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Biological Studies on the Main Fractions of Hematoporphyrin Derivative[1]

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ABSTRACT

The four main fractions of hematoporphyrin derivative were separated by high-pressure liquid chromatography. Each fraction was studied with respect to photosensitizing capabilities, fluorescence, and tumor tissue uptake in mice bearing EMT-6 tumors.

Animals received i.p. injections of 10 mg/kg of each fraction, and 24 h later tumors either were treated with 100 J/cm² of light (630 nm) to evaluate photosensitizing capabilities, or the animals were sacrificed and tumors removed for fluorescence and fraction uptake determination.

The results indicate that the fraction responsible for photosensitization has the highest tumor tissue uptake and retention. Furthermore, this fraction demonstrates the highest overall fluorescence localization in neoplastic tissue. The other poorly photosensitizing fractions have a lower overall fluorescence *in vivo* due to their poor tumor tissue localization.

INTRODUCTION

Due to their selective retention in tumors and their efficiency as photosensitizers, porphyrins are being evaluated by numerous groups for their effectiveness in the diagnosis and treatment of cancer (1–4). Recent work with HpD[2] has shown great promise in treating a wide variety of animal and human tumors with little damage to the adjacent normal tissues and host. This high therapeutic ratio and relative lack of morbidity of HpD-PDT have made this form of therapy very attractive.

HpD, whose tumor localizing properties were first described by Lipson (5) over 20 yr ago, is defined as the product resulting from the alkaline hydrolysis of a mixture of hematoporphyrin acetates (6). After the i.v. administration of labeled HpD, there is a gradual accumulation of radioactivity in tumor tissue (7). Although the mechanism of HpD's preferential retention is uncertain, it is known that soon after injection HpD localizes in most normal tissues. What is clear is that the total time the HpD is being retained in the malignant tissue is much longer than in the nonmalignant tissue from which it is generally cleared between 24 and 48 h (8). The recent development of instrumentation for exploring the localization of porphyrins in malignant tissues has provided the incentive for continued research into porphyrin photochemistry.

Singlet oxygen ($^1O_2$), the metastable excited state of triplet molecular oxygen, has been identified as the cytotoxic agent that is probably responsible for the photodynamic destruction of malignant cells exposed to light of the appropriate wavelength and intensity (9). This short-lived highly reactive molecule subsequently catalyzes the destruction of numerous cellular loci including mitochondria (10), microsomes (11), lysosomes (12), and transport and permeability factors associated with the cell membrane (13).

HpD is a complex mixture of porphyrins, and it is not always clear which of the components are responsible for cellular photosensitization *in vitro* or *in vivo*. Several investigators have attempted to determine which of these components is active with respect to fluorescence, photosensitization, and tumor localization using thin-layer chromatography (14), high-pressure liquid chromatography (15–19), and reverse-phase chromatography (20). Attempts to ascertain which of the porphyrins is active have been frustrated by difficulty in determining the relative purity of the various fractions. Even with apparently pure preparations of the individual HpD components, impurities have often complicated the interpretation of data.

In the present study, we have studied the four major fractions of HpD with respect to photosensitizing ability, fluorescence, and porphyrin localization in tumors of BALB/c mice bearing EMT-6 tumor. These comparisons were performed in order to further clarify which of the fractions are responsible for fluorescence as well as those responsible for sensitization and localization *in vivo*. An analysis of the individual chemical species was not carried out because they were too numerous and present in too low concentrations to make such analysis possible with our equipment.

MATERIALS AND METHODS

Hematoporphyrin Derivative. Photofrin II was obtained from Photofrin, Inc., Cheektowaga, NY, as an aqueous solution at a concentration of 2.5 mg/ml and stored in the dark at −70°C until used. For *in vivo* experiments, Photofrin II was diluted 1:4 with 0.9% NaCl solution and injected i.p.

HPLC. Analytical HPLC studies were carried out with a Beckman 324 gradient liquid chromatography system using a Beckman Ultrasphere Octyl C_8 column (5-μm particle size, 4.6 × 150-mm column dimension). The column was eluted at a constant flow rate of 2.5 ml/min. Photofrin II was applied directly to the column by injecting 2 ml at a concentration of 2.5 mg/ml for each run. The porphyrins were then eluted with a linear gradient application of 30% methanol:70% water, 30% methanol:70% isopropyl alcohol for 200 min. The column was then eluted with a linear gradient application of 30% methanol:70% isopropyl alcohol and 100% isopropyl alcohol for an additional 40 min. The four principle fractions were all separated on the column within 4 h. The fractions were collected at the following time intervals: Fraction I, 60 to 80 min; Fraction II, 95 to 110 min; Fraction III, 125 to 210 min; and Fraction IV, 210 to 240 min. Each band was collected, and the solvent was removed with a rotary evaporator and freeze dried. To determine the concentration, each fraction was dissolved in 0.1 N NaOH for 1 h at room temperature, and its absorbance was measured at 400 nm in comparison to a known concentration of Photofrin II. After the concentration was calculated, proper dilution was carried out with saline to obtain a final concentration of 2.5 mg/ml used for *in vivo* studies.

Animal and Tumor System. All mice were 14 to 16 wk old and weighed between 30 and 35 g at the time of treatment. The following tumor system was used: a EMT-6 (experimental mammary tumor) undifferentiated sarcoma obtained from the Frederick Cancer Institute, Frederick, MD, arising in the flank of a BALB/c mouse (21). Tumors were harvested fresh from mice and minced using fine scissors. Transplanted tumors were initiated intradermally in the right flank of each mouse by injecting 0.1 ml of fresh tumor inoculum prepared with a concentration of 5 × 10⁵ viable tumor cells/ml suspended in RPMI (GIBCO, Grand Island, NY). Cell viability was assessed by the ability to resist cell lysis and exclude trypan blue dye (GIBCO). The mouse tumors were generally palpable at 5 days and reached a size of 5 to 7

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[2] The abbreviations used are: HpD, hematoporphyrin derivative; HPLC, high-pressure liquid chromatography; PDT, photodynamic therapy; Hp, hematoporphyrin; HVD, 2(4)-hydroxyethyl-4(2)-vinyldeuteroporphyrin; DVFM, digital video fluorescence microscopy; DHE, dihematoporphyrin ether.

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mm at 10 to 14 days, at which time treatment was started. At this size, the small tumor was homogeneously white, and spontaneous tumor necrosis was minimal or absent.

Procedure for Photosensitization Studies. When tumors were of the appropriate size (as indicated above), the animals were shaved in the tumor area and given i.p. injections of the individual fractions in doses equal to 10 mg/kg of body weight. The remainder of the experiment was done in the dark, including housing of the animals. Control tumor-bearing animals were those that received light without porphyrin and porphyrin without light. Twenty-four h postinjection of material, the experimental animals were treated with the laser light delivery system (see below). The mouse was anesthetized with ketamine hydrochloride (Parke-Davis) and covered with a metal shield with a circular hole exposing the tumor. Animals were sacrificed 24 h after photodynamic therapy by halothane (Halocarbon Laboratories, Inc., Hackensack, NJ) anesthesia. Tissue was excised immediately and fixed in 3% glutaraldehyde:5% formalin in phosphate buffer, pH 7.4. Samples were then dehydrated in graded alcohols, cleared in xylene, and embedded in paraffin. Six-μm sections were cut, stained with hematoxylin:eosin, cleared of paraffin in xylene, and dried. Sections were examined with an Axiomat microscope (Zeiss) and photographed with Panatomic X film (Eastman Kodak).

Laser Light Delivery System. Laser irradiations were performed with a Coherent (Palo Alto, CA) Innova 20 argon ion laser stimulating a Coherent PRT-95 dye laser. The dye laser was tuned to emit radiation at 630 nm. The wavelength was verified using a Jobin Yvon No. 5/354 UV monochromator (Longjuneau, France). The radiation was then coupled into a 400-μm fused silica fiber optic using a Spectra-Physics (Mountain View, CA) Model 316 fiber optic coupler. The output end of the fiber was terminated with a microlens that focused the laser radiation into a circular field of uniform light intensity. Laser irradiation emanating from the fiber was monitored with a Coherent Model 210 power meter before and after treatment.

Mice were placed underneath an aperture that controlled the area of light illumination on the tumor site. The area of illumination was 1 cm^2. Total light dose was 100 J/cm^2 with a power density of 150 $milliwatts/cm^2$. The intensity of light on the tumor surface was calculated by measuring the intensity of the light emitted from the laser and dividing this number by the area treated in cm^2. The total light dose was calculated as intensity in W/cm^2 multiplied by the treatment time in s and expressed in J/cm^2.

Fluorescence Studies. Animals destined for fluorescence studies were sacrificed 24 h postinjection of 10 mg/kg of the individual fractions. Tumors were excised, immediately embedded in Tissue Tek II (Miles Laboratories, Inc., Naperville, ILL), and frozen at −70°C. Six-μm sections were cut on a cryostat, placed on acid-cleaned slides, and stored at −70°C until fluorescence microscopy was performed.

Fluorescence Microscopy. Frozen histological sections of tumor were viewed and recorded using epifluorescence. Light from a 100-W mercury lamp was filtered through a G 546 band pass (530 to 570 nm) exciter filter (Zeiss) and directed to the tissue by a chromatic beam splitter. The fluorescence was filtered through a LP-590 long pass barrier filter and then directed to a low light level video camera, Venus Scientific No. TV2M (Zeiss). The video signal was recorded on video tape by a GYYR DAS-MkII video tape recorder.

Image Processing. The image processing system used to analyze the fluorescence recorded above has been described earlier (22). The signal from the video tape recorder in playback mode was fed into an image array processor with synchronization between the two units provided by a time base corrector. A LSI-11/23 minicomputer was used to control the image processor.

The video signal was acquired by the image processor under control of software loaded in the computer. Thirty-two frames were acquired from each video scene and then averaged to give an increase in the signal:noise ratio. This acquired image was used by the computer for measurement of the average Gy level (corresponding to fluorescence) within a zone defined by a cursor on the image display monitor. Measurements were made in all areas of the tumor tissue. Generally, the tumor was arbitrarily divided into 4 equal areas, and within each area 10 measurements were made. The average of the 40 measurements is presented in the tables. Tumors from 50 animals were examined: 10 animals received each of the individual 4 fractions, and 10 animals were used as controls.

Localization and Uptake Studies. Animals destined for localization and uptake studies were sacrificed 24 h postinjection of 10 mg/kg of individual fractions. Tumors were excised and immediately frozen at −70°C until extraction procedures were performed. The extraction procedure used has been previously well described by Kessel (23). Briefly, tumor tissue was quickly thawed and weighed (approximately 300- to 600-mg wet weight). Extractions were carried out by disrupting tumor tissue in 2.5 ml of sodium phosphate buffer (pH 3.5) using a glass homogenizer. The homogenate was shaken for 5 min at 22°C with 2.5 volumes of methanol:chloroform (1:1) and subsequently centrifuged (1000 × *g*, 10 min, room temperature). The lower fluorescent phase was removed and evaporated under nitrogen, the residue was taken up in 100 μl of methanol, and insoluble materials were removed by brief centrifugation (12,000 × *g*, 30 s, room temperature). Porphyrin uptake was estimated from the absorbance of a 2-ml aliquot of the methanol extract scanned from 350 to 650 nm using a Beckman DU-7 spectrophotometer. The concentration of each fraction was determined by comparing its absorbance at 400 nm with a known concentration of Photofrin II (20). Absorption spectra were obtained in solution for each fraction which showed a broad peak of maximal absorption between 380 and 420 nm. Values listed in Table 2 are expressed in terms of μg of porphyrin per g of tumor tissue (wet weight).

RESULTS

Our HPLC analysis (Fig. 1) shows that HpD (Photofrin II) contains the same main fractions reported by other investigators (24–27). Fraction I is obviously Hp based on its location on the chromatogram. Fraction II is composed of the isomers of HVD. Fraction III contains a large number of components (Moan has previously reported in Ref. 17) including protoporphyrin. Fraction IV contains all components eluted after Fraction III. Reanalysis of Fraction IV results in a chromatogram almost identical to that of Photofrin II and probably includes small amounts of all the previous fractions strongly bound to the column.

Photosensitizing Efficiency. Inspection of the tumors 24 h post-PDT revealed no evidence of necrosis in control animals which received 100 J/cm^2 of light. In those animals that received either Fraction I or II, gross inspection of the tumors revealed no visual evidence of necrosis and only minimal superficial necrosis at the surface of the tumor upon histological examination. In contrast, those animals that received Fraction III had 100% destruction of their tumor. Histologically, these tumors were completely hemorrhagic with all tumor cells destroyed. Those tumors that received Fraction IV demonstrated hemorrhagic and coagulation necrosis in over 75% of the tumor.

Fluorescence. Animals were sacrificed at 24 h postinjection

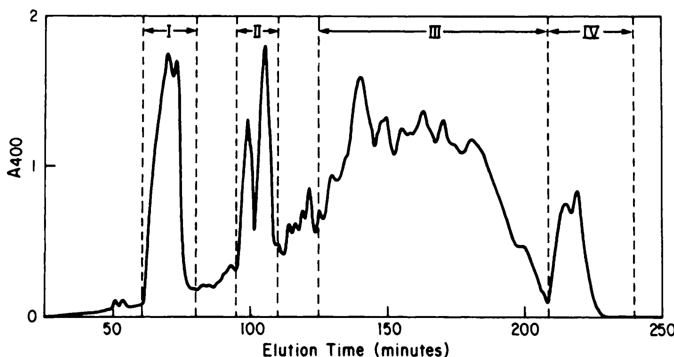

Fig. 1. HPLC elution profile of Photofrin II on a C_8 column. Absorbance at 400 nm was monitored.

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of 10 mg/kg of each fraction, and the total fluorescence was measured by computer-enhanced DVFM. Relative to tumor-bearing non-HpD control animals, the increase in total tumor tissue fluorescence was 2.12 and 2.26 times control for Fractions I and II, respectively. Animals that received Fractions III and IV had a total tumor fluorescence of 3.42 and 2.58 times control (Table 1).

Tumor Localization and Uptake. Animals were sacrificed at 24 h postinjection of 10 mg/kg of each fraction, and the porphyrins were extracted, expressed in terms of μg of porphyrin per g of tumor (wet weight), and compared in terms of their increase over control non-HpD tumors. Control animals had an average of 1.02 μg of porphyrin/g of tumor. Animals that received Fractions I and II had an average of 1.68 and 1.54 μg of porphyrin, respectively, or 1.65 and 1.51 times control animals. Animals that received Fraction III and Fraction IV had an average of 7.07 and 3.60 μg of porphyrin, respectively, or 6.93 and 3.51 times control animals (Table 2).

DISCUSSION

Photofrin II, the commercially available "enriched" fraction of HpD, was separated into four main fractions by HPLC. Our HPLC chromatographic analysis of Photofrin II agrees with the previously published work of others (24–27). Furthermore, our photosensitization studies document that the fraction responsible for photochemistry lies in the broad band containing protoporphyrin (Fraction III) also in agreement with the work of previous investigators. The exact chemical structure of this photosensitizing fraction is still under considerable debate at the present time. Studies by Dougherty *et al.* (28) in 1983 with fast atom bombardment, mass spectrometry, and nuclear magnetic resonance spectra led to the conclusion that the active ingredient was most likely a structural isomer of DHE. Kessel (29) has recently reported on a series of hydrolysis experiments conducted in solvents inhibiting porphyrin aggregation, and he found the hydrolysis pattern to be most consistent with the presence of a diporphyrin ester structure.

Table 1 *Total tumor fluorescence using computer-enhanced DVFM*

Component	Mean absolute fluorescence (pixels)[a]	Av.	Increase from control (times)
Control (non-HpD)	2367, 2459 2280, 2313 2357, 2347 2325, 2345 2347, 2401	2354	1.0
Fraction I	5053, 5042 4779, 5377 4904, 4956 4953, 5011 4953, 4932	4996	2.12
Fraction II	5356, 5380 5304, 5242 5311, 5368 5255, 5256 5347, 5300	5312	2.26
Fraction III	8067, 8059 8080, 8113 8057, 8047 8025, 8045 8068, 8092	8065	3.42
Fraction IV	6160, 6110 6104, 6042 6053, 6050 6068, 6068 6063, 6050	6076	2.58

[a] The image is divided into small regions called picture elements or pixels for short. At each pixel location, the image brightness is sampled and quantized. This step generates an integer value or Gy level at each pixel representing the brightness or darkness of the image at that point. The computer divides the image into 256 different Gy levels from which a threshold value is chosen. All brightness above the threshold Gy level is counted by the computer.

Table 2 *Tumor localization and uptake of the individual fractions*

Component	Uptake of porphyrin (μg/g tumor)	Av. porphyrin (μg/g tumor)	Increase from control (times)
Control (non-HpD)	1.05 1.02 0.98 1.07 0.99	1.02	1.0
Fraction I	1.64 1.73 1.92 1.55 1.57	1.68	1.65
Fraction II	1.31 1.54 1.56 1.66 1.62	1.54	1.51
Fraction III	7.19 6.93 7.14 7.07 7.02	7.07	6.93
Fraction IV	3.64 3.57 3.33 3.64 3.74	3.60	3.51

Using the methodology described by Kessel (23), we found that the total porphyrin accumulation by EMT-6 tumors 24 h after injection of Fraction III, as described above, to be on the average approximately 7.07 μg of porphyrin per g of tumor tissue. This is an approximate 7-fold increase from control animals. This would seem to confirm this fraction as being the major tumor localizing fraction of HpD. Our study shows that the relative efficiencies of the individual fractions in sensitizing tumors to photoinactivation follow the same pattern as tumor uptake and retention. It is important to optimize localization as well as sensitizing effect, and it therefore appears necessary that these two quantities should be studied independently. Other investigators have reported that the uptake of the HpD components increased with decreasing polarity (13, 24). The excellent tumor uptake of the more hydrophobic Fraction III shown in our study is consistent with these observations. Another factor which has been shown to be of importance for the cellular uptake and photosensitizing properties of porphyrin is their tendency to dimerize and aggregate in aqueous solutions. This tendency to aggregate has also been shown by other investigators to increase with decreasing polarity (15, 30). Thus we conclude that the photosensitizing effect of HpD is primarily due to this fraction.

The relative fluorescence yield of the individual fractions is shown in Table 1. Data shown in Table 1 indicate that Fraction III results in the greatest overall tumor fluorescence *in vivo* (approximately 3.42 times control animals). This is interesting in light of the fact that other investigators have shown the predominant fluorescent species *in vitro* (15) to correspond with Hp and HVD. Kessel and Cheng (31) examined the HpD fraction containing DHE and reported that this material had the lowest quantum fluorescent yield of all HpD components.

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Our experiments show that Fractions I and II which correspond to Hp and HVD are more fluorescent when one considers their fluorescence in terms of μg of porphyrin taken up in the tumor. Our data show that these fractions are able to more than double the total tumor fluorescence with only minor increases in tumor uptake (1.65 and 1.51 times control for Fractions I and II). It would appear that the reason for Fractions I and II resulting in such poor overall fluorescence in tumors is due to the poor uptake and retention of these fractions *in vivo*.

Use of Fraction III results in an increase in tumor fluorescence 3.42 times control desite the fact that the total porphyrin content of the tumor increases almost 7 times the control. We are thus left with the question of how a poorly fluorescent porphyrin results in the greatest overall tumor fluorescence. Kessel (23) has recently proposed a sequence of events which appears to answer this question. He suggested that the administration of HpD *in vivo* leads to the accumulation at the tumor loci of the DHE (Fraction III). Subsequently, gradual intracellular hydrolysis yields the highly fluorescent Hp and HVD components which are responsible for the overall tumor fluorescence. These conclusions were based on an experiment where Kessel (23) injected pure DHE into mice and subsequently used the extraction procedure described above and found the resulting HPLC analysis to include large amounts of the highly fluorescent Hp and HVD isomers. An almost identical HPLC profile was obtained when HpD was administered. Dougherty (6) has also described experiments in which Hp and HVD were found in neoplastic tissue even though these components are not tumor localizers. He also concluded that these fractions must therefore result from the degradation of the tumor-localizing component of HpD, which is by itself in the unhydrolyzed form, a poorly fluorescent porphyrin. It is clear that the hematoporphyrin derivtive is a complex mixture of porphyrins, and models which treat this material as a single compound are incomplete.

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