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PHOTOSENSITIZING EFFICIENCY OF TWO REGIOISOMERS OF THE BENZOPORPHYRIN DERIVATIVE MONOACID RING A (BPD-MA)

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Abstract—Benzoporphyrin derivative, monoacid ring A (BPD-MA), currently in clinical trials as a photosensitizer for photodynamic therapy for cancer, consists of two regioisomers (A1 and A2) present in equal proportions. The contribution of the regioisomers to the overall photosensitizing potency of BPD-MA was tested *in vitro* and *in vivo*. The *in vitro* photosensitizing potencies of BPD-MA-A1 and -A2 were tested in a standard cytotoxicity assay using M1 (rhabdomyosarcoma of DBA/2 mice) tumor cells and were found to be equivalent. The *in vivo* photosensitizing efficacies of the regioisomers were tested in the M1 tumor model in DBA/2 mice and were also found to be equivalent. Biodistribution of the regioisomers in mouse plasma, tumor and liver was studied in M1 tumor-bearing DBA/2 mice at 15 min and 3 hr post intravenous injection of [^{14}C]BPD-MA-A1/A2 at 4 mg/kg body weight. Plasma and extracts from tumor and liver were analysed by HPLC and tested for radioactivity. The two regioisomers were eliminated from plasma and liver at different rates, which resulted in A1 : A2 ratios of 1 : 0.28 in plasma and 1 : 0.75 in liver at 3 hr post injection. The differential elimination was not observed to any significant degree in the tumor, where even at 3 hr post injection the A1 : A2 ratio was 1 : 1.15. Therefore, we concluded that in tumor tissue, at 3 hr post injection, the time at which laser photodynamic therapy is carried out, both regioisomers were present in about equal proportions. Further, both regioisomers were fully active as determined by an *in vitro* cytotoxicity assay following extraction.

Photodynamic therapy (PDT‖) for cancer is a new experimental modality which seems to offer considerable promise for the treatment of some types of cancer [1, 2]. It is based on the use of light-activated drugs (mainly porphyrins and phthalocyanines), which are able to absorb the energy of light and either use it directly in chemical reactions with adjacent molecules or transfer it to molecular oxygen activating it to the singlet oxygen state. The exact mechanism of photodynamic damage is not known; however, the role of singlet oxygen and possibly other toxic oxygen species is postulated [3].

At present the only photosensitizer which has been tested extensively in clinical trials (phase III) is Photofrin® (QLT Phototherapeutics Inc., Vancouver, B.C. Canada/American Cyanamid, Pearl River, NY, U.S.A.). Although there is general agreement that Photofrin® is both efficacious and safe as a treatment for a number of cancer indications [4–7], considerable research continues on the development of new photosensitizers with improved characteristics over Photofrin® [8].

For the last few years we have tested analogues of the benzoporphyrin derivative, a chlorin type photosensitizer. One analogue especially, namely benzoporphyrin derivative, monoacid ring A (BPD-MA), was found to be a very potent photosensitizer *in vitro* [9] and *in vivo* [10]. This analogue also showed some promise as a bone marrow purging agent for the treatment of leukemia [11] and as an antiviral agent for blood and blood products [12]. Its advantages over Photofrin® are that it is a pure chemical (not a mixture like Photofrin®); it absorbs light of a longer wavelength than does Photofrin® (690 nm as compared to 630 nm), free from interference by hemoglobin absorption; and it does not appear to cause prolonged skin photosensitivity [13]. When injected intravenously into tumor-bearing mice, it reaches higher concentrations in tumors than in tissues such as skin and muscle, and is eliminated rapidly from the body [14]. All these characteristics offer great promise for clinical use of BPD-MA in PDT. This photosensitizer is currently in phase I clinical trials for patients with recurrent cutaneous neoplasms.

As BPD-MA exists in the form of two regioisomers which differ by the position of the ester group (either at ring C or D; Fig. 1), it was important to test the contribution of each regioisomer to the overall photosensitizing efficacy of BPD-MA. This paper reports the photosensitizing equivalency of the two regioisomers.

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‖ Abbreviations: PDT, photodynamic therapy; BPD-MA, benzoporphyrin derivative, monoacid ring A; NOE, Nuclear Overhauser Enhancement; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; DME, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; and ATCC, American Type Culture Collection.

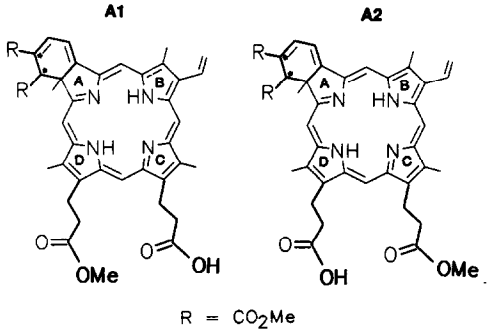

Fig. 1. Structures of regioisomers A1 and A2 of BPD-MA. Asterisks mark the position of the label in [^{14}C]BPD-MA.

MATERIALS AND METHODS

Regioisomers of BPD-MA. Benzoporphyrin derivate (BPD) was synthesized in the laboratory of Dr. David Dolphin (Department of Chemistry, University of British Columbia), originally in a diacid form [15]. The incomplete hydrolysis of the diester form (precursor of the diacid) results in formation of the monoacid forms [10]. BPD-MA exists as two regioisomers which differ in the location of the one acid and one ester group at the C and D rings of the porphyrin macrocycle (Fig. 1). The regioisomers can be separated by HPLC into two separate peaks (Fig. 2). The ratio between the first (BPD-MA-A1) and the second (BPD-MA-A2) peak was 1:1.06 (based on peak area).

Separation and identification of the regioisomers were carried out by Dr. E. Sternberg, Department of Chemistry, University of British Columbia. Separation was achieved on 0.5 mm preparative thin-layer chromatography plates (Si02-60, Merck) using a methylene chloride/ethyl acetate/acetic acid solvent system as a developer. Identification of the regioisomers was obtained by an extensive evaluation of Nuclear Overhauser Enhancement (NOE) studies [16].

BPD-MA and isolated regioisomers were stored in dimethyl sulfoxide (DMSO) at a concentration of 8 mg/mL, frozen at −70°. The dilutions for cytotoxicity assays (in culture medium) and for injections into the animals (in phosphate-buffered saline, PBS) were prepared immediately before use. The stocks and the dilutions were protected from light at all times.

[^{14}C]*BPD-MA.* The synthesis of ^{14}C-labeled BPD-MA, carried out in Dr. Dolphin's laboratory, was described earlier [10]. ^{14}C was incorporated in the cyclohexadiene ring attached to ring A in positions 2 and 3. The specific activity was 60.8 μCi/mg. Radiopurity was determined by HPLC analysis and was 94.2%. The stock in DMSO (at 7.1 mg/mL) was stored at −70°. Dilutions in PBS (500 μg/mL) were prepared immediately before injection into animals.

In vitro *cytotoxicity assay.* The *in vitro* photosensitizing efficacy of BPD-MA regioisomers was studied in a standard cytotoxicity assay, carried out using M1 (a rhabdomyosarcoma cell line from DBA/2 mice) tumor cells. The cells were obtained from freshly excised tumors grown subcutaneously in the flanks of DBA/2 mice, and cultured to near confluency in 96-well plates in Dulbecco's Modified Eagle's Medium (DME), usually for 72–96 hr. The

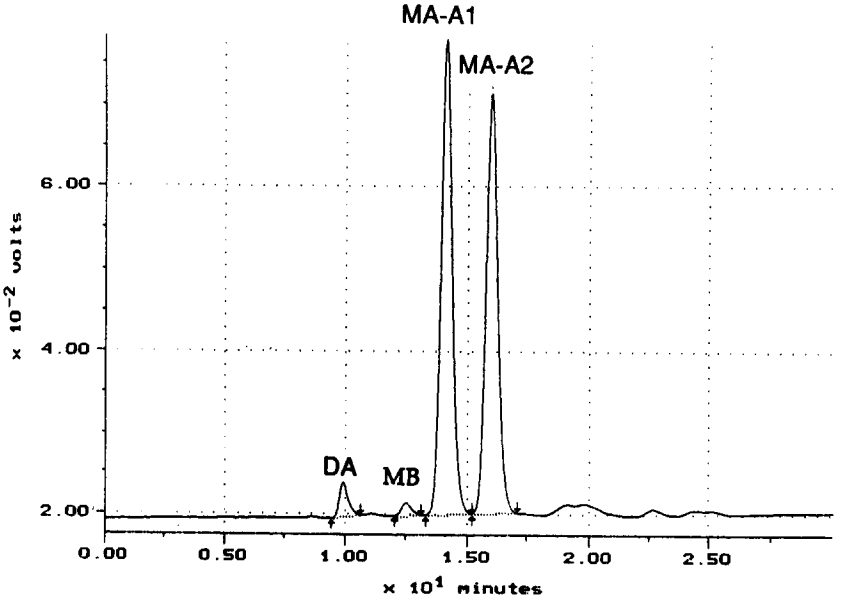

Fig. 2. HPLC chromatogram of a standard BPD-MA. The contaminating impurities, BPD-DA (diacid, ring A; 3.95%) and BPD-MB (monoacid, ring B; 1.23%), were identified by means of appropriate standards. For the description of the method see the text.

detailed protocol for the cell culture and assay conditions was described earlier [9]. Briefly, cells were incubated for 1 hr at 37° with either of the regioisomers in concentrations ranging from 2 to 30 ng/mL in culture medium, in the absence of serum. Following incubation, the excess drug was removed and the cells were exposed for 1 hr to fluorescent light at a dose of 10.08 J/cm^2. The viability of cells was determined after overnight incubation in medium containing 5% fetal bovine serum (FBS) by means of a colorimetric method, utilizing the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [17] as described earlier [9].

BPD-MA extracted from tissues was tested for phototoxic activity at dilutions of 1:500–1:8000, made immediately before assay. The modified cytotoxicity assay was carried out as described earlier [15] using L1210 cells (a mouse lymphocytic leukemia; ATCC CCL 219) at 10^6 viable cells/mL as target cells. This assay was performed to determine whether BPD-MA, extracted from tumor tissue, carried with it materials which might be toxic to cells in culture. This assay was performed with appropriate dark controls and compared to standard doses of BPD-MA in the presence or absence of light.

In vivo *tumor photosensitization.* The *in vivo* efficacy of BPD-MA regioisomers in photosensitizing tumors was tested in a mouse tumor model using M1 tumors, grown intradermally in the flanks of DBA/2 mice, as described earlier [10]. When tumors reached 4–5 mm in diameter, the animals were shaved in the tumor region and injected intravenously with either of the regioisomers, at a dose of 2 mg/kg body weight. Ten DBA/2 male mice (Charles River Laboratories, St. Constant, Quebec, Canada) were injected with each regioisomer. The animals were kept for 3 hr in the dark after which the tumor sites were exposed to 690 ± 2 nm laser light delivered via a fiberoptic system. The total dose delivered was 150 J/cm^2. Following light irradiation, the animals were housed under normal conditions. They were observed daily for 20 days and reappearance or continued absence of tumor was noted. The data thus obtained were compared with retrospective data compiled in our laboratory, obtained under the same conditions using BPD-MA and 70 mice.

[^{14}C]*BPD-MA biodistribution studies in mice.* Six DBA/2 male mice, bearing the M1 tumor grown subcutaneously in their flanks (as described earlier [14]) were used in this study. When tumors reached between 8 and 10 mm in diameter, animals were injected intravenously with [^{14}C]BPD-MA at 4 mg/kg body weight. Three animals were killed at 15 min and at 3 hr post injection. The radioactivity in blood, plasma and tissue samples was determined by liquid scintillation counting (Packard Tri-Carb 4550) in Econofluor (New England Nuclear, Boston, MA, U.S.A.) after solubilization in Protosol (New England Nuclear) as described earlier [14]. The counts per minute were converted to disintegrations per minute by means of a quench curve.

Preparation of [^{14}C]*BPD-MA extracts from tissue homogenates.* Tumors and livers (not blanched) excised from mice killed at 15 min or 3 hr post injection of [^{14}C]BPD-MA were homogenized in 1 mL of ice-cold water and frozen at −20° until further processing. Immediately before extraction, the homogenates were thawed and transferred to 7 mL amber vials. To each vial, 2 mL of ethyl acetate was added and the pH was adjusted to 5.0 using 10% acetic acid. The content of the vials was mixed using a vortex for 1 min, sonicated for 30 min in ice water, using an Ultrasonic Cleaner (Mettler Electronics) at setting 10, and then the vials were placed on a rotator for 1 hr at 4°. The organic phase was separated by centrifugation at 3000 rpm for 10 min and collected in a glass conical tube. The sediment was extracted again with 1 mL of ethyl acetate for 1 hr and then overnight. All three supernatants were pooled and the solvent was evaporated by bubbling with nitrogen. The solid residue was reconstituted in 100 μL of DMSO/tube. The extracts from the same type of tissue, obtained at the same time post injection were pooled in order to obtain enough material for tests. The radioactivity in the extracts thus obtained and in tumor and liver homogenates before and after extraction was determined by liquid scintillation counting. The unextracted radioactivity, remaining in the tissues following extraction, represented 10.4 ± 2.2% (SD) of the total radioactivity before the extraction.

In vitro *experiments with mouse plasma.* BPD-MA from DMSO stock was added to freshly obtained mouse heparinized plasma at 20 μg/mL and was incubated at 37° for up to 24 hr. Samples for the HPLC analysis were obtained immediately (0 hr) and after 15-min, 1-hr, 3-hr and 24-hr incubations.

HPLC analysis of BPD-MA in plasma and tissue extracts. The HPLC method for the purification of BPD-MA was developed originally by the Metabolism Research and Bioanalytical Support Departments of the American Cyanamid Co. (Pearl River, NY, U.S.A.) and was somewhat modified by us for these studies. The HPLC analysis of BPD-MA was carried out using a Waters 625 BioLC System, 484 Absorbance Detector and Baseline 815 chromatography software (Version 3.3; 1989, Millipore Corp., Milford, MA, U.S.A.) and a reverse phase ultrasphere C-8 column (Beckman). All reagents used were HPLC grade (BDH Inc., Vancouver, B.C., Canada). The mobile phase was composed of 0.08 M $(NH_4)_2SO_4$:acetonitrile:tetrahydrofuran:acetic acid (52:28:28:5), and the pH was adjusted to 3.0 with phosphoric acid. The flow rate was 1 mL/min. The eluent was monitored at 410 nm. All samples were filtered through Sep-Pak® C18 before being injected into the column. The peaks were identified by their retention time determined by means of the appropriate standards. The analyses and comparisons were carried out on the basis of the percentage of total peak area represented by the individual peaks on the chromatogram.

Plasma samples (100 μL) were mixed with 0.5 mL of acetonitrile, which caused the precipitation of protein and extracted BPD-MA. The organic phase was then separated by centrifugation, evaporated to dryness under nitrogen and the solid residue was reconstituted in 100–200 μL of the mobile phase and injected into the column in 50–100 μL aliquots. Extracts from tissue homogenates in DMSO were

injected directly into the column in 20–100 μL aliquots. On occasions when the radioactivity was determined in the eluate from the column, 0.5-mL samples were collected and counted in 5 mL Aquasol (New England Nuclear) in a liquid scintillation counter. Counts per minute were converted to disintegrations per minute by means of a quench curve, and the radioactivity in each 0.5-mL fraction was expressed as a percentage of the total radioactivity injected onto the column.

Light source and conditions for in vitro *studies.* The cells in 96-well plates were irradiated with fluorescent light produced by a bank of four fluorescent tubes as described earlier [9]. The light intensity, measured with an IL 1350 Photometer at the plane of exposure, was 2.8 mW/cm^2. The spectrum of light was between 300 and 800 nm, with the highest radiant power at 600 nm (General Electric Bulletin, unpublished data).

Light source and conditions for in vivo *studies.* A Spectra-Physics (Series 2000, Mountain View, CA, U.S.A.) 5 W argon ion pumped dye laser (Coherent, model 599, Palo Alto, CA, U.S.A.) and DCM dye (610–720 nm, Excitron Chemical Co., Dayton, OH, U.S.A.) were used. The light at 690 nm was delivered via a fiberoptic coupling system and microlens fibers (Quadra Logic Technologies, Vancouver, B.C., Canada). The illuminated spot size was 10 mm in diameter. The light intensity at the surface of the tumors was 89 mW/cm^2 (as measured by a Gentec TMP-A photometer with a PS-10 detector head; Gentec Inc., Sainte-Foy, Quebec, Canada). Mice were immobilized in special holders for the duration of the laser treatment (28 min).

RESULTS

Photosensitizing efficacy of BPD-MA regioisomers in vitro. The results of cytotoxicity assays, using the M1 tumor cell line, showed that both regioisomers of BPD-MA were equally potent as photosensitizers (Fig. 3). Under the test conditions, the concentrations required to kill 50% of M1 cells (LD_{50}), obtained from the concentration–response curve, were 5.8 ± 0.8 and 6.4 ± 1.1 (SD) ng/mL for A1 and A2, respectively. These were comparable to values for BPD-MA containing equal amounts of the two regioisomers [9].

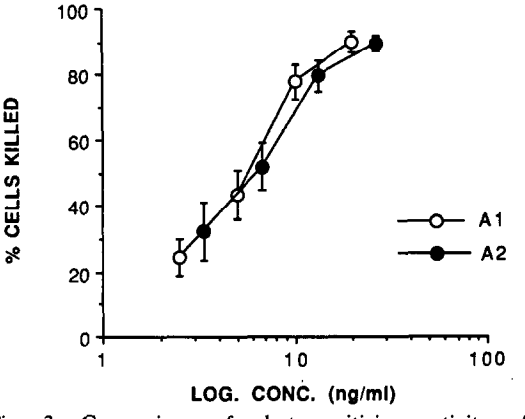

Fig. 3. Comparison of photosensitizing activity of regioisomers of BPD-MA, A1 and A2, on M1 tumor cells as tested in an *in vitro* cytotoxicity assay. The cells (at 10^5 cells/well) were incubated for 1 hr at 37° with various concentrations for A1 or A2 and then exposed to broad spectrum light (10.08 J/cm^2). Cells killed are presented as a percentage of control cells treated with light alone and expressed as means ± SD of six experiments.

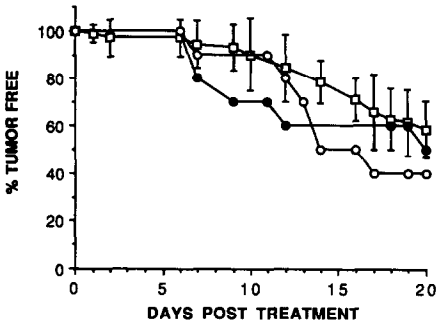

Fig. 4. Comparison of *in vivo* photosensitizing efficiency of A1 (●) and A2 (○) regioisomers of BPD-MA, tested in the M1 tumor model in DBA/2 mice. Each drug was injected intravenously at 2 mg/kg body weight, and at 3 hr post injection tumors were irradiated with 690 nm laser light at a dose of 150 J/cm^2. The number of animals tumor-free between day 1 and day 20 post treatment was determined daily, and expressed as a percentage of the starting number of animals. The data obtained with each of the regioisomers (10 mice/group) were superimposed on the data (mean ± SD) obtained in 70 mice with BPD-MA (□) (A1:A2 = 1:1.05), under the same conditions.

Photosensitizing efficacy of BPD-MA regioisomers in vivo. Mice bearing M1 tumors were given BPD-MA or one of the regioisomers administered intravenously, and 3 hr later the tumors were exposed to 690 nm laser light.

On the first day following laser treatment all treated animals, regardless of the tested compound (BPD-MA, BPD-MA-A1 or BPD-MA-A2), developed eschars at the treatment site due to tumor photosensitivity caused by the drug. Edema was present and no tumor was visible or palpable. No tumors were observed during the next 4 days. However, between days 5 and 7 post treatment tumors started to recur in all groups. Figure 4 presents the number of tumor-free animals between day 1 and day 20 post treatment. The results showed no significant difference in photosensitizing efficacy between the individual regioisomers as compared to the mixture. This demonstrates an equal contribution from both regioisomers to the photosensitizing potency of BPD-MA.

Biodistribution of BPD-MA regioisomers in mouse plasma, tumor and liver. HPLC analysis of [^{14}C]-BPD-MA, combined with analysis of radioactivity in the fractions eluted from the column, showed that the two regioisomers were present in the same proportion (A1:A2 = 1:1.05) as in non-labeled

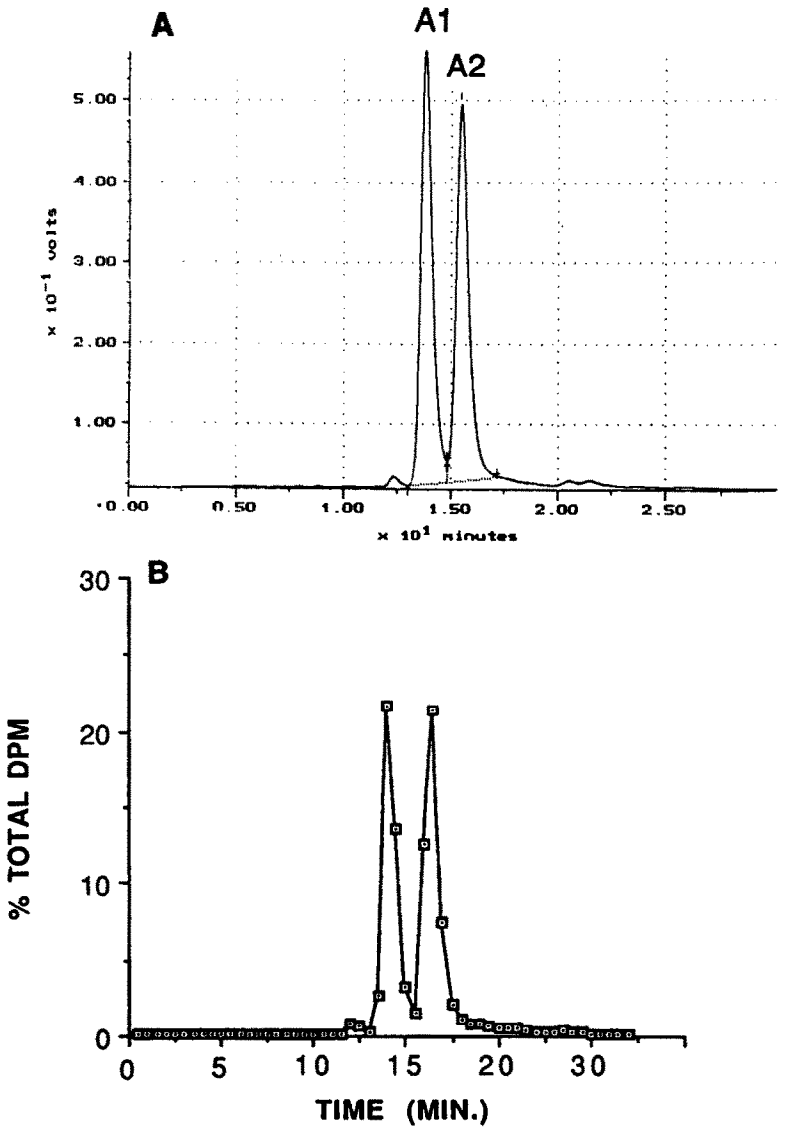

Fig. 5. HPLC analysis of [^{14}C]BPD-MA. (A) HPLC chromatogram of [^{14}C]BPD-MA-A1 and -A2 (A1 : A2 = 1 : 1.05). (B) Corresponding radioactivity in 0.5-mL fractions (rate: 1 mL/min) was expressed as a percentage of total radioactivity (1,689,541 dpm) in the analysed sample.

BPD-MA and that the radioactivity was distributed equally between both peaks (Fig. 5).

HPLC analysis of mouse plasma samples obtained at 15 min and 3 hr post injection of [^{14}C]BPD-MA showed an increasing difference between the amounts of A1 and A2 regioisomers present in plasma. At 15 min post injection the A1 and A2 peaks represented 39.5 and 42.4% of the total peak area, respectively, giving an A1 : A2 ratio of 1 : 1.07. At the same time the diacid peak represented 8.2% of the total peak area. At 3 hr post injection plasma concentrations of the two regioisomers were very different. The A1 and A2 peaks represented 51.2 and 14.1% of the total peak area, respectively, giving an A1 : A2 ratio of 1 : 0.28. The radioactivity in the HPLC fractions confirmed the difference between the two peaks (Fig. 6). At this time the diacid peak represented 9.3% of the total peak area. The radioactivity associated with the diacid peak, at both time points, established that this was a metabolite of the ^{14}C-labeled BPD-MA. These results indicated that the A2 regioisomer was either being cleared from blood faster than A1, or that it was being metabolized selectively in blood. Further experiments were carried out *in vitro* in mouse plasma to test these possibilities.

In vitro experiments carried out with mouse plasma (Table 1) showed a different hydrolysis pattern when compared to the *in vivo* studies. *In vitro*, there appeared to be some preference for hydrolysis of the ester at ring D, which is opposite to the apparent *in vivo* preference for hydrolysis at

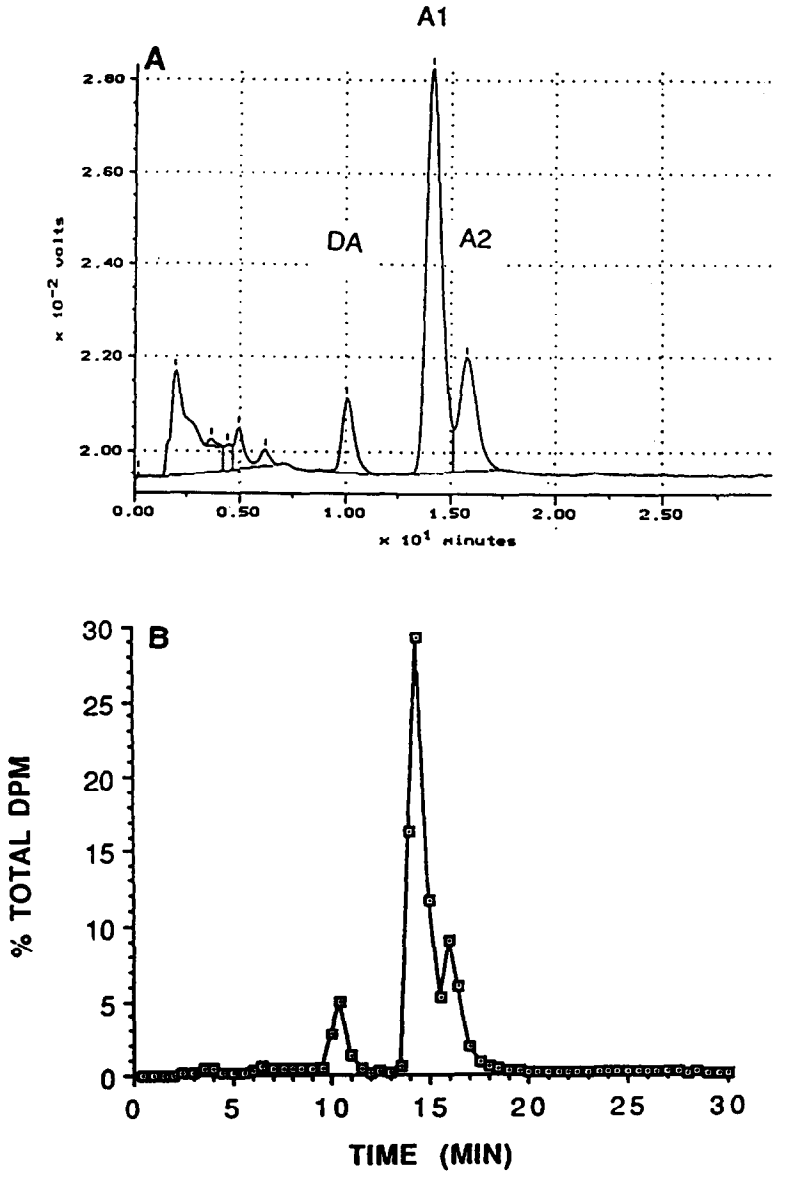

Fig. 6. HPLC analysis of mouse plasma at 3 hr post injection of [^{14}C]BPD-MA at 4 mg/kg body weight. (A) HPLC chromatogram. A1 and A2 are regioisomers of BPD-MA. The peak area for the diacid (DA), A1 and A2 represented 9.3, 51.2 and 14.1% of the total peak area, respectively. (B) Corresponding radioactivity in 0.5-mL fractions (rate: 1 mL/min) was expressed as a percentage of the total radioactivity (18,744 dpm) in the analyzed sample. For more details see the text.

ring C. *In vitro* the diacid, formed during the time of incubation, accumulated, and appeared to be the only major product. These results indicated that selective metabolism of the A2 regioisomer by plasma enzymes was not the cause of its relatively rapid disappearance from blood *in vivo*.

The extraction method used for tumor and liver tissue was very efficient as only about 10% of the original radioactivity remained in tissue homogenates following extraction. HPLC analysis of extracts from M1 tumors at 15 min and 3 hr post injection of [^{14}C]-BPD-MA showed that even at 3 hr, unlike the plasma levels at this time, the two regioisomers were present in essentially equal proportions (A1 : A2 = 1 : 1.15), as in the parent drug. Similarly, most of the radioactivity (86%) was associated with the peak fractions (Fig. 7).

The cytotoxicity assay, using L1210 cells and the material extracted from tumors at 15 min and 3 hr post injection, showed that phototoxicity to this cell line correlated absolutely with the radioactivity extracted from the tissue, indicating that all of the ^{14}C material had remained as active drug (Fig. 8).

HPLC analysis of extracts from mouse liver obtained at 15 min and 3 hr post injection of [^{14}C]-BPD-MA showed that both BPD-MA regioisomers

Table 1. HPLC analysis of mouse plasma samples following *in vitro* incubation with BPD-MA at 20 μg/mL

Incubation time	% Total peak area		
	DA	A1	A2
0 hr	3.5	43.7	49.8
15 min	6.9	41.9	48.1
1 hr	15.2	37.3	43.8
3 hr	30.6	28.1	38.0
24 hr	51.3	13.7	26.8

Freshly obtained mouse heparinized plasma was incubated with BPD-MA at 37° and the samples for HPLC analysis were obtained between 0 and 24 hr. The plasma concentrations of the diacid (DA) and of the two regioisomers (A1 and A2) of BPD-MA were measured by the peak areas and expressed as a percentage of the total peak area.

were present at both time points tested, in about equal proportions but at lower concentrations at 3 hr than at 15 min. The peak ratios of A1:A2 were 1:1.03 and 1:0.75 at 15 min and 3 hr, respectively. A BPD-diacid peak was present at both time points and, like the monoacid forms, its concentration was lower at 3 hr and represented about 48% of that at 15 min post injection (Fig. 9). The distribution of the radioactivity in liver extracts differed between the time points. At 15 min post injection 85% of the radioactivity was associated with both regioisomers of BPD-MA, 7% was associated with BPD-diacid and about 2% eluted from the column during the first 7 min. At 3 hr post injection, only 69% of radioactivity was associated with the regioisomers, 13% was associated with BPD-diacid and 6% eluted during the first 7 min. The increase of radioactivity in the early fractions indicated formation of some highly polar metabolites.

DISCUSSION

The chemical characteristics, mainly the presence of the same chromophore group, enabled both BPD-MA regioisomers to absorb the same spectrum of light (results not shown) and thus the same amount of light energy, which could be used for photochemical reactions, such as production of singlet oxygen [3]. However, in a biological environment the spatial arrangement of the side groups is often critical for the pharmacological effect(s) of the molecule. Although the mechanism of photodynamic damage to cells is presumed to be non-specific (i.e. it is not receptor mediated, or location dependent for its effect), there are other factors, such as the ability to cross cell membranes or to localize at a particular vulnerable site in the cell, which depend on the characteristics of a photosensitizer and therefore affect the results of photosensitization [reviewed in Ref. 18]. It was therefore important to test the efficiency of photosensitization of each of the regioisomers of BPD-MA in order to ascertain their contribution to the overall photosensitizing efficiency of BPD-MA.

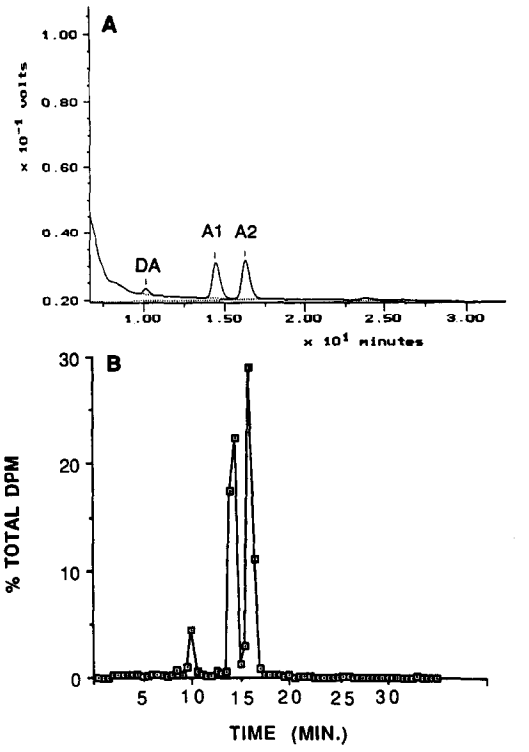

Fig. 7. HPLC analysis of tumor extract obtained from M1 tumors in DBA/2 mice excised at 3 hr post injection of [^{14}C]BPD-MA at 4 mg/kg body weight. (A) HPLC chromatogram. A1 and A2 are regioisomers of BPD-MA. The peak areas for the diacid (DA), A1 and A2 represented 0.5, 4.2 and 4.8% of the total peak area, respectively. (B) Corresponding radioactivity in 0.5-mL fractions (rate: 1 mL/1 min) was expressed as a percentage of the total radioactivity (21,007 dpm) in the analysed sample. For more details see the text.

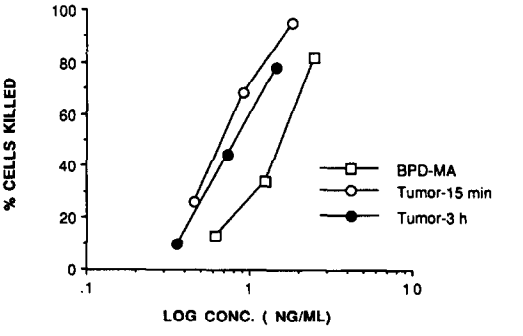

Fig. 8. Light-activated cytotoxicity of tumor extracts obtained from M1 tumors in DBA/2 mice excised at 15 min and 3 hr post injection of [^{14}C]BPD-MA at 4 mg/kg body weight. The concentration of BPD-MA in extracts was determined based on the radioactivity. The extracts, diluted 500- to 8000-fold, were incubated in 96-well plates with L1210 cells (10^5/well) for 1 hr at 37° following which they were exposed to broad spectrum light (10.08 J/cm^2). The appropriate dark controls were made and the dark toxicity was corrected. The percentage of cells killed at various concentrations of the extracts or standard BPD-MA was calculated in relation to the cells exposed to light alone.

The cytotoxicity test carried out *in vitro*, using M1 tumor cells, showed that both regioisomers were equally potent photosensitizers (Fig. 3). This indicated that although the presence of one ester group in the BPD molecule affected the photosensitizing potency (monoacid forms of BPD are significantly more potent than diacid forms [9]), the position of the ester at rings C or D of the porphyrin did not. This would indicate further that at the cellular level the position of the ester in BPD-MA is not critical for the ability of the molecule to reach cellular target sites.

Studies with the M1 tumor model in DBA/2 mice, at a range of doses that permitted calculation of the ED_{50}, showed that both regioisomers of BPD-MA were equally efficient in photosensitizing tumor cells *in vivo* (Fig. 4). Within margins of error, the same number of animals were tumor-free within the 20-day post treatment period, whether A1 or A2 regioisomers were injected and compared to the larger base of data obtained with BPD-MA. The results suggested that both regioisomers were able to reach the target sites in the tumor *in vivo* and were present there in an active form at the time of laser light activation.

To confirm the presence of active regioisomers in tumors and to trace the fate of both regioisomers in the body, DBA/2 mice were injected with [^{14}C]-BPD-MA at 4 mg/kg body weight. ^{14}C-Labeled BPD-MA was proved by HPLC analysis to be equivalent to unlabeled BPD-MA, in that it was composed of the two regioisomers in the same proportion as unlabeled BPD-MA and the radioactivity was associated with both regioisomers (Fig. 5). Therefore, tracing [^{14}C]BPD-MA in the body allowed us to trace the fate of the regioisomers. It was somewhat surprising that one of the regioisomers (A2) cleared from the blood at a faster rate than the other (A1) and yet this difference was not reflected in the tumor content of the two isomers which remained in essentially equivalent concentrations up to 3 hr post injection. The 3-hr values for A1 and A2 in plasma isolated from mice showed that A2 levels were considerably lower than A1 (51.2 to 14.1% of the total peak areas: Fig. 6). The presence of a small peak of radioactive BPD-DA at this time indicated that clearance of A2 from blood could be mediated via stereospecific esterases which converted A2 to the diacid form which is cleared more rapidly from blood than are the monoacid forms of BPD [9]. To determine whether this mechanism accounted for the disparity in A1:A2 levels at 3 hr, an experiment was carried out in mouse plasma *in vitro* over a 24-hr period. The results (Table 1) show that there was probably an esterase-mediated conversion of A1 and A2 to BPD-DA (at 24 hr 51.3% of radioactivity was recovered in the diacid fraction). However, no stereospecific preference for the A2 regioisomer was observed. Indeed, at 24 hr, the A1 isomer was present at much lower levels than A2 (13.7% of the radioactivity in comparison to 26.8%). Therefore, the *in vivo* observations made at 3 hr post injection suggest that the very low levels of A2 may be due to selective clearance rather than selective hydrolysis.

The HPLC analysis of M1 tumor extracts obtained at 15 min and at 3 hr post injection showed both regioisomers present in about equal proportions. The 3 hr point was of special importance as this was the laser treatment time. At this time there was very little of the diacid present in the extracts and the radioactivity was associated mainly with the regioisomers of BPD-MA (Fig. 7). This suggested that both regioisomers were present in the extracts in their most active form. The photosensitizing activity was confirmed by a cytotoxicity assay *in vitro* using L1210 cells and extracts of the tumors. This cell line is very sensitive to photodynamic treatment, which allowed us to use dilutions of extracts well below the concentrations at which any dark effects, attributable to cell components rather than BPD, might be seen. The results showed that at both 15 min and 3 hr post injection, the amount of BPD-MA, as determined by radioactivity, corresponded to the light-activated cytotoxicity, which proved that both regioisomers were present in an active form (Fig. 8).

Liver was chosen for metabolite testing because BPD-MA is eliminated from the body mainly via the bile and the feces [14]. HPLC and radioactivity analyses of extracts from mouse liver obtained at 15 min and 3 hr post injection of [^{14}C]BPD-MA showed a shift of radioactivity at 3 hr as compared to 15 min, from fractions containing unchanged drug to fractions eluting early from the column. This suggested that both BPD-MA regioisomers were metabolized to a highly polar metabolite(s) eluting early from the column (Fig. 9). The diacid appeared to be a transient intermediate metabolite since only a minimal accumulation was observed at 3 hr post injection. The reduction of radioactivity in liver extracts with time indicated rapid elimination of both A1 and A2 regioisomers of BPD-MA and their metabolites from the liver. Rapid elimination of labeled BPD-MA from liver was indicated in earlier studies [14] by rapid transfer of radioactivity between the liver and the gall bladder.

The results shown here indicate that the first step in the metabolism of BPD-MA (A1 and A2) is the hydrolysis of the ester group resulting in the diacid form of BPD. This was demonstrated in the *in vitro* plasma studies (Table 1). Combined HPLC and radioactivity analyses of liver extracts, over time, suggested that the diacid form of BPD was transient and that it was converted rapidly to a highly polar metabolite (results not shown).

The presence of both regioisomers of BPD-MA in about equal proportions in the tumor at 3 hr post injection suggested not only the absence of stereospecific hydrolysing enzymes in the tumor tissue but also a rapid accumulation of both regioisomers in the tumor tissue, since soon after injection blood would be supplying the regioisomers in unequal proportions to tumor tissue. This rather surprising observation implies that deposition of this photosensitizer occurs rapidly after intravenous injection, and that very little exchange between tumor-associated drug and plasma-associated photosensitizer takes place.

In conclusion, both BPD-MA regioisomers were shown to be equally potent photosensitizers of tumor cells *in vitro* and *in vivo*. They accumulated rapidly,

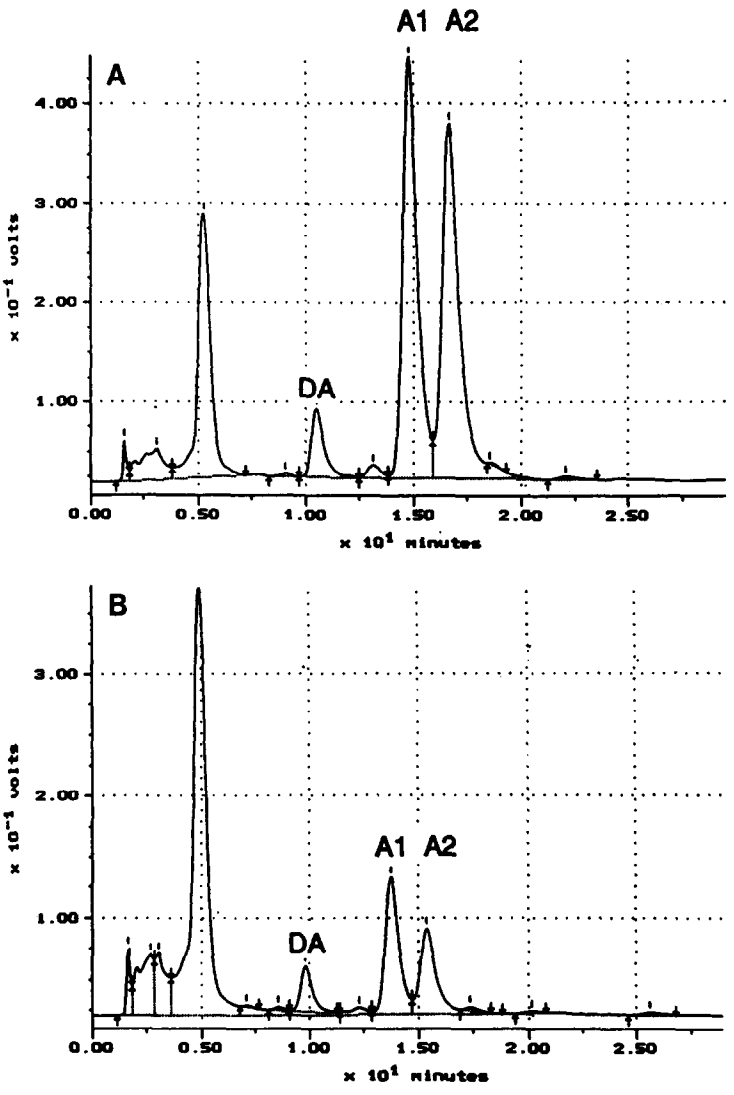

Fig. 9. HPLC analysis of liver extract obtained from livers of M1 tumor bearing DBA/2 mice, excised at 15 min (A) and 3 hr (B) post injection of [^{14}C]BPD-MA at 4 mg/kg body weight. A1 and A2 are regioisomers of BPD-MA. The peak areas, expressed as a percentage of total peak area, were at 15 min: 4.7, 34.2 and 35.2%, and at 3 hr: 4.1, 14.9 and 11.3% for the diacid (DA), A1 and A2 regioisomers, respectively. No radioactivity peak was observed in the fractions eluting at 5 min. The same peak was observed also in extracts from control livers (obtained from non-injected animals).

with equal efficiency in the tumor, and both were present in an active form during laser treatment at 3 hr post injection.

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REFERENCES

1. Wilson BC and Jeeves WP, Photodynamic therapy of cancer. In: *Photomedicine* (Eds. Ben-Hur E and Rosenthal I), Vol. 2, pp. 127–177. CRC Press, Boca Raton, FL, 1987.
2. Dougherty TJ, Photosensitizers: Therapy and detection of malignant tumors. *Photochem Photobiol* **45**: 879–889, 1987.
3. Spikes JD, Photobiology of porphyrins. In: *Porphyrin Localization and Treatment of Tumors* (Eds. Doiron DR and Gomer CJ), pp. 19–39. Alan R. Liss, New York, 1984.
4. Hayata Y and Konaka C, Photodynamic therapy of neoplastic disease. In: *Photodynamic Therapy of Neoplastic Diseases* (Ed. Kessel D), Vol. 1, pp. 43–64. CRC Press, Boca Raton, FL, 1990.
5. Wening BL, Kurtzman DM, Grossweiner LI, Mafee MF, Harris DM, Lobraico RV, Prycz RA and Appelbaum EL, Photodynamic therapy in the treatment

of squamous cell carcinoma of the head and neck. *Arch Otolaryngol Head Neck Surg* **116**: 1267–1270, 1990.
6. McCaughan JS Jr, Photodynamic therapy of skin and esophageal cancers. *Cancer Invest* **83**: 407–416, 1990.
7. Buchanan RB, Carruth JAS, McKenzie AL and Williams SR, Photodynamic therapy in the treatment of malignant tumours of the skin and head and neck. *Eur J Surg Oncol* **15**: 400–406, 1989.
8. Kreimer-Birnbaum M, Modified porphyrins, chlorins, phthalocyanines, and purpurins: Second-generation photosensitizers for photodynamic therapy. *Semin Hematol* **26**: 157–173, 1989.
9. Richter AM, Waterfield E, Jain AK, Sternberg ED, Dolphin D and Levy JG, *In vitro* evaluation of phototoxic properties of four structurally related benzoporphyrin derivatives. *Photochem Photobiol* **52**: 495–500, 1990.
10. Richter AM, Waterfield E, Jain AK, Allison B, Sternberg ED, Dolphin D and Levy JG, Photosensitising potency of structural analogues of benzoporphyrin derivative (BPD) in a mouse tumour model. *Br J Cancer* **63**: 87–93, 1991.
11. Jamieson CHM, McDonald WN and Levy JG, Preferential uptake of benzoporphyrin derivative by leukemic versus normal cells. *Leuk Res* **14**: 209–219, 1990.
12. Neyndorff HC, Bartel DL, Tufaro F and Levy JG, Development of a model to demonstrate photosensitizer-mediated viral inactivation in blood. *Transfusion* **30**: 485–490, 1990.
13. Richter AM, Yip S, Waterfield E, Logan PM, Slonecker CE and Levy JG, Mouse skin photosensitization with benzoporphyrin derivatives and Photofrin®: Macroscopic and microscopic evaluation. *Photochem Photobiol* **53**: 281–286, 1991.
14. Richter AM, Cerruti-Sola S, Sternberg ED, Dolphin D and Levy JG, Biodistribution of tritiated benzoporphyrin derivative (^{3}H-BPD-MA), a new potent photosensitizer, in normal and tumor-bearing mice. *J Photochem Photobiol [B]* **5**: 231–244, 1990.
15. Richter AM, Kelly B, Chow J, Liu DJ, Towers GH, Dolphin D and Levy JG, Preliminary studies on a more effective phototoxic agent than hematoporphyrin. *J Natl Cancer Inst* **79**: 1327–1332, 1987.
16. Silverstein RM, Clayton GC and Morrill TC, *Spectroscopic Identification of Organic Compounds*. John Wiley, New York, 1981.
17. Mosman T, Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**: 55–63, 1982.
18. Jori G, *In vivo* transport and pharmacokinetic behaviour of tumor photosensitizers. In: *Photosensitizing Compounds: Their Chemistry, Biology and Clinical Use* (Ciba Foundation Symposium 146), pp. 78–94. Wiley, Chichester, 1989.