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Spectral characterization of the benzoporphyrin derivative monoacid ring-A photoproduct formed in fetal calf solutions during irradiation with 694 nm continuous-wave radiation [1]

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Abstract

Benzoporphyrin derivative monoacid ring A (BPD-MA) is a second-generation photosensitizer for photodynamic therapy (PDT) that has shown good results in phase I clinical trials. Similar to other porphyrin derivatives, BPD-MA readily photobleaches during in-vivo PDT treatment. This study investigated the photodegradation of BPD-MA in fetal calf serum (FCS) solutions in vitro. Absorption and fluorescence spectra from dilute solutions of BPD-MA in 10% FCS were recorded before and immediately after irradiation with light at 694 nm. After irradiation, the appearance of a new fluorescence emission band at 650 nm and changes in the fluorescence excitation spectra indicate the formation of a photoproduct. Photoproduct formation was observed only when BPD-MA was bound to FCS and in oxygenated solutions. The spectroscopy of the photoproduct is consistent with the reaction of an oxygen species with the ring B vinyl group, forming a hydroxyaldehyde photoproduct. Monitoring the increase in photoproduct fluorescence during treatment may provide an in-vivo dosimeter to measure PDT efficacy.

Keywords: Benzoporphyrin derivative monoacid ring A; Photoproduct; Spectroscopy; Photodynamic therapy; Singlet oxygen

1. Introduction

Benzoporphyrin derivative monoacid ring A (BPD-MA) is a second-generation photosensitizer for photodynamic therapy (PDT) of cancer currently undergoing phase I clinical trials [1–3]. Although the primary interest in BPD-MA has been as a photosensitizer for cancer therapy, BPD-MA has also shown potential as an antiviral agent [4], for the treatment of atherosclerosis [5] and the treatment of psoriasis [1–3]. BPD-MA in-vivo phototoxicity is believed to proceed by a type II mechanism (singlet oxygen generation) and has greater phototoxicity than either hematoporphyrin derivative or Photofrin [6]. Drug clearance, measured by skin phototoxicity, is also much faster for BPD-MA (about 5 days) compared with either hematoporphyrin derivative or Photofrin (about 6 weeks).

Fluorescence measurements in vivo have also shown that, during PDT treatment, BPD-MA is easily photobleached (photodegraded) when irradiated with light at 694 nm. Protoporphyrin IX, a porphyrin molecule similar in structure to BPD-MA, forms several photoproducts when photodegraded in organic solutions. The goal of this study was to investigate in vitro the photodegradation and possible photoproduct formation of BPD-MA in solutions containing fetal calf serum (FCS) after 694 nm irradiation. Dilute solutions of BPD-MA in 10% FCS were selected as approximating an in-vitro biologically relevant environment.

2. Materials and methods

2.1. Chemicals

BPD-MA was kindly supplied by Quadra Logic Technologies, Inc. (Vancouver, Canada) and used without further purification. Stock solutions containing 1 mg BPD-MA ml^{-1} in dimethylsulfoxide (DMSO) (Aldrich Gold Label) were prepared and stored at 4 °C. For cell incubations, solutions containing 0.1 μg BPD-MA ml^{-1} in media were freshly prepared from stock solutions immediately before use. For the photodegradation experiments, solutions containing 100 ng BPD-MA ml^{-1} in 10% FCS (Gibco) in Delbucco's phos-

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phate-buffered saline (DPBS) (Gibco) were freshly prepared from stock solutions before each experiment for all fluorescence measurements and containing 1.0 μg ml^{-1} for all absorption measurements.

2.2. Spectroscopy

Absorption and fluorescence spectra were obtained for each solution prior to and immediately after irradiation with 694 nm irradiation. Absorption measurements were obtained with a HP-8452A spectrometer (Hewlett–Packard, Palo Alto, CA). Fluorescence spectra were obtained with a Spex Fluoromax spectrometer (Spex Industries, Edison, NJ). The bandwidth of both instruments was 2 nm.

A continuous-wave Ar-ion laser pumped dye laser (Innova 100 pump laser with a CR-599 dye laser head, Coherent Inc., Palo Alto, CA) tuned to 694 nm was used for all irradiations. This wavelength was purposely selected so that these results could be compared with those from previous experiments which employed a ruby laser. The output from the dye laser was coupled to a 1 μm fiber which homogenized the beam. Lenses and a variable diaphragm were then used to collimate the beam. The resulting beam of 2.5 cm diameter completely irradiated each solution being investigated.

2.3. Cell cultures

Cell cultures of ATCC HTB 9-5637 human bladder carcinoma cells (American Type Culture Collection, Rockville, MD, USA) in the subconfluent stage were incubated with 1 μg BPD-MA ml^{-1} in media for 3 hours. The cells were then washed 3 times with DBPS. Fluorescence spectra of the intact cells still attached to the plate were recorded prior to and after irradiation with 60 J cm^{-2} radiation at 694 nm. The cells were covered with DPBS throughout the irradiations and also during measurements.

3. Results

Absorption spectra from solutions of 1 μg BPD-MA ml^{-1} in DMSO and also in DPBS without and with FCS (1% and 10%) are shown in Fig. 1. Three major effects were observed upon addition of FCS to aqueous solutions of BPD-MA; there was an increase in the overall absorbance, the absorbance at 440 nm which was observed as a shoulder in the aqueous solution spectra became more prominent than the 400 nm Soret band, and the major long-wavelength absorbance band was blue shifted from 694 to 688 nm. The absorption of BPD-MA in aqueous solutions containing 10% or higher concentrations of FCS closely resemble the absorption of BPD-MA in DMSO. This indicates that, in 10% FCS solutions, BPD-MA is in a hydrophobic microenvironment.

Fig. 2 shows the absorption spectra of 1 μg BPD-MA ml^{-1} in 10% FCS before and after irradiation with 60 J cm^{-2} radiation at 694 nm. After irradiation the major BPD-MA

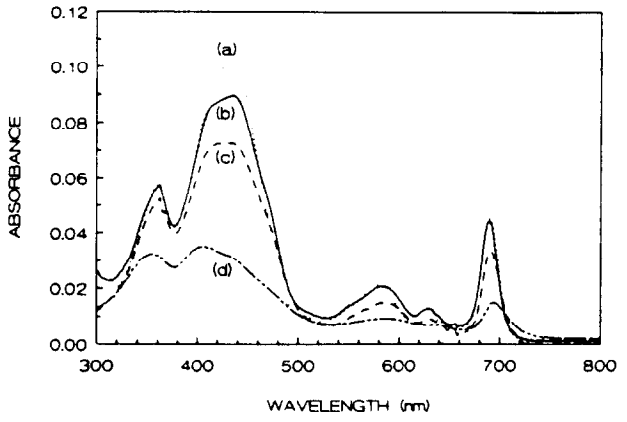

Fig. 1. Absorption spectra from solutions of 1 mg BPD-MA ml^{-1} in DMSO (curve (a)), 10% FCS in PBS (curve (b)), 1% FCS in PBS (curve (c)) and PBS alone (curve (d)).

absorption bands at 400–450 and 688 nm decreased together with the minor absorption bands at 580 and 625 nm, indicative of BPD-MA photobleaching. New absorption bands were observed at 320 and 650 nm, attributed to the formation of a photoproduct. Similar kinetics were not observed when BPD-MA was irradiated in PBS solutions, in homogeneous organic solutions [7] or in degassed FCS solutions.

Fluorescence emission spectra from solutions of 100 ng BPD-MA ml^{-1} in 10% FCS using 450 nm excitation are shown in Fig. 3. Only one fluorescence band, at 694 nm, was observed for the control BPD-MA solutions. After 60 J cm^{-2} irradiation, a dual fluorescence was observed. The 694 nm associated with BPD-MA fluorescence decreased from its pre-irradiation intensity and a new band, associated with the photoproduct fluorescence appeared at 650 nm. Similar changes in the fluorescence spectra prior to and after irradiation were also observed from intact cells incubated with BPD-MA (Fig. 3(b)).

Fluorescence excitation spectra of 100 ng BPD-MA ml^{-1} in PBS and its photoproduct are shown in Fig. 4. The emission wavelength for the BPD-MA spectrum was 710 nm and the emission wavelength for the photoproduct spectrum was 650

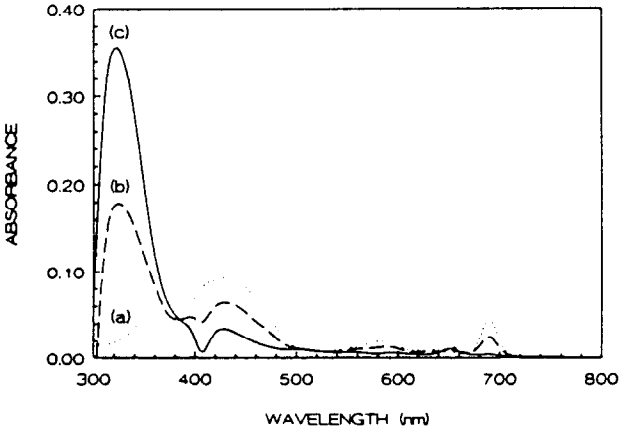

Fig. 2. Absorption spectra of BPD-MA in 10% FCS before (curve (a)) and immediately after 20 J cm^{-2} irradiation (curve (b)) and after 60 J cm^{-2} irradiation (curve (c)) with 100 mW cm^{-2} radiation at 694 nm.

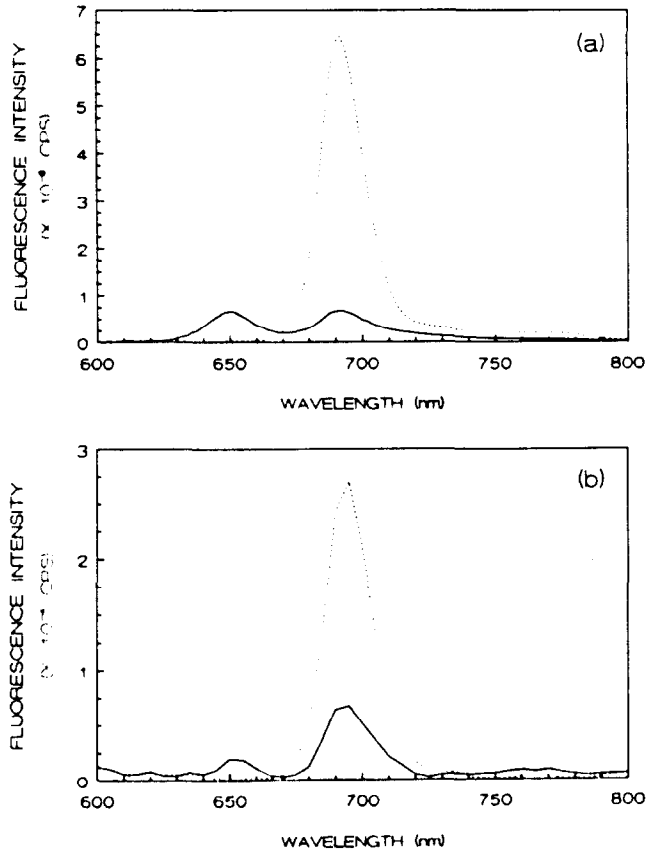

Fig. 3. (a) Fluorescence spectrum from BPD-MA from 100 ng ml^{-1} solutions of BPD-MA in 10% FCS before (· · · · ·) and after (——) irradiation with 60 J cm^{-2} radiation at 694 nm; (b) fluorescence spectrum from a monolayer of intact ATCC HTB 9,5637 human bladder carcinoma cells incubated with 0.1 mg BPD-MA ml^{-1} before (· · · · ·) and after (——) irradiation with 60 J cm^{-2} radiation at 694 nm.

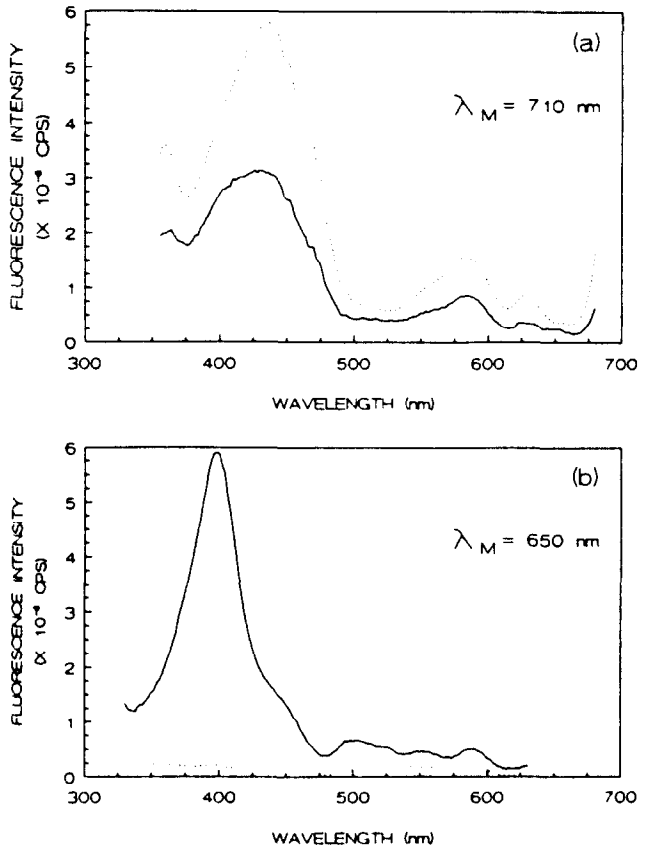

Fig. 4. Fluorescence excitation spectrum of 100 ng BPD-MA ml^{-1} in 10% FCS prior to (· · · · ·) and after (——) irradiation with 60 J cm^{-2} radiation at 694 nm. The emission monochromator was set at (a) 710 nm to detect BPD-MA fluorescence and (b) 650 nm to detect the fluorescence of the photoproduct.

nm. Although the fluorescence excitation spectrum of BPD-MA decreased in intensity after irradiation, no new spectral features were observed. In contrast, the fluorescence excitation spectrum of the photoproduct after irradiation was quite different from the pre-irradiated BPD-MA spectrum. After irradiation, a clearly defined Soret band at 400 nm was observed for the photoproduct together with new excitation bands at 550 and 590 nm.

4. Discussion

The spectroscopy and photophysics of porphyrins are highly influenced by their microenvironment. The photophysics of BPD-MA bound to proteins and/or lipoproteins are different from the kinetics observed in homogeneous organic solutions. The low concentrations of BPD-MA and high concentrations of FCS were deliberately used in this study to approximate relevant in-vivo treatment conditions where nearly all the serum BPD-MA is bound to proteins and/or lipoproteins [8,9].

The absorption and fluorescence spectra of BPD-MA in 10% FCS are similar to spectra obtained in organic solvents, indicating that bound BPD-MA is in a hydrophobic environment. Changes in the absorption and fluorescence spectra before and after irradiation indicate that a BPD-MA photoproduct was readily formed in these solutions. Although no photoproduct formation has been reported for BPD-MA in homogeneous organic solutions, previous in-vitro experiments have shown that photoproduct formation occurred only when BPD-MA was bound to FCS and the photoproduct formation was highly oxygen dependent. These results indicate that the photoproduct was formed between a reactive oxygen species and BPD-MA monomers bound to FCS or cellular targets.

Reaction of singlet oxygen with another porphyrin derivative, protoporphyrin IX (PPIX), has been extensively reported [10–14]. Several reaction mechanisms leading to different photoproducts have been proposed for PPIX. A hydroxyaldehyde photoproduct, accompanied by the reduction of the pyrrole ring, is formed in organic solvents by the cycloaddition of singlet oxygen to one of the vinyl groups. A free-radical mechanism may also exist where a reactive oxygen species reacts with one of three vinyl groups via a dioxetane intermediate, forming a formyl group, with no change in the porphyrin ring structure. This mechanism may occur either via a superoxide anion or reaction of singlet oxygen with the substrate, forming a reactive oxygen species which subsequently reacts with PPIX. New spectral features are

observed in the absorption and fluorescence emission spectra when the hydroxyaldehyde photoproduct is formed whereas no spectral changes are observed when the formyl photoproduct is formed.

The predominant mechanism for the formation of the BPD-MA photoproduct is not known. The spectroscopy of the observed BPD-MA photoproduct formed in FCS solutions (major absorption bands at 400 and 650 nm and with Q bands at 550 and 590 nm; fluorescence maximum at 650 nm) is consistent with the formation of a hydroxyaldehyde photoproduct. However, this may not be the only photoproduct formed. The fluorescence excitation and emission spectra of a formyl photoproduct would be indistinguishable from the corresponding spectra of BPD-MA. Cycloaddition of singlet oxygen to form the hydroxyaldehyde photoproduct is more likely in these oxygen-rich solutions and then a free-radical mechanism, forming a formyl photoproduct.

Under high irradiances, the facile reaction of BPD-MA with singlet oxygen may lead to oxygen depletion within the irradiated area [15–17], limiting the effectiveness of BPD-MA as a type II photosensitizer. Preliminary in-vivo rabbit experiments using BPD-MA demonstrated that, at high irradiances, fractionation of the treatment dose may be more effective than continuous irradiation. More photoproduct fluorescence at 650 nm was observed from skin sites treated with fractionated irradiation doses, suggesting increased reoxygenation of the treated sites during the "dark" periods of the fractionated treatment regime.

This study showed that BPD-MA, when bound to proteins and/or lipoproteins in vitro, reacts with the singlet oxygen or a reactive oxygen species produced during PDT treatment, forming a photoproduct. The competition for singlet oxygen between a PDT effect and photoproduct formation must be considered when designing an effective in vivo PDT treatment regime. Photoproduct fluorescence has been observed both in vitro using 10% FCS solutions and also in vivo in rabbits. It should be much easier to follow in vivo the increase in the photoproduct fluorescence at 650 nm during PDT than the decrease in the BPD-MA fluorescence at 694 nm. Once quantitated, the photoproduct fluorescence might provide a sensitive internal dosimeter of BPD-MA PDT efficacy.

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