which contain 2-carbalkoxyethyl groups. It was found that facile hydrolysis of these esters could readily be conducted, leaving any carbalkoxy groups associated with the Diels-Alder product obtained from a dicarbalkoxyalkyne virtually completely unhydrolyzed. This resulted in four species of compounds, BPD-MA, BPD-MB, BPD-DA and BPD-DB as depicted in Figure 1; this figure taken from U.S. Patent No. 5,171,749. In this depiction, R^1 and R^2 are carbalkoxy groups, typically carbomethoxy or carboethoxy, and R is alkyl (1-6C).

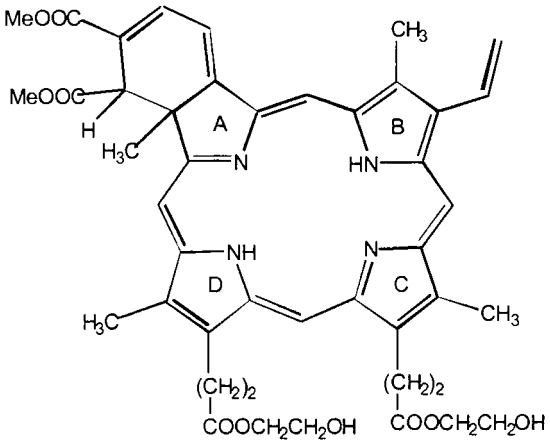
[0004] BPD- MA was found to have particularly useful properties for PDT and is currently in clinical development. However, there remains a need for additional specific forms of photoactive agents which expand the repertoire of photoactive compounds for the variety of indications to which PDT is applied, as noted above. The present invention provides compounds in which rings C and D contain ethylene glycol esters of the carboxyalkyl substituents. These compounds have pharmacokinetic properties which are advantageous in certain instances where PDT is employed.

[0005] Levy JG *et al* have described the clinical status of benzoporphyrin derivative. Obochi Mok *et al* have described prolonged skin allograft survival after photodynamic therapy associated with modification of donor skin antigenicity. Waterfield JD *et al* have described evaluation of the immunotoxicity of benzoporphyrin derivative (BPDMA) in mice.

Disclosure of the Invention

[0006] The compounds for use according to the invention are useful new additions to the repertoire of compounds that find application in photodynamic therapy and related methodologies that employ photoactive compounds. The presence of ethylene glycol esters in these molecules provides them with characteristics that permit expansion of the scope of conditions under which such photoactive compounds are employed and fine tuning of the treatment.

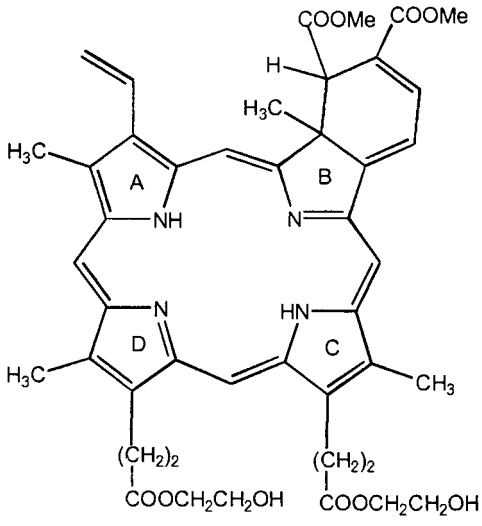
[0007] The present invention provides a compound of the formula



A-EA6

(1)

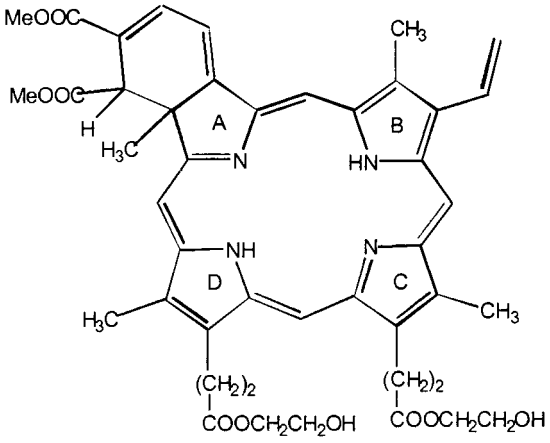
and



B-EA6

(2)

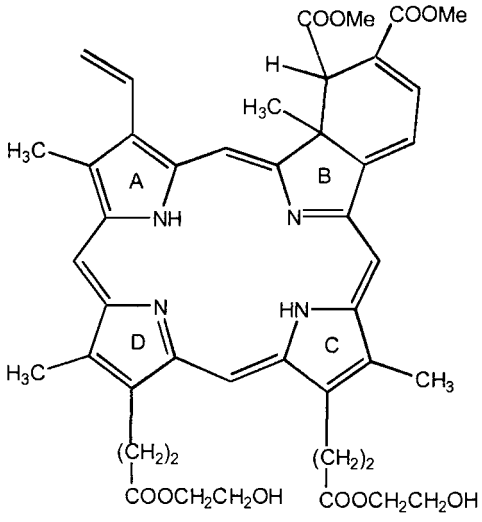
or the metallated or labelled or conjugated form thereof;
for use in a method of photodynamic therapy to modulate an immune response. The present invention also provides the use of a compound of the formula



A-EA6

(1)

and



B-EA6

(2)

or the metallated or labelled or conjugated form thereof;
in the manufacture of a photoactive compound for use in photodynamic therapy to modulate an immune response.

[0008] The compounds of formulas 1 and 2 are derived from protoporphyrin IX. The invention also includes the use of isomers of the various forms of formulas 1-2 which result from the unrearranged Diels-Alder condensation products (i.e. the 1,4-diene) as described in US Patent 4,883,790.

Brief Description of the Drawings

[0009]

Figure 1 shows the compounds of the prior art, BPD-MA, BPD-MB, BPD-DA and BPD-DB.

Figure 2 shows the kinetics of uptake of B-EA6 by L1210 cells.
Figure 3 shows the kinetics of release of B-EA6 by L1210 cells.
Figure 4 shows a graphic depiction of the pharmacokinetics of B-EA6 *in vivo*.
Figure 5 shows a comparison of the kinetics of uptake of B-EA6 by normal splenocytes and L1210 cells.
Figure 6 shows the time course of PDT using B-EA6 in mice as compared to mice treated with BPD-MA and BPD-MB.
Figure 7 shows the effect of B-EA6 on microvasculature in mice.
Figure 8 shows a comparison of the spectra in plasma of BPD-MA and B-EA6.
Figures 9A and 9B show the cytotoxic effect of photodynamic treatment using A-EA6 in comparison with BPD-MA in L1210 cells and in dendritic cells.
Figure 10 shows the comparative effects of A-EA6 and BPD-MA in decreasing the surface expression of MHC I receptors.
Figure 11 shows the effect of photodynamic therapy using A-EA6 and BPD-MA on stress and mitogenic pathway kinases in HL60 cells.
Figure 12 shows the comparative effect of PDT using A-EA6 and BPD-MA on caspase activation in HL60 cells.
Figure 13 shows the comparative effect of PDT using A-EA6 and BPD-MA on DNA fragmentation in HL60 cells.

Modes of Carrying Out the Invention

[0010] The compounds for use according to the invention are related to those disclosed in the BPD patents cited above, but differ in that they contain esters of ethylene glycol in the substituents on rings C and D. These compounds can be prepared by simple hydrolysis of the carbalkoxyalkyl or carbalkoxyl substituents and reesterification of the resulting carboxyl groups in the C and D rings of the benzoporphyrins, or can be obtained directly by transesterification.

[0011] It will be noted that compounds 1 and 2 are individual species of the genus, described in the above-referenced U.S. patents, obtained through a process which comprises a Diels-Alder reaction with protoporphyrin IX. Since protoporphyrin IX is not symmetric with respect to the A and B rings, two possible products result depending on whether the Diels-Alder addition occurs in the A or B ring.

[0012] As used herein, the term "alkyl" refers to a saturated straight or branched chain hydrocarbon which may, if it contains a sufficient number of carbon atoms, be cyclic or contain a cyclic portion. Typical examples are methyl, ethyl, t-butyl, cyclohexyl, and the like.

[0013] A "hydrocarbon radical" refers to a monovalent substituent containing only carbon and hydrogen which may be straight or branched chain, saturated or unsaturated, aromatic or nonaromatic or both, and cyclic or noncyclic. Thus, a hydrocarbon radical of 1- 10C could include cyclopentylethyl, 2- pentenyl, 3- butynyl, 2, 4- dimethylhexyl, and the like.

[0014] Cyclic amines include pyridyl, pyrimidyl, thiazolyl, quinolyl, and so forth. Thus, they may include single ring or fused ring systems and may contain additional heteroatoms.

[0015] It will be noted that the compounds for use according to the invention contain at least one chiral center and thus may exist in various stereoisomeric forms. If desired, such stereoisomers, including enantiomers, may be separated using techniques standard in the art; however, racemic mixtures or mixtures containing more than one diastereomer may also be used. The compounds as indicated in formulas 1-2, therefore, are representative of the individual optical isomers, enantiomers or diasteriomers as the case may be, as well as mixtures of these individual chiral isomers.

[0016] If desired, the compounds for use according to the invention can prepared in metallated forms by treating the tetrapyrrole-type nucleus with an appropriate ion such as magnesiurn ion, zinc ion, stannous ion and the like, to obtain a metal complex. The metal ion may also be a radiolabel. Generally, the metal ion is inserted using the appropriate salts under conditions standard in the art. For example, zinc ion can be introduced by treating the compound with zinc acetate in 1:1 methylene chloride:methanol.

[0017] The compounds may also contain label, including radioisotopes, chromophores, and fluorescent labels. Radioisotope labeling is generally useful when the compounds are to be followed *in vivo* or used to label specific moieties. Useful cationic moieties that are radioisotopes include technetium, gallium and indium. In addition, radioisotopes of heteroatoms, such as ^{131}I or ^{32}P, in the molecule itself, or inclusion of ^{14}C may be used to label the molecule.

[0018] As further described in the BPD-related patents set forth above, the compounds for use according to the invention may be coupled, if desired, to a targeting agent which will direct the molecule to a specific tissue or organ. Such targeting agents include antibodies, receptors, receptor-ligands and the like. Linkage of the targeting agent to the compound is conducted using standard techniques. By a "conjugated form" is meant a compound of formulas 1-2 coupled to a targeting agent, as above described. Both A-EA6 and B-EA6, compounds for use in the invention, have been prepared. Both are effective photosensitizers; it appears that A-EA6 is the easier to formulate.

[0019] The various forms of the compounds can be used in the photodynamic therapy techniques generally known in the art. As set forth in the Background section above, photodynamic therapy can be conducted using a plethora of protocols and for a variety of indications. In addition, compounds of this type exhibit pharmacological activity in the absence of light in some instances. Standard pharmaceutical compositions, including liposomal compositions as pre-

ferred, are used as desired in such applications.

[0020] The following examples are intended to illustrate the invention. The Examples illustrate and demonstrate the surprising pharmacokinetic properties of two members of the species of the invention, A-EA6 and B-EA6. Hence, the small class of compounds contained in the present invention offers valuable additions to the repertoire of photodynamic agents useful in treating the various conditions to which this therapy has been directed.

[0021] In one embodiment of the invention, a compound of the invention is for use in prevention of an immune response.

[0022] In one embodiment of the invention, a compound of the invention is for use in a method of photodynamic therapy to modulate an immune response in a subject afflicted with arthritis.

[0023] In one embodiment of the invention, a compound of the invention is for use in a method of photodynamic therapy, wherein the photodynamic therapy comprises administration of the compound to a subject followed by irradiation of a location in the subject to modulate an immune response in the subject.

Example 1

Preparation of Two Forms of EA6

[0024] A. To prepare B-EA6, the starting material is BPD-DB as the dimethyl ester -- i.e., BPD-DB as shown in Figure 1 wherein R^1 and R^2 are both COOMe an R" is vinyl.

[0025] To 2.0 g (2.7 mM) BPD-DB in 50 mL ethylene glycol and 100 mL dichloromethane was added 1.0 mL sulfuric acid. The reaction was stirred for 18 hr. at room temperature. Then the reaction was added to a stirring mixture of 100 mL 5% aqueous ammonium acetate and 100 mL dichloromethane. The organic layer was isolated and then washed twice with 50 mL water. The solvent was removed by rotary evaporation. The dark green residue was then chromatographed on 75 g alumina (deactivated with 5% water) and eluted with a gradient of 0.5%-5.0% methanol in dichloromethane. The solvent from the fractions containing product was then removed by rotary evaporation. The residue was dried *in vacuo* overnight to provide 2.02 g (89%) of the analytically pure green sold title compound.

[0026] B. In a manner similar to that set forth in paragraph A, but substituting BPD-DA for BPD-DB, the isomeric form, A-EA6 was prepared.

Example 2

Comparison of Uptake and Release of B-EA6 and BPD-MA by L1210 Cells

[0027] BPD- MA or B- EA6 were incubated at 3 μg/ml in the presence of 10% fetal bovine serum with 10/mL of L1210 cells, a murine leukemia cell line. Intracellular content of the photosensitizers was measured by fluorescence of cell lysates at various times. The maximum concentration reached was 145.9 ng/ 10^6 cells for B- EA6 and 149.5 ng/ 10° cells for BPD- MA. The time course of uptake is shown in Figure 2 as a percentage of cell content at 60 min by which time uptake had reached a maximum in both cases. As shown. B- EA6 is taken up more rapidly and reaches 80% of its maximum concentration after only 5 min and reached its maximum uptake within 15 min.

[0028] The kinetics of release of these drugs from L1210 cells was measured by preloading the cells at 3 μg/ml for 1 hr and then placing the cells in drug-free medium containing 10% fetal bovine serum. Remaining intracellular drug content was measured at various time points by lysing the cells and measuring fluorescence. As shown in Figure 3 (again as a percent of starting intracellular content), BPD-MA and B-EA6 showed different kinetics of release. Initial release of B-EA6 was much more rapid, but release was more complete in the case of BPD-MA.

[0029] It was unexpected that the *in vitro* pharmacokinetics of B-EA6 were more rapid than those of BPD-MA. While the higher retention of B-EA6 could be attributed to its increased size as compared to BPD-MA, the faster transfer through the cellular membrane was unexpected.

Example 3

Comparison of *In Vivo* Pharmacokinetics

[0030] Either BPD-MA or B-EA6 was administered by intravenous injection into DBA/2 mice at a dose of 4 mg/kg using 3 mice per time point. The drug content of plasma, skin, liver and kidney was determined by fluorescence in the tissue extracts. Figure 4 shows the results plotted as a percentage of the concentration in the relevant tissue 15 min postinjection. As seen in Figure 4, neither BPD-MA nor B-EA6 accumulated in plasma, liver or kidney; however, BPD-MA accumulated in skin within the first 3 hr; B-EA6 does not.

[0031] The more rapid accumulation of B-EA6 as compared to BPD-MA, as here confirmed *in vivo* by more rapid clearance from all tissues, constitutes an advantage. The treatment with light can be carried out soon after injection of

the photosensitizer and due to the rapid clearance, no prolonged skin or eye photosensitivity will be exhibited. Thus, the subjects treated can resume normal lives without special precautions such as avoiding bright light and wearing dark eyeglasses.

[0032] The half-life of B-EA6 and BPD-MA in various tissues was then computed in the time-frame 15 min-3 hr and the results are shown in Table 1:

Table 1: Tissue Half-Lives of B-EA6 and BPD-MA

	T½* (15 min-3 hours)	
Tissue	**B-EA6**	**BPD-MA**
Liver	0.6	2.4
Spleen	0.8	10.9
Kidney	0.8	5.6
Skin	1.9	0**
Muscle	11.1	ND†
Plasma	0.6	2.0

* shown in hours

** BPD-MA concentration in the skin increased for up to 3 hr

† ND = not determined

[0033] The half-life of BPD-MA in this time-frame could not be computed in skin since its concentration increased during the 3 hr period. As shown in Table 1, generally, B-EA6 has a much shorter half-life than BPD-MA in most tissues. The lack of accumulation of B-EA6 in normal skin as compared to BPD-MA was unexpected, and indicates more rapid clearance than that of BPD-MA. As set forth above, this is advantageous as skin photosensitivity is the only recognized side effect of photodynamic therapy utilizing photosensitizers.

[0034] The pharmacokinetics were also determined using an *in vivo* mouse tumor model. Groups of 10 DBA/2 mice containing M1 rhabdomyosarcoma tumors were injected intravenously with a liposomal formulation of BPD-MA at various dosages of 0.75-1.5 mg/kg. The tumors were irradiated with 690 nm laser light at 50 or 150 J/cm^2 at various times after injection. The results, as shown in Table 2, were determined in terms of the percentage of mice in each group that were tumor-free on day 7 after injection.

Table 2: Results of Bioassay

PDT Conditions			Percent Tumor Free on Day 7*	
Drug Dose (mg/kg)**	**Time post IV (min)**	**Ligh*** dose (J/cm^2)**	**BPD-MA**	**B-EA6**
0.75	15	50	(4/5)	50%
	30	50	70%	0%
1.0	15	50	100%	90%
	30	50	90%	0%
1.5	180	150	70%	0%

* tumor model = MI tumor in DBA/2 mice - each PDT condition was tested in 10 animals

** the drugs were liposomally formulated and injected intravenously

*** 690 nm laser light.

[0035] As shown in Table 2, BPD-MA treated mice showed substantial survival rates when postinjection times ranged from 15-180 min. On the other hand, B-EA6 treated mice showed no response at 30 min or 180 min; however, significant responses were obtained when irradiation was supplied after only 15 min.

[0036] These data demonstrate that PDT using B-EA6 will be effective in early treatment with light. The lack of effect of later times postinjection indicates, again, rapid clearance of B-EA6 which is advantageous for the reasons set forth above.

Example 4

Determination of LD_{50} With and Without Serum

[0037] Either B-EA6 or BPD-MA was incubated for 1 hr with L 1210 cells at a range of concentrations and exposed to 9 J/cm^2 broad spectrum light. This determination was made in the absence of serum and in the presence of 10% serum. The results are shown in Table 3.

Table 3: LD_{50} Values

	No serum	10% serum
BPD-MA	3.7 ng/ml	54.0 ng/ml
B-EA6	4.7 ng/ml	19.7 ng/ml

[0038] As shown, BPD-MA and B-EA6 have comparable LD_{50} values in the absence of serum; however, in the presence of serum, B-EA6 shows a substantially better retention of effectiveness.

[0039] In most instances, the presence of serum greatly reduces the photoactivity of agents used in PDT, such as BPD-MA. Surprisingly, B-EA6 shows more affinity for cell membranes than for plasma components and is thus very slightly affected by the presence of serum in the cellular environment. Thus, *in vivo*, its activity may be higher than that of BPD-MA and other compounds of this family.

Example 5

In Vitro Efficacy of B-EA6

[0040] The ability of B-EA6 to exert a cytotoxic effect on L1210 cells *in vitro* was further tested by incubating the cells with B-EA6 at various concentrations for 1 hr in the absence of serum. After excess drug was removed, the cells were exposed to 9 J/cm^2 broad spectrum light (380-750 nm) and cell survival was determined by the MTT assay (Mosmann, T. et al. J Immunol Meth (1983) 65:55-63). The percentage of killed cells was calculated in reference to survival of cells exposed to light only. At a concentration of approximately 7 ng/ml, 80% of the cells were killed; at 15 ng/ml, almost 100% of the cells did not survive. As stated above, the LD_{50} for B-EA6 is approximately 4.7 ng/ml.

[0041] The somewhat lower effect of B-EA6 as compared to BPD-MA *in vitro* makes even more unexpected the comparatively higher activity of B-EA6 as compared to BPD-MA *in vivo* in the presence of serum as demonstrated in Example 4.

Example 6

Selectivity of B-EA6 for Tumor Cells

[0042] The ability of L1210 cells to accumulate B-EA6 was compared to the ability of splenocytes to do so. B-EA6 at 3 μg/ml was incubated with each cell type and the cell content of B-EA6 was determined by fluorescence in cell lysates. Figure 5 shows a comparison of uptake for the two cell types in ng/10^6 cells. As shown, L1210 cells were able to take up approximately 140 ng/10^6 cells reaching this value after approximately 20 min. Splenocytes, on the other hand, accumulated less than 20 ng/10^6 cells after an hour of incubation.

[0043] DBA/2 mice bearing M1 (rhabdomyosarcoma) tumor, grown subcutaneously in their flanks, were used as a model to show that B-EA6 demonstrated selectivity for tumors. Mice were administered 0.75 mg/kg of B-EA6 in a liposomal formulation intravenously. After 15 min, a 1 cm area which included a 5 mm diameter tumor was exposed to 50 J/cm^2 of 70 mW light of 690 nm wavelength from an argon-pumped dye laser. The exposure effectively eliminated the tumor, but did not affect the surrounding normal skin. Thus, B-EA6 demonstrates tumor specificity.

Example 7

Immunomodulation by B-EA6

[0044] Balb/C mice (5-8 mice per group) were tested using the delayed skin photosensitivity assay also called the contact hypersensitivity (CHS) assay. The mice were painted in the flank with the sensitizing agent dinitrofluorobenzene (DNFB) and 5 days later, one ear is challenged with DNFB, while the other serves as a control. The swelling is an

indicator of immune response. Mice were injected intravenously with 1 mg/kg liposomal B-EA6 and either irradiated with 15 J/cm^2 light over the whole body or exposed to ambient light. The ability of this treatment to prevent the immune response as demonstrated by inhibition of ear swelling was determined. The results showed that administering B-EA6 combined with either after irradiation with 15 J/cm^2 whole body light or with ambient light decreased swelling in the test ear as compared to untreated mice. The swelling in both cases was only approximately 60% of the that shown in mice without treatment.

[0045] In an additional assay to determine immunomodulation, murine peritoneal macrophages were isolated, purified and activated by recombinant interferon-γ (100 U/ml). The activated cells were incubated for 1 hr at 37°C with B-EA6 at a range of concentrations and then exposed to 690 nm LED light at 5 J/cm^2. Expression levels of MHC I, MHC II, CD54, CD80 and CD86 were determined 24 hr later using FITC conjugated antibodies and a cell sorter. The results are shown in Table 4 for B-EA6 at 0.5 ng/ml in comparison to similar experiments using BPD-MA at 2.5 ng/ml.

Table 4: Effect of Low-Dose PDT with B-EA6 on Expression Levels of Cell Surface Antigens by Murine Peritoneal Macrophages

Compound	MHC Class I	MHC Class II	CD54 (ICAM-1)	CD80 (B7-1)	CD86 (B7-2)
BPD-MA (2.5 ng/ml)	99.1 ± 4.3%	79.3 ± 10.1%	105.4 ± 3.0%	93.5%	99.2%
BPD-B-EA6 (0.5 ng/ml)	100.4%	71.8%	106.9%	102.3%	92.2%

[0046] The results in the table are given as a percent of expression as compared to cells treated with light only. As shown, BPD-MA and B-EA6 were both able to reduce expression of MHC II, but not the remaining surface markers. Thus, although B-EA6 has advantageous pharmacokinetics, it retains the immunomodulatory activity of BPD-MA and other compounds of this group.

Example 8

Effect of B-EA6 in an Arthritis Model

[0047] MRL- lpr mice spontaneously develop arthritis; this was enhanced by intradermal injection of Freund's Adjuvant. Various numbers of MRL- lpr mice were treated with PDT on days 0, 10, and 20 after injection of the adjuvant. PDT consisted of 0.5 mg/kg liposomal B- EA6 injected intravenously followed by exposure of the ventral part of the mice to red (560- 900 nm) light at 80 J/cm^2 at 1 hr post- B- EA6 injection. The mice were observed and symptoms scored every 5 days for 30 days. The results are shown in Figure 6 in comparison to mice similarly treated with BPD- MA and BPD-MB. As shown in Figure 6, whether measured by the incidence of clinical symptoms (i.e., the percentage of mice exhibiting these symptoms) or by the change in bimaleolar ankle width in millimeters, B- EA6 (shown as solid circles) was effective in preventing the sequellae of adjuvant injection.

[0048] Again, the retention of immunomodulatory activity of B- EA6 is demonstrated.

Reference Example 9

Effect of B-EA6 on Microvasculature

[0049] The mouse cremaster muscle model was used. B-EA6 was administered intravenously at 2 mg/kg and starting at 5 and 15 min postinjection, surgically exposed arterioles and venules were irradiated with light at an intensity of 25 J/cm^2 per 5 min beginning at 5 min and 15 min after injection of the B-EA6. The vessels were measured as red blood column diameter as a percentage of controls.

[0050] The results are shown in Figure 7. While transient vessel closure could be obtained when irradiation was started at 5 min, permanent closure was obtained when radiation was started after 15 min.

[0051] The enhanced capacity of B-EA6 to constrict or occlude vasculature, as demonstrated in this Example, in combination with more rapid pharmacokinetics, make B-EA6 particularly advantageous in treating neovascular diseases in the eye.

Example 10

Absorption Spectrum of B-EA6

[0052] BPD- MA and B- EA6 have similar absorption spectra in plasma before and after 4- hr exposure to fluorescent (380- 750 nm) light. A comparison of these spectra is shown in Figure 8. The similarity of the spectrum of B- EA6 to the spectrum of BPD- MA is advantageous since the use of BPD- MA as a therapeutic agent useful in PDT is well developed. The similarity in their spectra indicates that the same light sources can be used for B- EA6 as are successful in treatment with BPD- MA.

Example 11

In Vitro Cytotoxicity of A-EA6

[0053] In a manner similar to that set forth in Example 5, the cytotoxicity of A-EA6 *in vitro* on two different cell lines was tested and compared with BPD-MA. Either L1210 cells or the dendritic cell line D2SC/1 was incubated for one hour at 37°C with either A-EA6 or BPD-MA. After removal of excess drug, the cells were exposed to 690 nm light at 5 J/cm^2 light for dendritic cells and at 9°J/cm^2 light for L1210 cells. Cell survival was determined 18-24 hours later using the MTT colorimetric assay described in Example 5. Percent cells killed was calculated by reference to cells exposed to light only. As shown in Figure 9A, A-EA6 showed comparable cytotoxicity to BPD-MA with respect to L1210 cells in the absence of serum but was markedly more toxic in the presence of serum than BPD-MA. The open circles represent A-EA6 plus serum; the closed circles represent BPD-MA plus serum; open squares represent A-EA6 in the absence of serum; and closed squares represent BPD-MA in the absence of serum.

[0054] As shown in Figure 9B, in dendritic cells where BPD-MA has an LD_{50} of 6 ng/ml and A-EA6 has an LD_{50} of 2.7 ng/ml, A-EA6 was toxic at lower concentrations than BPD-MA in the presence of 5% fetal calf serum. In Figure 9B, closed circles represent BPD-MA and open squares represent A-EA6.

[0055] In a similar determination, but measuring MHC I receptors rather than cytotoxicity, A-EA6 was effective in decreasing expression of these receptors at lower concentrations. In this determination, dendritic cells were incubated for 1 hour at a drug concentration less than its LD_{50}; 2.5 ng/ml and 5 ng/ml for BPD-MA and 1 ng/ml and 2.5 ng/ml for A-EA6. The cells were treated with 690 nm light at 5 J/cm^2 and then labeled with the appropriate antibody 3 hours post-treatment and assessed by flow cytometry. The results were measured as the percent of the mean channel fluorescence intensity for light-treated control cells. These results are shown in Figure 10; BPD-MA gave an 18% and a 29% reduction, respectively, at 2.5 ng/ml and 5 ng/ml; A-EA6 lowered the channel fluorescence by approximately 25% at both 1 ng/ml and 2.5 ng/ml concentrations.

Example 12

Effect of A-EA6 on Intracellular Signaling

[0056] The conditions of the study set forth in Example 11 were repeated using HL-60 cells as the target and comparing the effects of A-EA6 and BPD-MA on cytotoxicity, on the mitogenic pathway kinase p70 S6K, and on the stress pathway kinases c-jun and HSP27. The results are shown in Figure 11. At sublethal concentrations, A-EA6 showed stronger activation of the stress pathway kinases and stronger inhibition of the mitogenic pathway kinases.

[0057] The effect on caspase activation in HL-60 cells was also measured. A-EA6 showed a stronger activation of caspases than did BPD-MA. This effect is desirable as it is associated with apoptosis. Using apoptosis to remove unwanted cells causes the least effect on surrounding normal cells and tissues. The comparison of A-EA6 with BPD-MA is shown in Figure 12.

[0058] Figure 13 shows a similar comparison when percent DNA fragmentation was measured in HL-60 cells. Again, A-EA6 was effective at lower concentrations than BPD-MA.

Reference Example 13

In Vivo Photodynamic Therapy Using A-EA6

[0059] In a protocol similar to that set forth in Example 3, either A-EA6 or BPD-MA was injected intravenously into mice harboring MI tumors at a dose of 1 mg/kg. This was followed by whole body irradiation with 50 J/cm^2 of 690 nm laser light at various times after administration of the drug. The number of tumor-free animals on day 7 was determined and the results are shown in Table 5.

Table 5		
Photosensitizer	**Irradiation time (post i.v.)**	**Day 7 tumor-free animals**
BPD-MA	15 min	10/10
	30 min	9/10
A-EA6	15 min	2/2
	30 min	6/6

[0060] These results show A-EA6 is at least as effective as BPD-MA in this assay.

Example 14

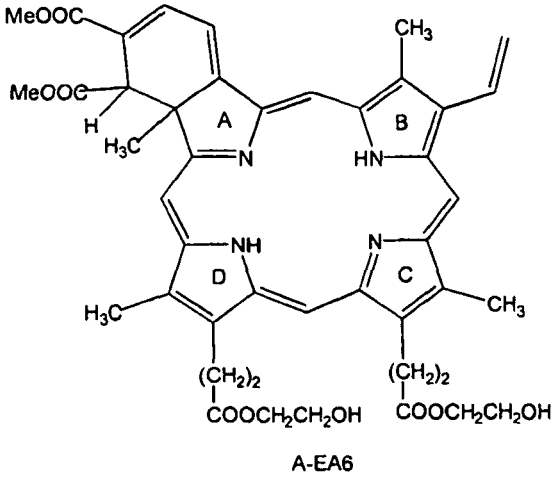
Immunomodulatory Activity

[0061] Flanks of control and test mice were painted with the antigen DMFB and their ears were challenged 5 days later by pasting with the same compound. Test animals were treated with whole-body PDT using BPD-MA or A-EA6, by injecting the photosensitizer intravenously and then exposing the animals to red LED light at 15 J/cm^2. The percent suppression of ear swelling was calculated in comparison to controls. The results are shown in Table 6 and indicate that A-EA6 had a stronger immunomodulatory effect in this assay than did BPD-MA.

Table 6		
Photosensitizer	**Dose (mg/kg)**	**Percent suppression**
BPD-MA	1.0	49%
A-EA6	1.0	68%
A-EA6	0.3	59%

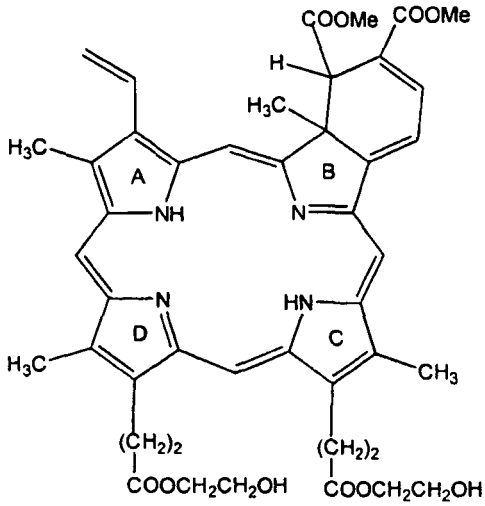
Claims

1. A compound of the formula



A-EA6

or



B-EA6

or the metallated or labeled or conjugated form thereof;

for use in a method of photodynamic therapy to modulate an immune response.

2. The compound for use of claim 1 wherein the modulation of an immune response is prevention of said response

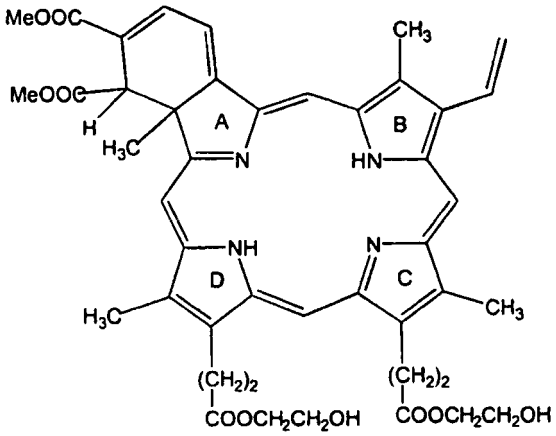
3. The compound for use of claim 1 or 2 wherein said immune response is in a subject afflicted with arthritis.

4. The compound for use according to any one of the preceding claims which is in a metallated form, and/or in conjugated form, and/or is labelled.

5. The compound for use according to any one of the preceding claims wherein the compound does not contain a metal ion.

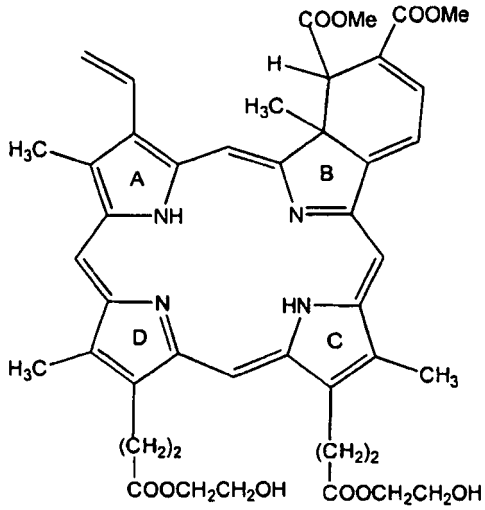
6. The compound for use of any one of the preceding claims wherein the photodynamic therapy comprises administration of the compound to a subject followed by irradiation of a location in the subject to modulate an immune response in the subject.

7. Use of a compound of the formula



A-EA6

or

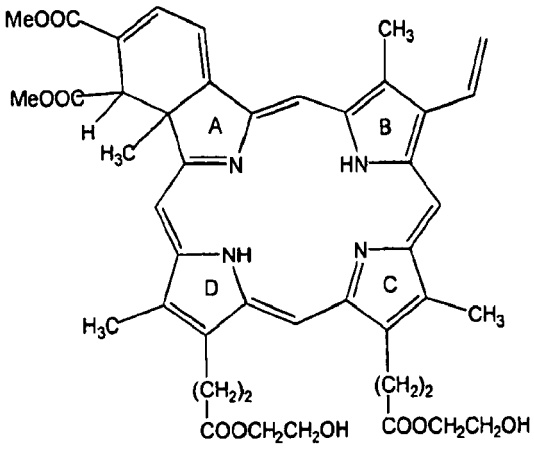


B-EA6

or the metallated or labeled or conjugated form thereof;
in the manufacture of a photoactive compound for use in photodynamic therapy to modulate an immune response.

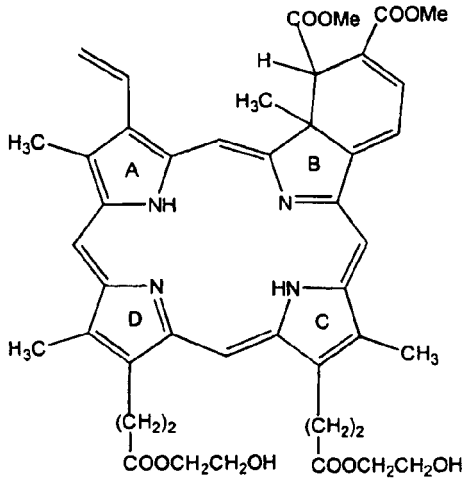
Patentansprüche

1. Eine Verbindung der Formel



A-EA6

oder



B-EA6

oder die metallierte oder markierte oder konjugierte Form davon,
zur Verwendung in einem photodynamischen Therapieverfahren, um eine Immunantwort zu modulieren.

2. Die Verbindung zur Verwendung nach Anspruch 1, wobei die Modulation einer Immunantwort die Verhinderung der Antwort ist.

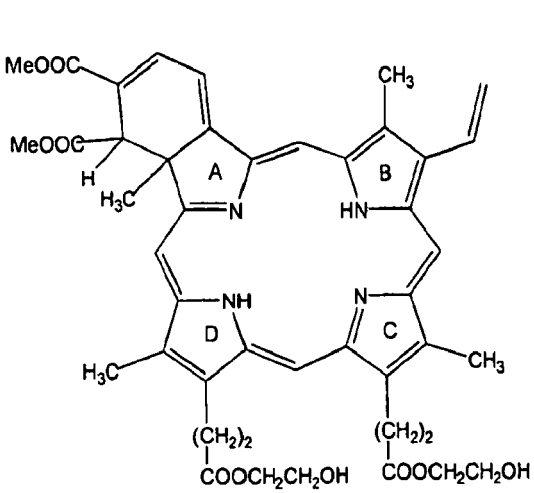
3. Die Verbindung zur Verwendung nach Anspruch 1 oder 2, wobei die Immunantwort in einem an Arthritis leidenden Subjekt stattfindet.

4. Die Verbindung zur Verwendung nach einem der vorstehenden Ansprüche, welche in einer metallierten Form und/oder in konjugierter Form vorliegt und/oder markiert ist.

5. Die Verbindung zur Verwendung nach einem der vorstehenden Ansprüche, wobei die Verbindung kein Metallion enthält.

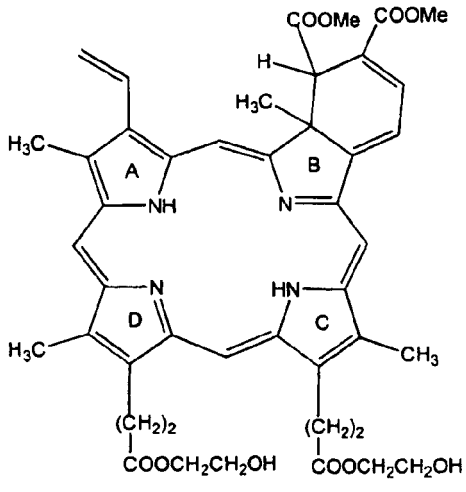
6. Die Verbindung zur Verwendung nach einem der vorstehenden Ansprüche, wobei die photodynamische Therapie das Verabreichen der Verbindung an ein Subjekt, gefolgt von Bestrahlen eines Ortes im Subjekt, um eine Immunantwort im Subjekt zu modulieren, umfasst.

7. Verwendung einer Verbindung der Formel



A-EA6

oder

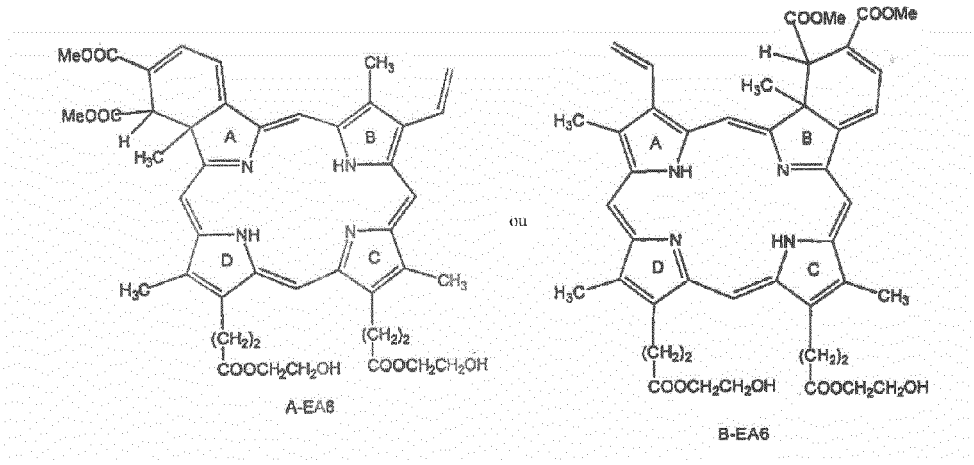


B-EA6

oder der metallierten oder markierten oder konjugierten Form davon;
zur Herstellung einer photoaktiven Verbindung zur Verwendung in der photodynamischen Therapie, um eine Immunantwort zu modulieren.

Revendications

1. Composé de formule



ou la forme métallatée ou marquée ou conjuguée de celui-ci ;
pour utilisation dans un procédé de thérapie photodynamique pour moduler une réponse immunitaire.

2. Composé selon la revendication 1 dans lequel la modulation d'une réponse immunitaire est la prévention de ladite réponse.

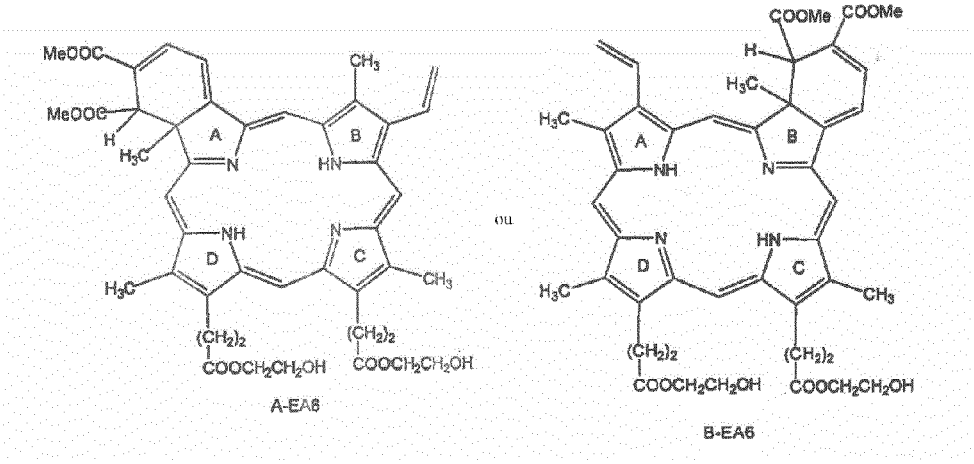
3. Composé selon la revendication 1 ou 2 dans lequel ladite réponse immunitaire a lieu chez un sujet souffrant d'arthrite.

4. Composé selon l'une quelconque des revendications précédentes qui est sous une forme métallatée, et/ou sous forme conjuguée, et/ou est marqué.

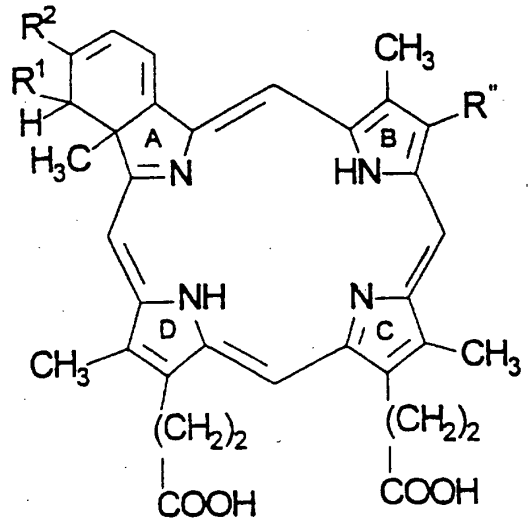
5. Composé selon l'une quelconque des revendications précédentes dans lequel le composé ne contient pas d'ion métallique.

6. Composé selon l'une quelconque des revendications précédentes dans lequel la thérapie photodynamique comprend l'administration du composé à un sujet suivie par une irradiation d'un endroit chez le sujet pour moduler une réponse immunitaire chez le sujet.

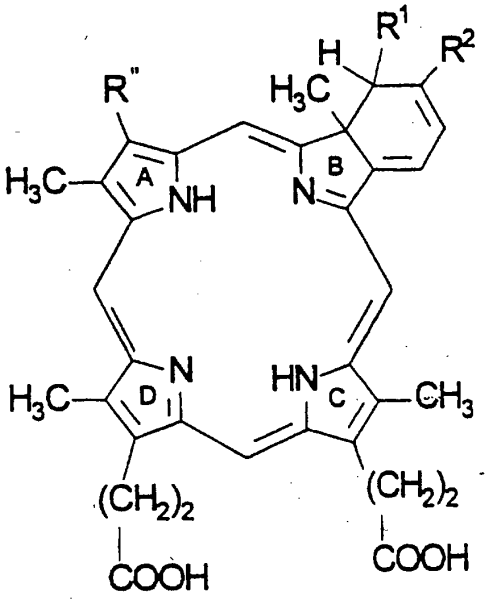
7. Utilisation d'un composé de formule



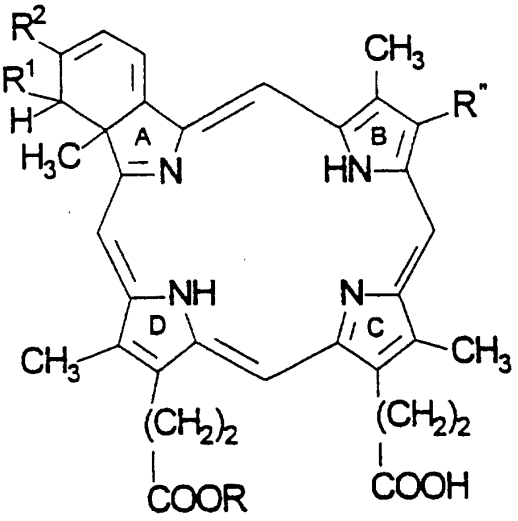
ou de la forme métallatée ou marquée ou conjuguée de celui-ci ;
dans la préparation d'un composé photoactif pour utilisation dans une thérapie photodynamique pour moduler une réponse immunitaire.



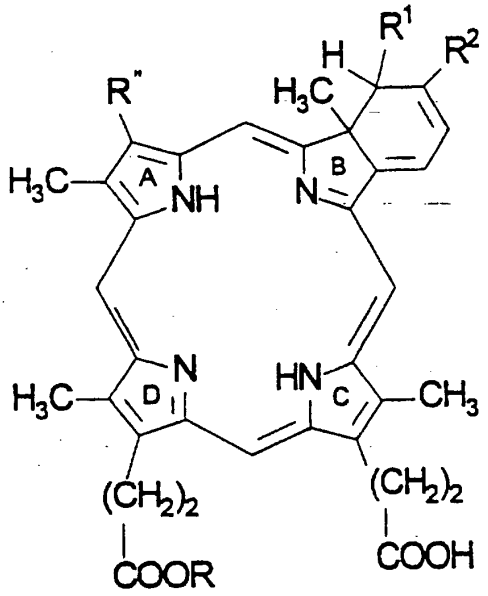
BPD-DA



BPD-DB



BPD-MA



BPD-MB

FIG. 1

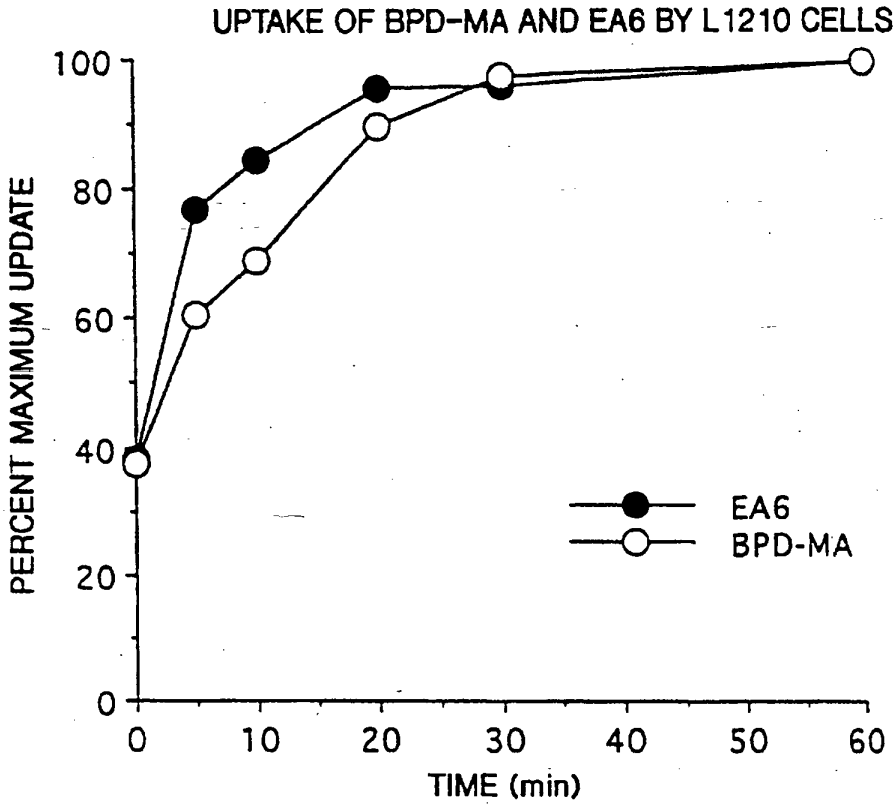

UPTAKE OF BPD-MA AND EA6 BY L1210 CELLS
PERCENT MAXIMUM UPDATE
100
80
60
40
20
0
0
10
20
30
40
50
60
TIME (min)
EA6
BPD-MA

FIG. 2

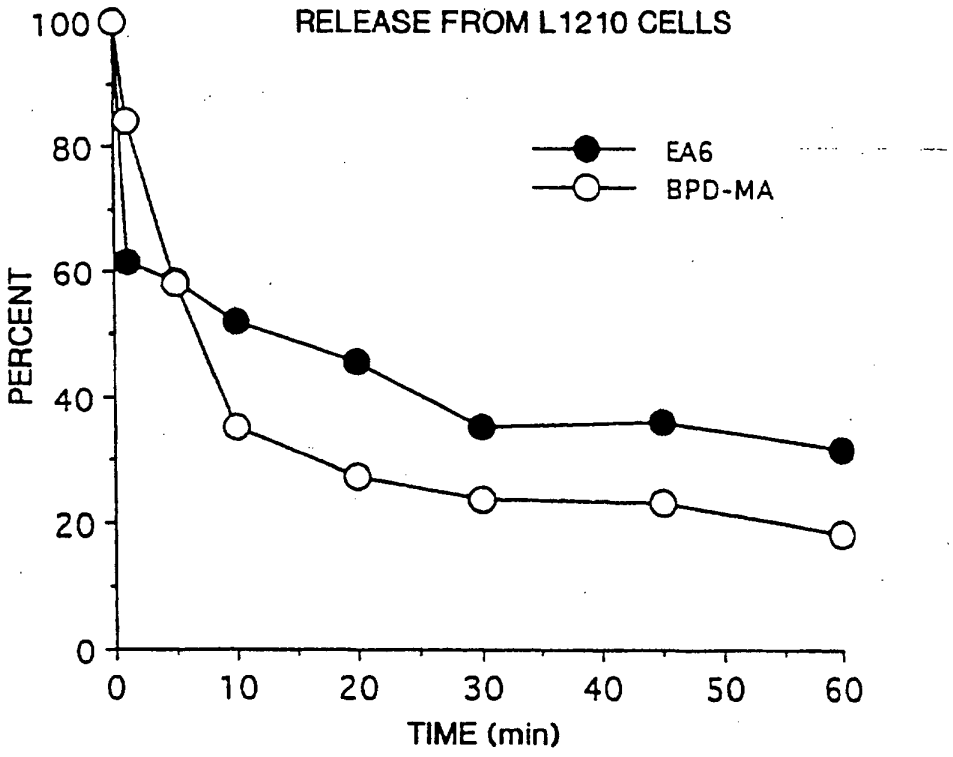

RELEASE FROM L1210 CELLS
PERCENT
100
80
60
40
20
0
0
10
20
30
40
50
60
TIME (min)
EA6
BPD-MA

FIG. 3

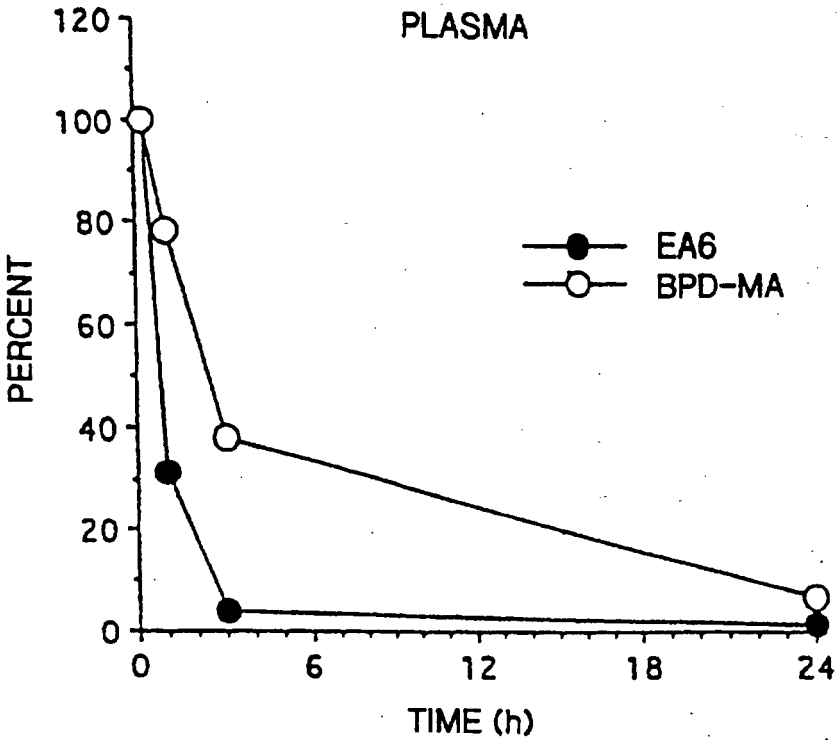

PLASMA
120
100
80
60
40
20
0
PERCENT
0
6
12
18
24
TIME (h)
EA6
BPD-MA

FIG. 4A

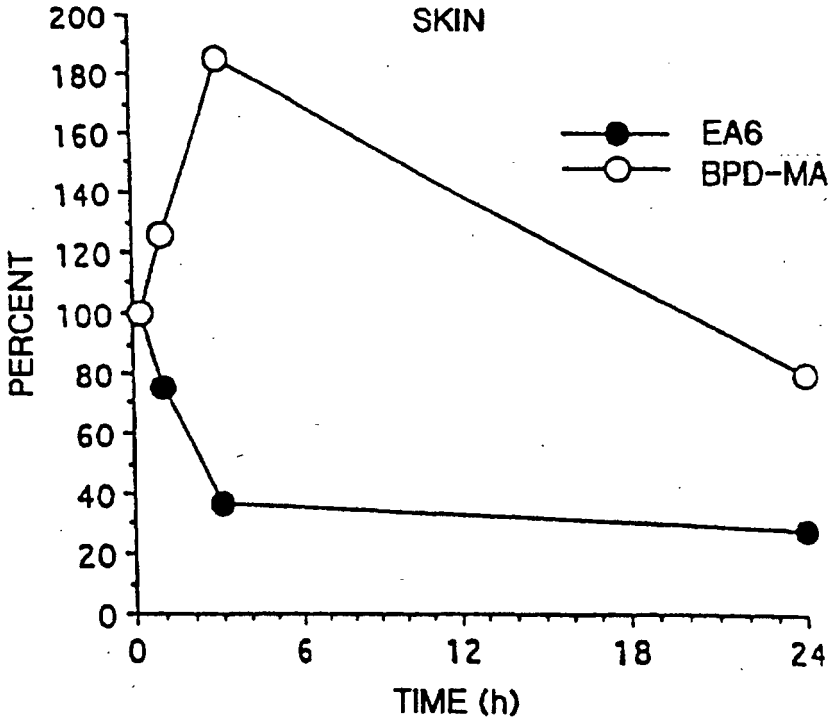

SKIN
200
180
160
140
120
100
80
60
40
20
0
PERCENT
0
6
12
18
24
TIME (h)
EA6
BPD-MA

FIG. 4B

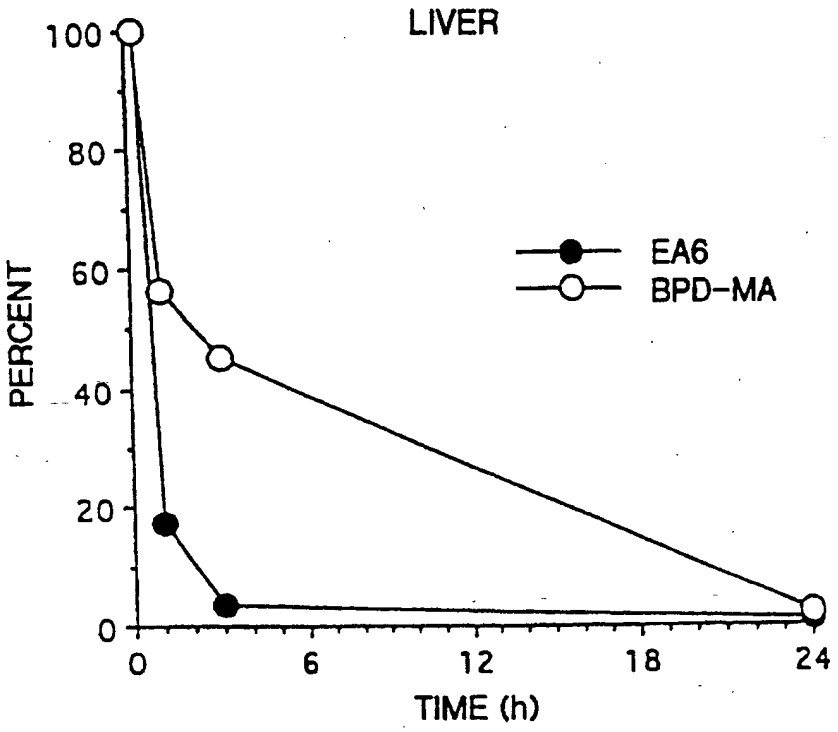

LIVER
100
80
60
40
20
0
PERCENT
EA6
BPD-MA
0
6
12
18
24
TIME (h)

FIG. 4C

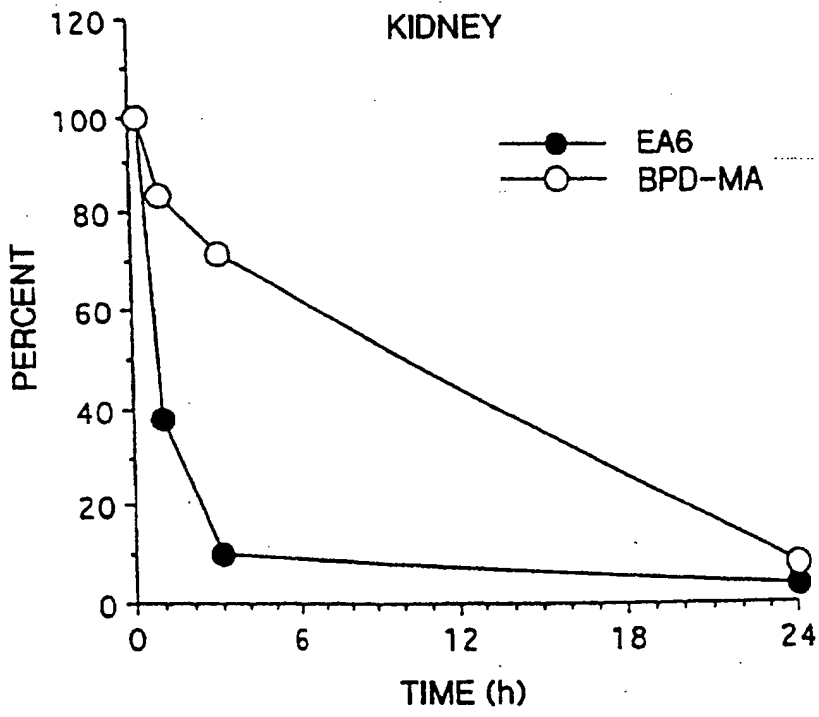

KIDNEY
120
100
80
60
40
20
0
PERCENT
EA6
BPD-MA
0
6
12
18
24
TIME (h)

FIG. 4D

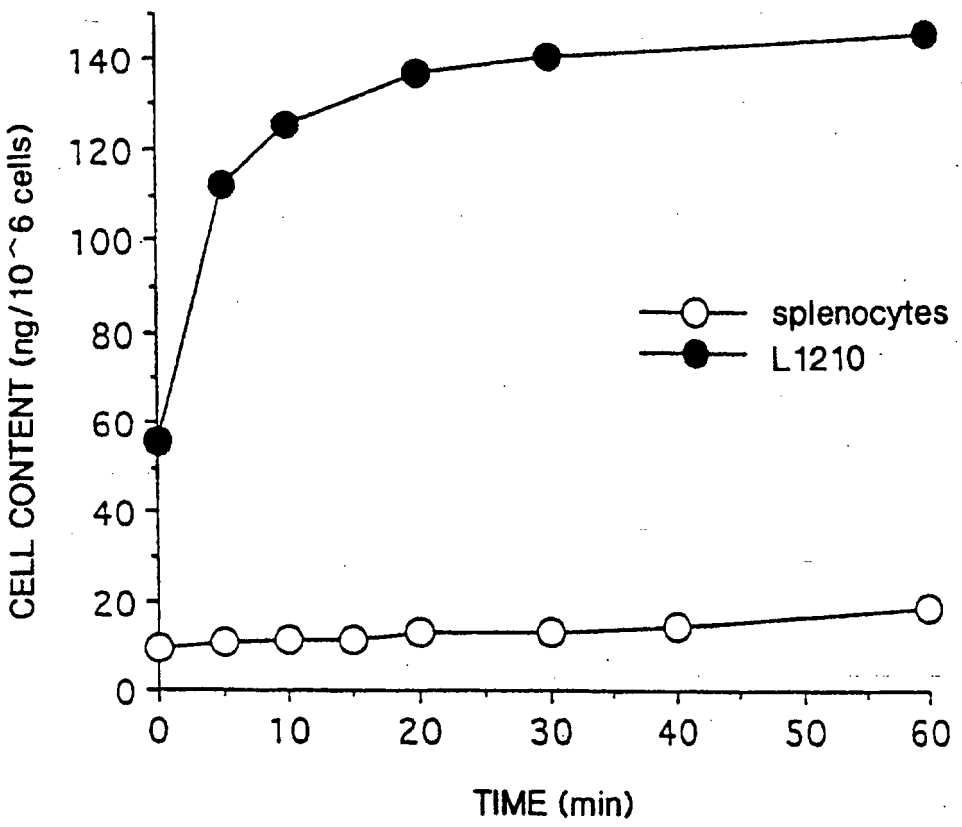

CELL CONTENT (ng/10^6 cells)
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
TIME (min)
splenocytes
L1210

FIG. 5

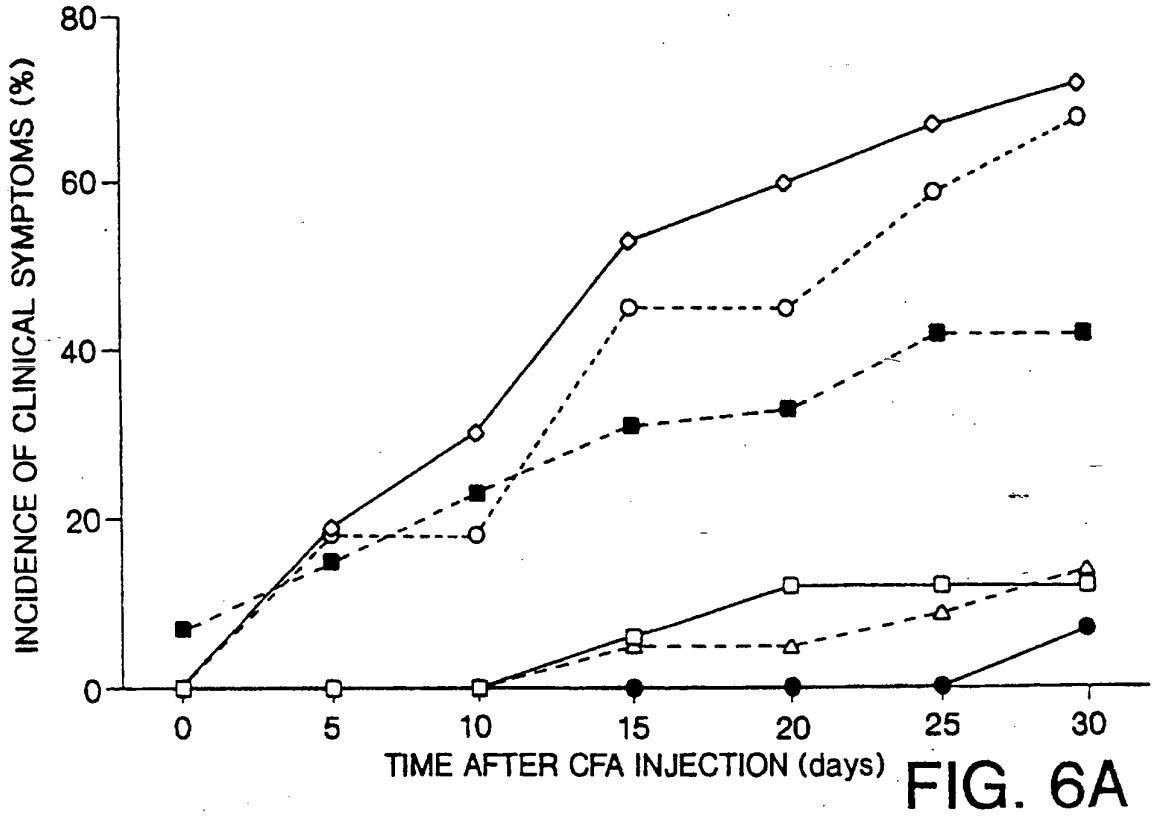

INCIDENCE OF CLINICAL SYMPTOMS (%)
80
60
40
20
0
0
5
10
15
20
25
30
TIME AFTER CFA INJECTION (days)

FIG. 6A

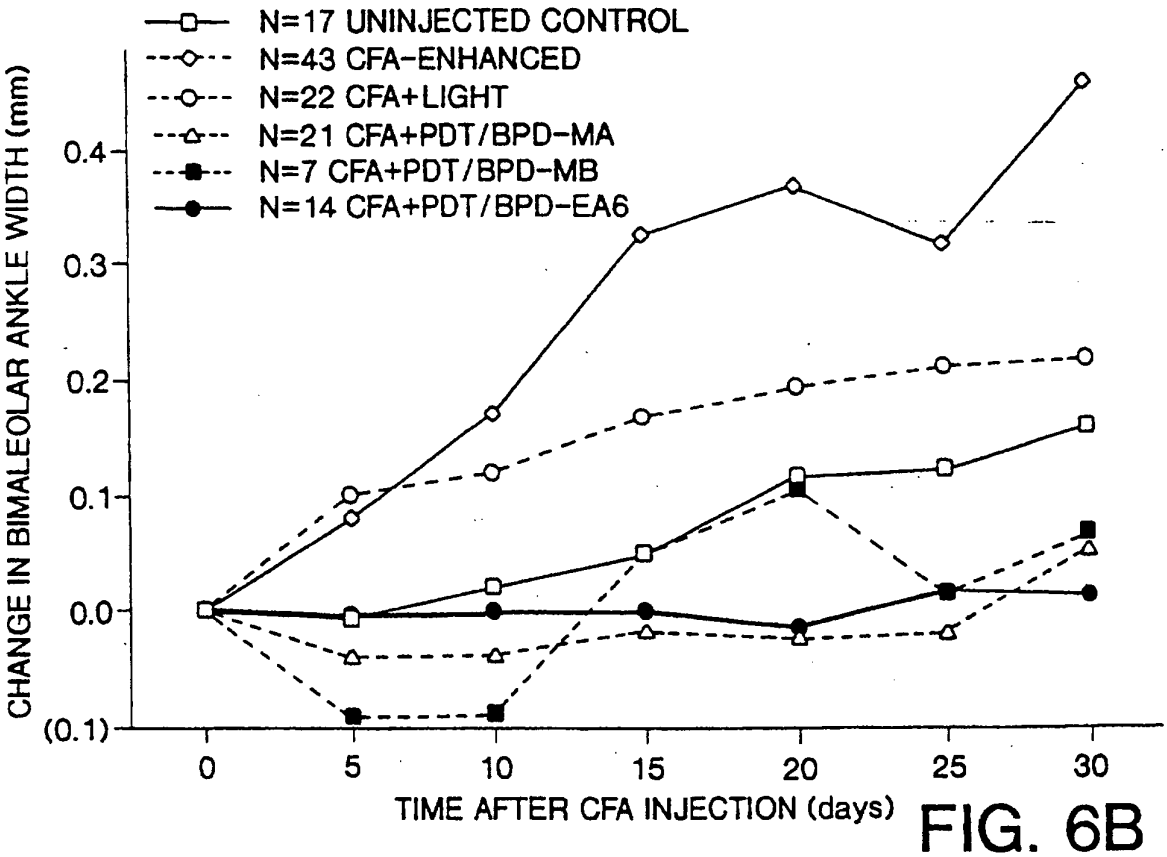

N=17 UNINJECTED CONTROL
N=43 CFA-ENHANCED
N=22 CFA+LIGHT
N=21 CFA+PDT/BPD-MA
N=7 CFA+PDT/BPD-MB
N=14 CFA+PDT/BPD-EA6
CHANGE IN BIMALEOLAR ANKLE WIDTH (mm)
0.4
0.3
0.2
0.1
0.0
(0.1)
0
5
10
15
20
25
30
TIME AFTER CFA INJECTION (days)

FIG. 6B

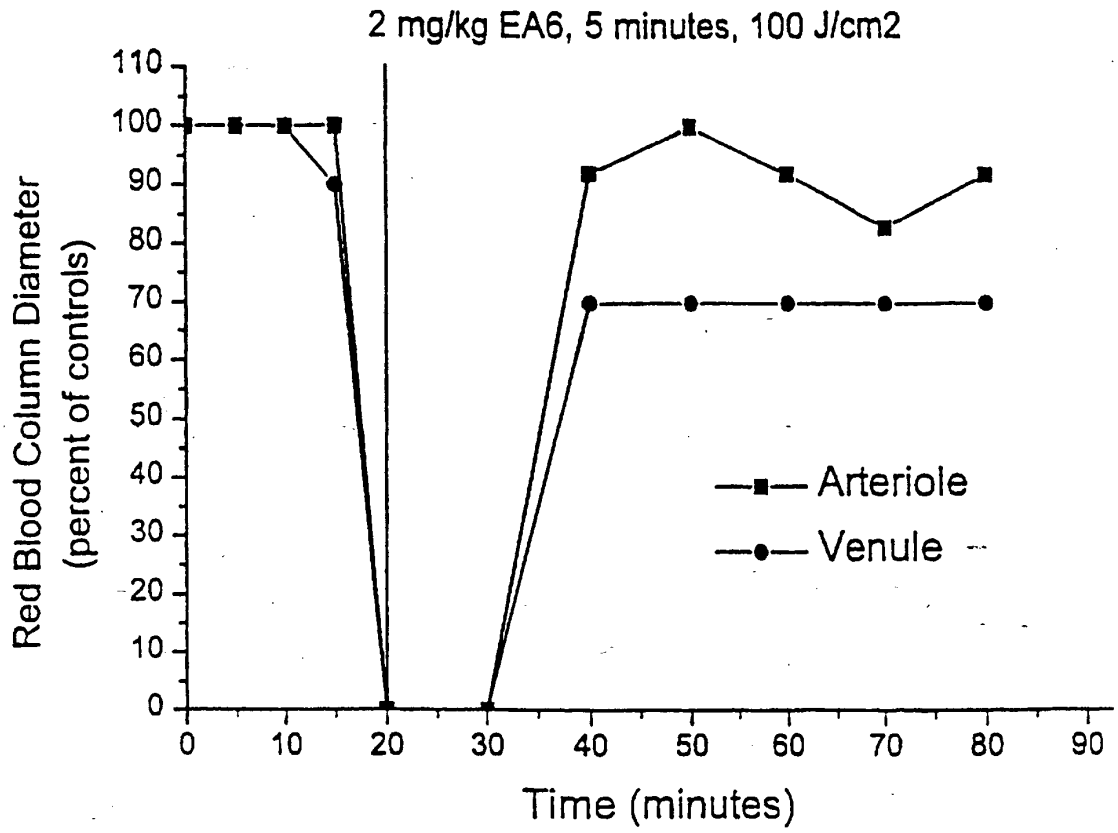

2 mg/kg EA6, 5 minutes, 100 J/cm2
Red Blood Column Diameter
(percent of controls)
110
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
Arteriole
Venule
Time (minutes)

FIG. 7A

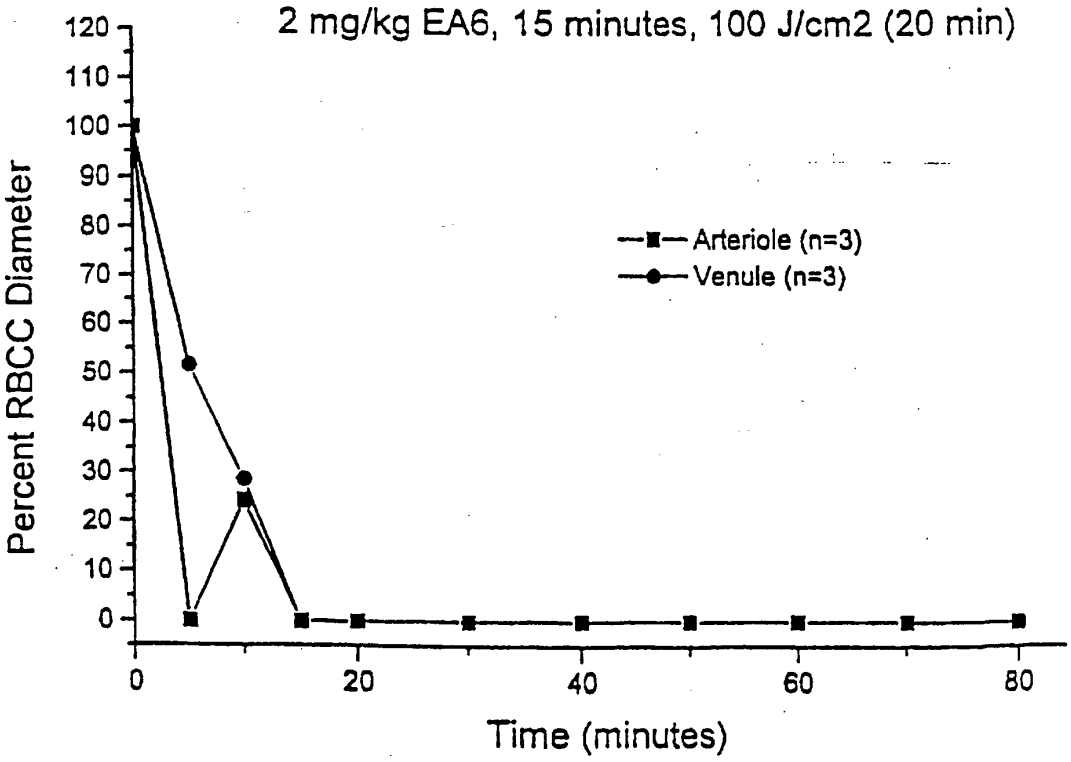

2 mg/kg EA6, 15 minutes, 100 J/cm2 (20 min)
Percent RBCC Diameter
120
110
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
Arteriole (n=3)
Venule (n=3)
Time (minutes)

FIG. 7B

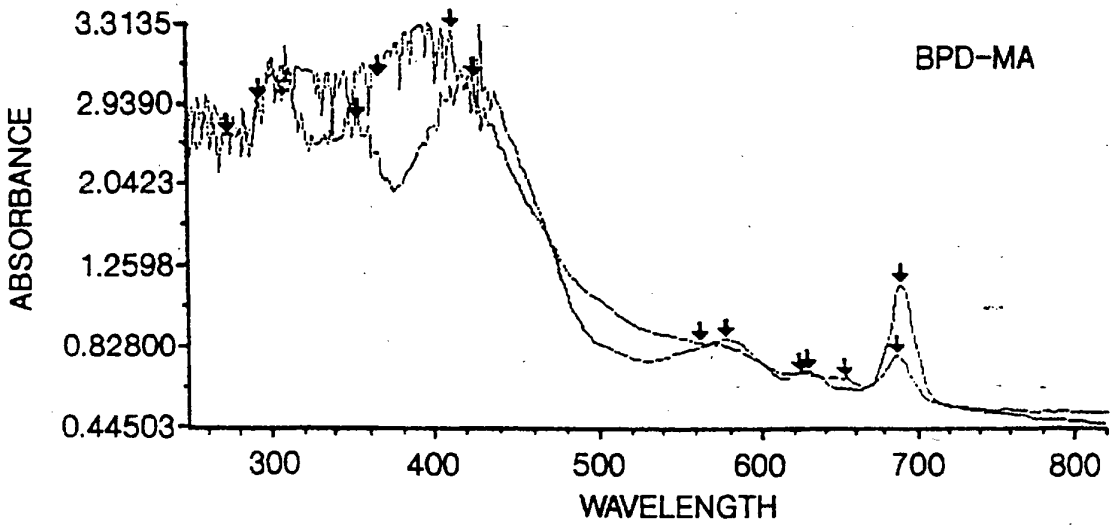

3.3135
2.9390
2.0423
1.2598
0.82800
0.44503
ABSORBANCE
300
400
500
600
700
800
WAVELENGTH
BPD-MA

FIG. 8A

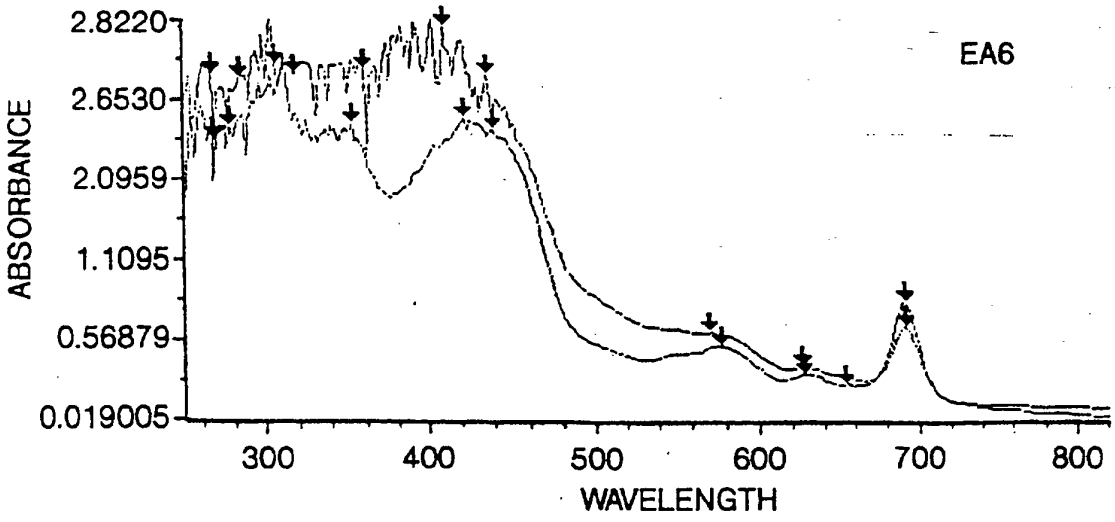

2.8220
2.6530
2.0959
1.1095
0.56879
0.019005
ABSORBANCE
300
400
500
600
700
800
WAVELENGTH
EA6

FIG. 8B

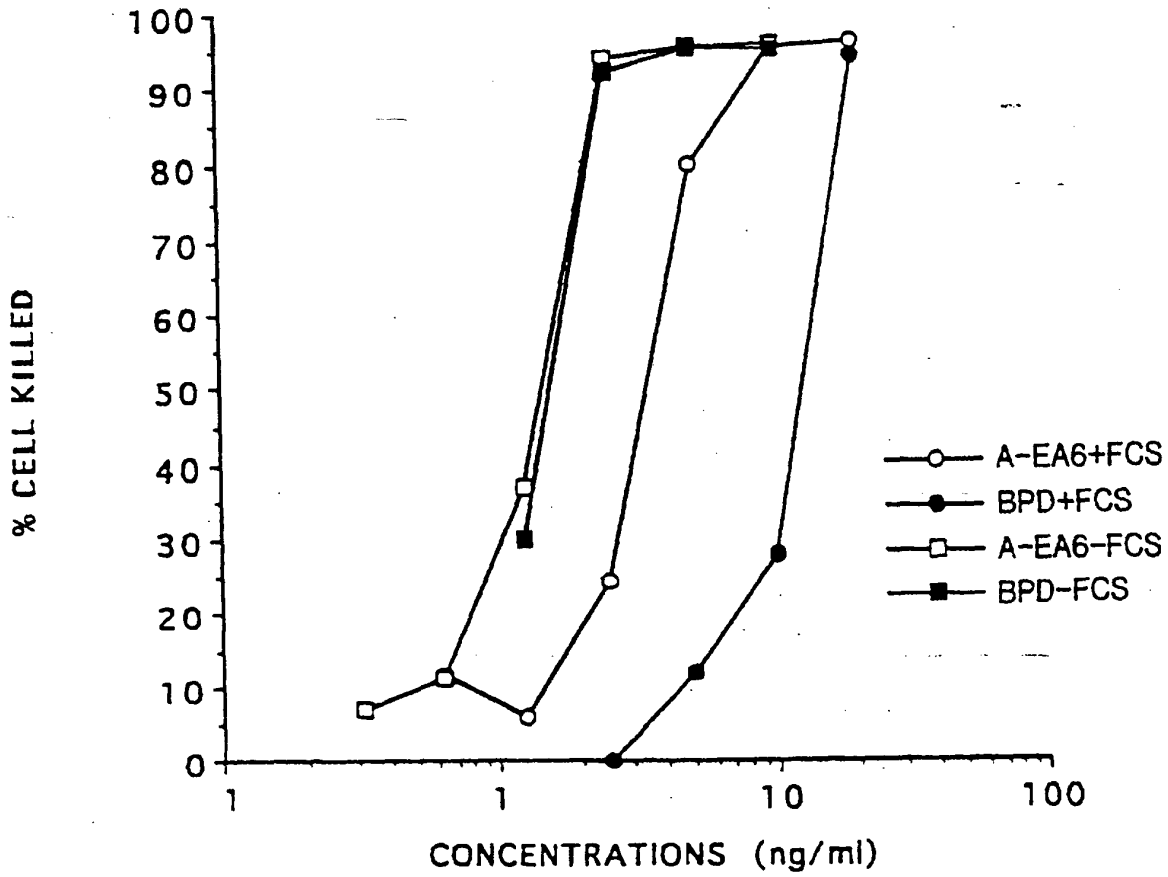

100
90
80
70
60
50
40
30
20
10
0
% CELL KILLED
1
1
10
100
CONCENTRATIONS (ng/ml)
A-EA6+FCS
BPD+FCS
A-EA6-FCS
BPD-FCS

FIG. 9A

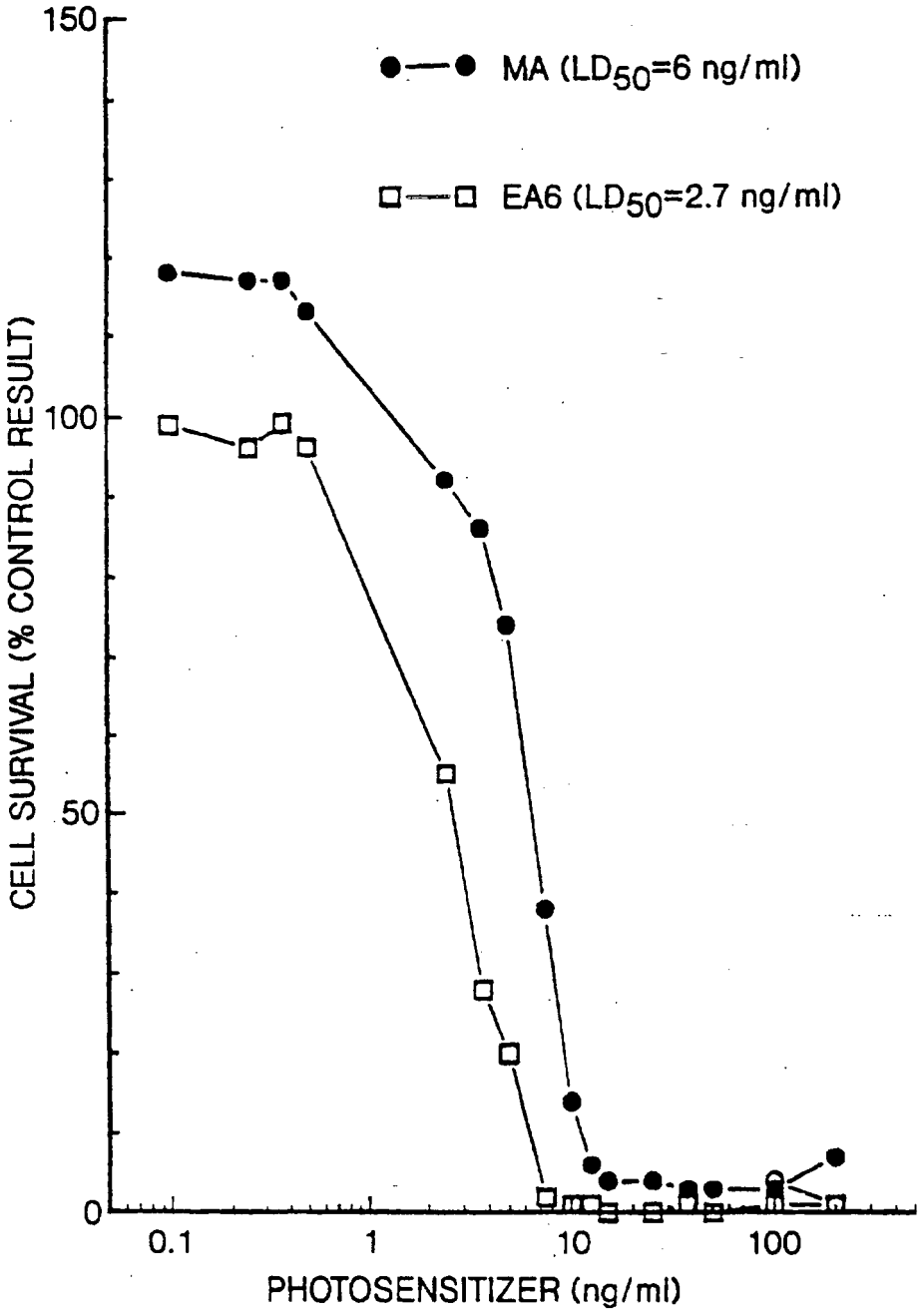

150
100
50
0
MA (LD_{50}=6 ng/ml)
EA6 (LD_{50}=2.7 ng/ml)
CELL SURVIVAL (% CONTROL RESULT)
0.1
1
10
100
PHOTOSENSITIZER (ng/ml)

FIG. 9B

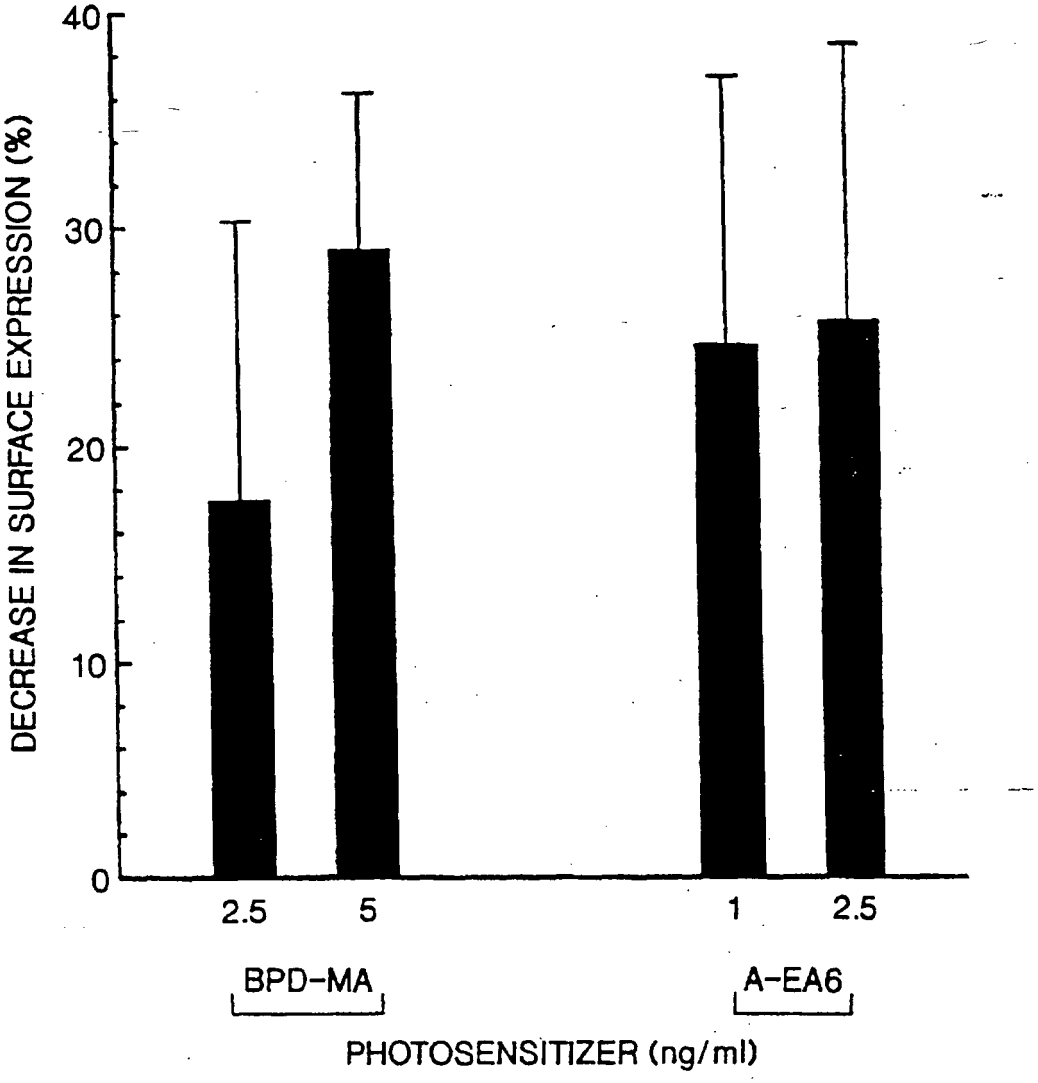

40
30
20
10
0
DECREASE IN SURFACE EXPRESSION (%)
2.5
5
1
2.5
BPD-MA
A-EA6
PHOTOSENSITIZER (ng/ml)

FIG. 10

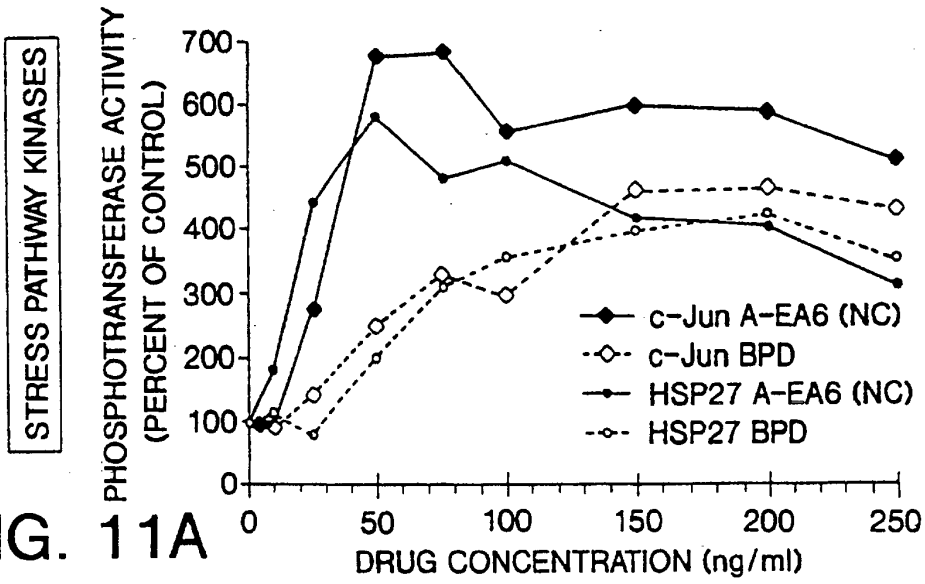


FIG. 11A

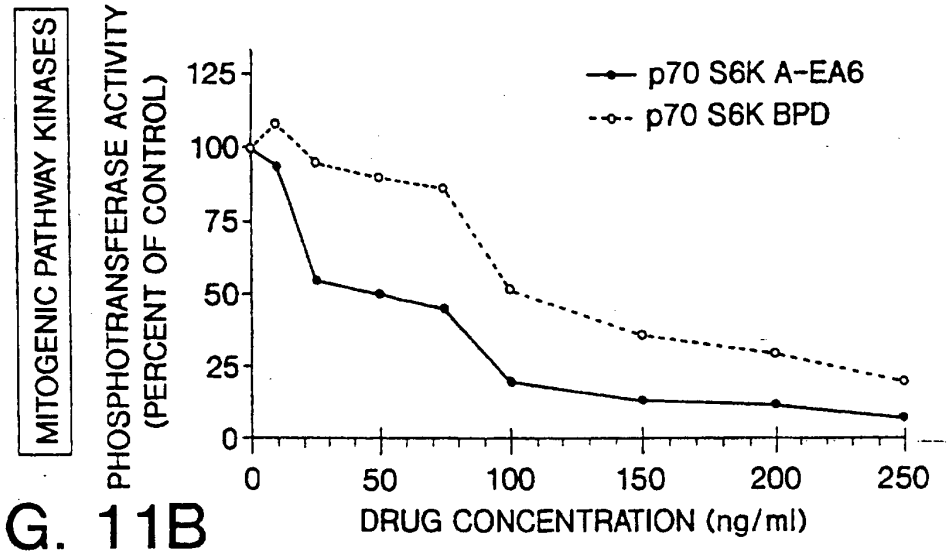


FIG. 11B

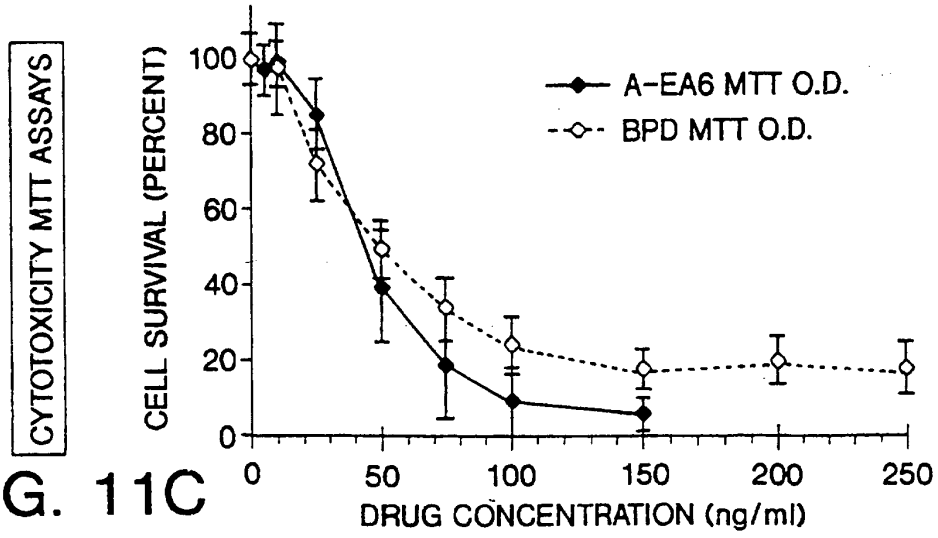


FIG. 11C

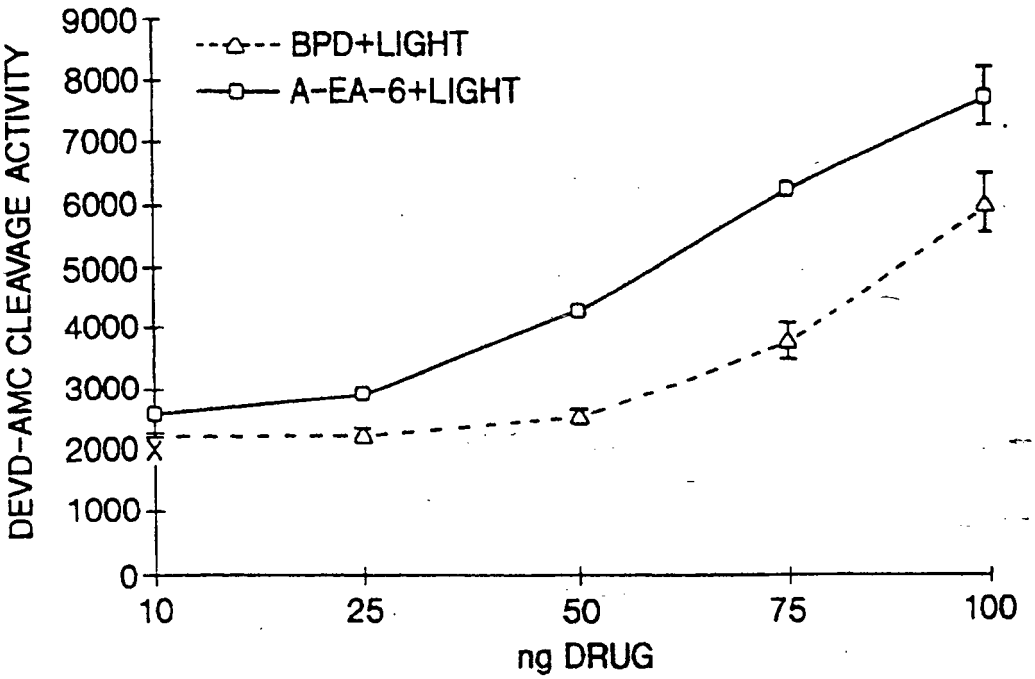

DEVD-AMC CLEAVAGE ACTIVITY
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
BPD+LIGHT
A-EA-6+LIGHT
10
25
50
75
100
ng DRUG

FIG. 12

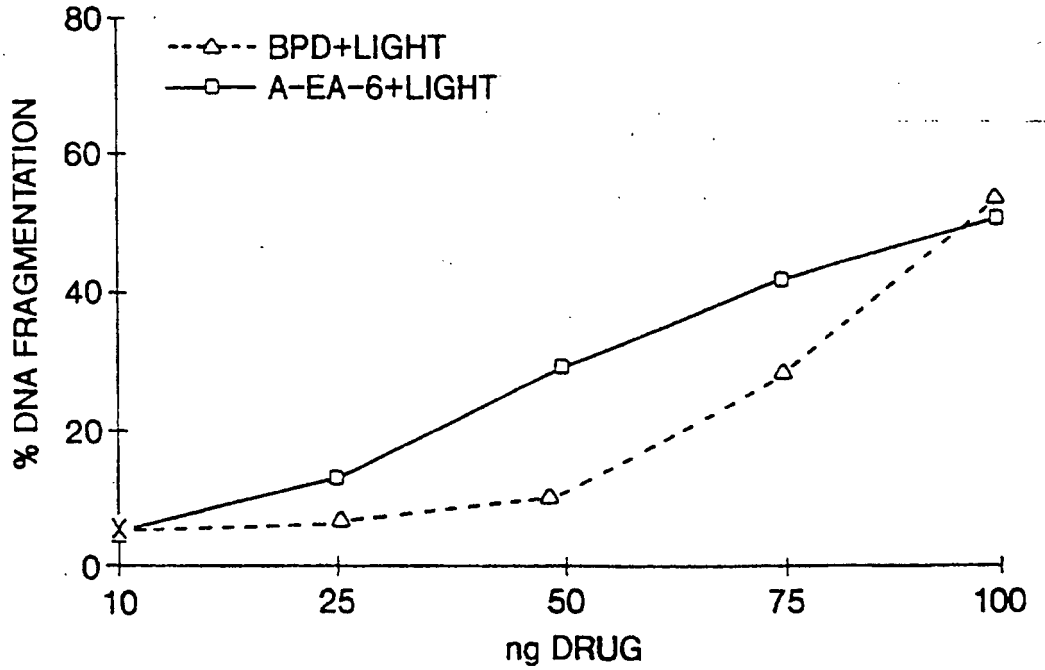

% DNA FRAGMENTATION
80
60
40
20
0
BPD+LIGHT
A-EA-6+LIGHT
10
25
50
75
100
ng DRUG

FIG. 13

REFERENCES CITED IN THE DESCRIPTION

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Patent documents cited in the description

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- US 5283255 A **[0003]**
- US 5399583 A **[0003]**
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- US 4920143 A **[0003]**
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Non-patent literature cited in the description

- **MOSMANN, T. et al.** *J Immunol Meth,* 1983, vol. 65, 55-63 **[0040]**