Nature of the Tumor-localizing Components of Hematoporphyrin Derivative

Panayiotis A. Scourides,1 Ralph M. Böhmer, Andrew H. Kaye, and George Morstyn

Melbourne Tumour Biology Branch, Ludwig Institute for Cancer Research [P. A. S., R. M. B., G. M.], and The Higginbotham Neurosciences Laboratory [A. H. K.], P. O. Royal Melbourne Hospital, Victoria 3050, Australia

ABSTRACT

Hematoporphyrin derivative linked by ether bonds (HE) was synthesized by unambiguous procedures in order to compare its properties to hematoporphyrin derivative (HpD). Reverse phase high performance liquid and gas filtration chromatography were used to compare the HE derivatives to HpD. The cellular uptake of HE derivatives was compared to HpD using the WEHI 3B (D+) cell line and was shown to be taken up to a degree and in a manner similar to HpD. The efficiency of HE porphyrins as photosensitizers was compared to HpD using the V79 cell line. HE porphyrins were more efficient in sensitizing the V79 cells than was HpD. The in vivo tumor localizing properties of HE porphyrins were compared to HpD in CBA mice bearing the C6 cerebral glioma, and sized by unambiguous procedures in order to compare its properties to BALB/c mice bearing the EMT6 mammary tumor. HE derivatives localized in both tumor models as effectively as HpD. We conclude that the properties of ether linked hematoporphyrin derivatives are very similar to properties of HpD.

INTRODUCTION

Photoirradiation therapy is a relatively new method of cancer treatment. It is a form of local treatment (1–3) that relies on the selective retention of a photosensitizer, HpD,2 by the tumor. Porphyrins absorb light and emit red fluorescent light which can be used for the detection of neoplasms (4). Porphyrins when exposed to light act as efficient photosensitizers and produce singlet oxygen which appears in part to mediate the tumor necrosis (5, 6). Successful treatment of cancers of various histologies has been reported including those of the lung, esophagus, stomach, and bladder (1).

At present either HpD or a partially purified form called Photofrin II are used for clinical therapy. HpD is prepared by a two-step procedure that involves treatment of HP dihydrochloride with 5% sulfuric acid in acetic acid to form an acetylated mixture which is then solubilized using alkali (2). Studies of the acetylated mixture prior to alkali treatment resulted in the identification (7–9) and synthesis of all the major components but these were nonlocalizing in vivo. Studies of the alkali treated form of HpD (10) identified the formation of the tumor-localizing components from the acetylated precursors during alkali hydrolysis. The tumor-localizing components were isolated and tested in vivo, and Berenbaum et al. (9) proposed that the structure of the tumor-localizing component was that of a dimer or oligomers linked via ether or carbon-to-carbon covalent bonds. Improved methods of isolating the tumor-localizing components were developed (11–13). Dougherty et al. (14) described the isolation of the tumor-localizing components and presented some evidence from fast atom bombardment mass spectrometry and nuclear magnetic resonance spectra that the localizing components were most likely isomers of dihematoporphyrin ether (Fig. 1A). More recently Kessel (15) presented results based on HPLC and spectroscopic data which suggested that the tumor-localizing component of HpD is an isomeric dihematoporphyrin ester. The presence of polymeric material was not ruled out. The proposed ester structure is shown in Fig. 1B. In view of these conflicting proposals concerning the nature of the tumor-localizing component of HpD we prepared by unambiguous procedures derivatives of HpD linked by ether bonds to study more directly one of the proposed structures for the tumor-localizing component(s) of HpD. We found that the properties of the ether linked HpD were similar to HpD.

MATERIALS AND METHODS

Porphyrins. HP (Fig. 2, II) was purchased from Roussel UCLAF (Sydney, Australia). PP (Fig. 2, I) was prepared from HP as described (16). Photofrin I was purchased from Photofrin Medical, Inc. (Cheektowaga, NY). HPDME (Fig. 2, IV) was prepared as described (7) and was purified by column chromatography on Kiesel Gel 60 (230–400 mesh) Merck from BDH Chemicals Pty, Ltd. (Melbourne, Australia). PPDME (Fig. 2, III) was prepared from PP and purified by column chromatography (7, 16).

PPDME dibromide adduct (Fig. 2, V) was prepared from PPDME by a modification of a published method (17). In a typical preparation, PPDME (25 mg) was dissolved in hydrogen bromide solution in glacial acetic acid (45% w/v; 2 ml) in a sealed flask with stirring at 21°C. After 2 h the solvents were removed under high vacuum to leave the required adduct (Fig. 2, V). Because of its reactive nature the dibromide adduct was used as prepared.

Preparation of Ether Linked HP Samples. HP samples were prepared by Methods A, B, C, and D and these are described fully in "Results."

The hydrolysis of the methyl esters of HE to their water soluble form was carried out by treatment of the stirred tetrahydrofuran solution of the esters with sodium hydroxide and water. In a typical hydrolysis HE methyl esters (0.1 g) were dissolved in tetrahydrofuran and to this were added 1 M sodium hydroxide (0.5 ml) and enough distilled water in portions with stirring to obtain a strongly alkaline, clear solution as a single phase. Occasionally dimethyl sulfoxide was also used in small amounts to aid solubility. The hydrolysis was carried out at room temperature, and its course was monitored by partitioning aliquots of the reaction mixture between equal volumes of chloroform and water. The esters remained in the organic layer while hydrolyzed porphyrins partitioned into water. The hydrolysis was complete when all porphyrins remained in the water layer, and the result was confirmed by HPLC. The hydrolyzed HE was isolated by adjusting the pH to 3.5–4.0 with 0.5 M hydrochloric acid and removal of the tetrahydrofuran under vacuum. The precipitated porphyrins were isolated by centrifugation, washed with water at the centripuge, and dried under vacuum.

Thin layer chromatography was carried out on HP-thin layer chromatography-Alufolien Kieselgel 60F 254, Merck (Melbourne, Australia), irrigated with dichloromethane, chloroform, methanol, and combinations of these.

HPLC. The equipment used consisted of a Model 510 pump and Lambda-Max Model 418 LC spectrophotometer both from Waters Associates (Melbourne, Australia) and a Rhodyne Model 7105 injector. Analytical HPLC was carried out on a Nova Pak C18 column, 3.9 mm x 15 cm, and preparative HPLC on a custom packed column, 19 mm x 15 cm, packed with the same Nova Pak C18 silica, both from Waters Associates. HpD and HE samples were chromatographed by gradient elution from eluent A (methanol:water, 70:30, at pH 4.0; the water component was a 300-mM solution of formic acid brought to pH 4.0 with triethyl amine, to eluent B (methanol:water, 90:10, pH 8.0); the water component was a 300-mM solution of formic acid brought to...
The aim of the first synthesis of HE (Method A), was to make ether derivatives of HP such as the dimethyl ether (23) and thioethers such as porphyrin c (17) have been described previously. Both of these procedures use the reactive dibromide adduct (Fig. 2, VI) to react with the alcohol or thiol, under unnecessarily harsh conditions, to form the corresponding ethers. We prepared the dibromide adduct (Fig. 2, VI) and monitored its reaction with methanol by HPLC and found the ether formation to be very fast, the reaction going to completion within 5 min at 21°C. The reactive nature of the dibromide adduct meant that HE could be prepared under mild conditions. We monitored its reaction with methanol by HPLC and found the ether formation to be very fast, the reaction going to completion within 5 min at 21°C. The reactive nature of the dibromide adduct meant that HE could be prepared under mild conditions.

RESULTS

Synthesis of HE Derivatives. Procedures for the synthesis of ether derivatives of HP such as the dimethyl ether (23) and thioethers such as porphyrin c (17) have been described previously. Both of these procedures use the reactive dibromide adduct (Fig. 2, VI) to react with the alcohol or thiol, under unnecessarily harsh conditions, to form the corresponding ethers. We prepared the dibromide adduct (Fig. 2, VI) and monitored its reaction with methanol by HPLC and found the ether formation to be very fast, the reaction going to completion within 5 min at 21°C. The reactive nature of the dibromide adduct meant that HE could be prepared under mild conditions. We used the dibromide adduct and prepared derivatives of HP covalently linked by ether bonds by four different procedures. The aim of the first synthesis of HE (Method A), was to make the synthesis unambiguous. The following example describes Method A.

HPDME (20 mg, dry) was dissolved in dichloromethane (1 ml) and was added to solid PPDME dibromide adduct (Fig. 2, VI) (25 mg) prepared as described above. The mixture was stirred at 21°C in a 25-ml conical flask for 5 min and then diluted with dichloromethane (3 ml); water (15 ml) was added and the mixture shaken. The dichloromethane layer was washed in a separating funnel with water until neutral pH and then evaporated under vacuum to obtain the methyl esters of HE. Porphyrin recovery from this reaction was greater than 90%.

Fig. 1. Ether (A) and ester (B) linked structures proposed for the tumor localizing component of HpD.

Fig. 2. Structures of porphyrins used in the synthesis of HE derivatives.
The reaction scheme in Fig. 3 shows the unambiguous synthesis of HE described in Method A. For this synthesis PP was converted to its dimethyl ester PPDME and HP to HPDME by previously reported procedures (7, 16) in order to protect the carboxylic acid groups and prevent ester formation. PPDME was converted to its dibromide adduct (Fig. 2, V) and the integrity of the methyl ester groups confirmed by the hydrolysis of a portion of the adduct (Fig. 2, V) to form HPDME, shown to be identical with authentic HPDME by thin layer chromatography comparison. A HPLC analysis of a typical preparation of the methyl esters of HE is shown in Fig. 4G. Normal phase HPLC (not shown) also confirmed the integrity of the methyl esters of the HE product obtained. We found that the aggregates of HpD are relatively stable to hydrolysis by alkali, as has been previously described (14, 24). We were able to selectively hydrolyze the methyl esters of HE and obtained these in the form of the free acids. We then confirmed that no ester formation could occur between the carboxylic and hydroxyl groups of HE during alkaline hydrolysis. This was proven by the hydrolysis of HPDME under the same conditions used for the methyl ester hydrolysis of HE. HPLC analysis of the product showed that only pure HP was produced (not shown). HPLC analysis of a sample of the HE derivative from Method A in its water soluble form is shown in Fig. 4A and the absorption spectrum in Fig. 5 (2). Having ensured that porphyrin-to-porphyrin covalent bonds were ether linkages in this preparation, we were able to further simplify their synthesis.

With Method B, since the hydrolysis of the dibromide adduct (Fig. 2, VI) produces HP (16) and the hydrolysis of Fig. 2, V produces HPDME, we were able to simplify the synthesis of HE. HPDME was formed from Fig. 2, V in situ by hydrolysis, and the HPDME reacted with unchanged Fig. 2, V to form the methyl esters of HE. This eliminated the need to prepare and purify HPDME. The following is a typical preparation of HE using Method B.

PPDME dibromide adduct (Fig. 2, V) (200 mg) was dissolved in dichloromethane (30 ml) precooled to −70°C and to this was immediately added saturated sodium chloride solution in water (40 ml). The mixture was sealed and shaken vigorously in a 250-ml flask for 120 min, then centrifuged and the water layer discarded. The organic layer was washed until neutral pH with water at the centrifuge and evaporated under vacuum to obtain the methyl esters of HE. Porphyrin recovery from this reaction was greater than 90%.

The product obtained by this method was similarly hydrolyzed to its water soluble form. HPLC analysis of a typical sample is shown in Fig. 4B and its absorption spectrum in Fig. 5 (3).

The published procedure for the synthesis of the dimethyl ether of hematoporphyrin (23) involves the use of the dibromide adduct (Fig. 2, VI) in the form of the free acid, and no simultaneous esterification occurs even under the excessively harsh
controlled hydrolysis of the dibromide adduct (Fig. 2, VI) of PP formed HP in situ which reacted with unchanged Fig. 2, VI to form HE in its water soluble form. This procedure eliminated the need to prepare and purify PPDME from PP and also the need to hydrolyze the HE obtained. The following is a typical example of the preparation of HE from the dibromide adduct (Fig. 2, VI) by Method C.

PP dibromide adduct (Fig. 2, VI) was prepared from PP (200 mg) as described above, the solvent was removed as described, and the gummy solid was dissolved in dichloromethane (40 ml) precooled to −70°C. To this was immediately added saturated sodium chloride solution in water (80 ml), and the sealed mixture was shaken vigorously for 10 min in a 500-ml flask. A further 100 ml of saturated sodium chloride were added, the precipitated porphyrin was isolated by filtration, and the solid was washed with water and dried under suction at the water pump, then under high vacuum (0.01 mm Hg). The yield of HE obtained was in the region of 60–70%. The HE product obtained by this method was analyzed by HPLC, Fig. 4C, and the shift in the position of the soret band (not shown) confirmed the presence of aggregates (12) and was similar to the spectrum from previous HE samples.

In view of the higher cost of PP, and with the knowledge that the hydroxyl groups of HP have been converted previously to the dichloride adduct (25) and to the dibromide adduct (Fig. 2, VI) (26), we decided to prepare HE from HP using the procedure described in Method C. Thus Method D differs from C only in the fact that the dibromide adduct Fig. 2, VI was prepared from HP instead of PP using the same procedure that was used for PP. A HPLC profile of HE obtained by Method D is shown in Fig. 4D and its absorption spectrum (not shown) confirmed the presence of aggregates and was similar to spectra of HE from Methods A and B (12).

Gel exclusion chromatography was used previously (11–14, 24) to obtain fractions of HPD enriched in the high molecular weight aggregates of composition similar to Photofrin II (14) that form the tumor-localizing component of HPD. We used the method of Cowled et al. (24) and Kessel and Cheng (12) and chromatographed the samples of HE described above. We found from HPLC analysis of the first band eluted from the HE samples that this band contained material considerably enriched in the aggregated porphyrins, Fig. 4F.

HE in the form of the free acid was subjected to gel filtration chromatography (24), and the porphyrin aggregate rich fraction was freed of the single porphyrin contaminants prior to recording its 13C nuclear magnetic resonance spectrum, Fig. 6. The peaks were referenced (to d6 dimethyl sulfoxide) and identified as follows: (6, ppm), (11.5), ring CH3; (21.9), CH2CH2CO2H; (23.5 and 23.8), CH3CHOH; (29.1), CH3CHO; (37.9), CH2CH2CO2H; (60.2 and 60.7), CH3CHOH; (65.9 and 66.8), CH3CHOH; (94–100), methine carbons; (134–148), pyrrole carbons; (174.8), CH3CH2CO2H. Dimethyl sulfoxide is at 38.9–40.5 ppm.

The 13C NMR spectrum of HP (not shown) was also recorded under the same conditions and showed most of the above peaks but with some important differences. The peak due to CH3CHOH in HP was not resolved into two peaks unlike what is seen in the HE spectrum. The peak due to CH3CHOH in the HP spectrum was considerably more intense and was not resolved into two, unlike the result in the HE spectrum. The peak due to the CH3CHO- carbon in the HE spectrum was absent in the HP spectrum confirming that the hydroxyl group has been derivatized. The peaks due to the CH3CHO- carbon in the HE spectrum were absent in the HP spectrum.

The fast atom bombardment mass spectrum of HE prepared by Method D was recorded on the methyl ester form of HE and is shown in Fig. 7. The major peaks observed are due to single porphyrin species such as PPDME at 591 mass numbers and hydroxyethylvinyldeuteroporphyrin dimethyl ester at 609 mass numbers, with a weak peak at 627 mass numbers for HPDME. In the higher mass range the peak at 1217 mass numbers is 24–25% of the peak, at 609, and other peaks of interest are shown at 1234 and 1235 mass numbers.

The fast atom bombardment mass spectrum of HE in the free acid form was recorded on HE prepared by Method C. All peaks in this spectrum were weak including those of PP and hydroxyethylvinyldeuteroporphyrin. However, in the high mass range of the spectrum peaks were present at 1178, 1179, and 1161 mass numbers; the spectrum is shown in Fig. 8.

Cellular Uptake of Porphyrins. The cellular uptake of HE porphyrins was studied and compared using the WEHI 3B (D+) cell line and a flow cytometer as described above. As shown in Fig. 9, column A, all HE preparations showed similar uptake kinetics resulting in high fluorescence levels in the cells after 20 min exposure to HE. There was an initial rapid phase and a second slower phase. This pattern of uptake was similar to that previously reported for HPD (18). After incubation with each porphyrin preparation the rate of decrease of fluorescence was shown to be similar for cells incubated with each HE sample, Fig. 9, column B, as were the final fluorescence levels, and were comparable to results obtained with HPD under similar conditions.

In Vitro Cell Kill. The effectiveness of HE porphyrins as photosensitizers was compared by studying their efficiency in photosensitizing V79 cells. The results from such an experiment are shown in Fig. 10. When V79 cells incubated with HE at 10 mg/liter and HE prepared by Method D were kept in the dark, no direct toxicity was apparent in any of the samples tested, Fig. 10A. However, when the cells were incubated with the porphyrins, resuspended in prophyrin-free medium, and exposed to irradiation for 10 min, HE caused complete cell kill at 10 mg/liter, and HPD at 20 mg/liter, Fig. 9B. The result shown for HE obtained from Method D was characteristic of all HE samples obtained by the four methods described.

In Vivo Tumor Localization Studies. In both in vivo model systems administration of HE resulted in excellent tumor localization as measured by fluorescence. The HE and HPD preparations, as measured by fluorescence, were selectively localized in the intracerebral tumor with only slight fluorescence in the brain adjacent to the tumor and no detectable localization as measured by fluorescence.
fluorescence in the normal brain. All HE samples were taken up to a similar extent as HpD although there were small differences in the time course, Fig. 11. The uptake of HE (from Method C) at 2 h was significantly greater than that of HE from Methods B and D and HpD.

The localization of HE in the systemic mammary tumor was also quantitated on the basis of fluorescence, as described (20), and the fluorescence found 72 h after administration of HE prepared by Methods B and C, respectively, were 43 and 34 (see Fig. 11 for comparison).

**DISCUSSION**

**Chemical Characterization of HE.** The preparation of hematoporphyrin dimethyl ether (23) from the dibromide adduct (Fig. 2, VI) proceeded without simultaneous esterification, although the free acid form of the porphyrin was used under unnecessarily harsh conditions. Despite this we made the HE synthesis entirely unambiguous and prevented ester formation by protecting the carboxylic acid groups as the methyl esters (Method A). Similar compositions could be obtained when the synthesis of HE was simplified (Methods B, C, and D).

As mentioned earlier the dibromide adduct of PP has been shown to be very reactive and was shown to lead to ether bond formation during hydrolysis depending on the reaction conditions. Since HP is also prepared by the hydrolysis of the dibromide adduct (16), it may perhaps account for previous reports (27-29) suggesting that HP localizes in tumors.

It is worth noting that the reactive nature of the dibromide adduct could lead to widely different compositions in HE derivatives prepared from it, and the reaction conditions are critical. The results obtained from the HPLC comparison of HE and HpD samples show a striking similarity in composition between the HE samples and HpD. HPLC shows the major
The Tumor-localizing Components of HpD

**Fig. 9.** In vitro cellular uptake by WEHI 3B D+ cells (A) and release of HP derivatives (B) 1, HE derivative from Method C; 2, HE derivative from Method B; 3, HE derivative from Method A.

**Fig. 10.** In vitro photosensitization of V79 cells by HpD ( ) and HE ( ) (from Method D). A, effect of these derivatives on V79 cells without irradiation; B, result after irradiation for 10 min.

The fast atom bombardment mass spectrum of HE from Method A shows the expected mass peak due to the HP dimer at 1235 (M) as well as peaks at 1236 (M+1) and 1237 (M+2). A strong mass peak is seen at 1217 corresponding to the loss of a water molecule.

The 13C nuclear magnetic resonance spectrum of HE shows all the required structural information to confirm the presence of ether linked hematoporphyrins. The peaks observed in our spectrum are in agreement with the results of Dougherty et al. (14), although the use of different solvents and conditions results in differences in chemical shifts. Our spectrum allowed us to observe double peaks for some of the carbons. These are most probably due to the isomers formed by ether formation which can occur in one of three different ways, bond formation between the hydroxyl groups of the two 8-, or the two 3-, or a 3- with an 8-hydroxyethyl group.

Biological Properties of HE. The results obtained from the study of cellular uptake and release of the HE samples showed that all HE samples were similar to HpD. The rate at which impurities found in HpD; that is, HP, HVD, and PP are also the major contaminants in HE samples. Further confirmation of the similarity of HE samples and HpD was obtained by gel exclusion chromatography in which HE has a high molecular weight component as does HpD.

The absorbance spectra of all HE samples show these to be composed of aggregated porphyrins showing the characteristic shift in the Soret band to shorter wavelengths and a broadening of this band, as observed in HpD samples (12).
HE porphyrins were released from cells when washed with fresh medium was found to exhibit HpD-like kinetics, and the final porphyrin levels remaining associated with cells were again similar to those observed when HpD was used under identical conditions.

The in vitro comparison of the efficiency of HE and HpD in sensitizing cells to kill showed HE samples to be efficient sensitizers capable of sensitizing cells more efficiently than HpD, as was shown in Fig. 10.

The in vivo studies showed HE samples to be efficient tumor localizers as determined by fluorescence, Fig. 11. The kinetics of uptake of HE porphyrins by the glioma tumor has been shown to be very similar to that of HpD, resulting in about the same porphyrin levels in the tumor as observed following HpD administration. HpD and HE also localized well to the EMT6 mammary tumor.

We conclude that the tumor localizing aggregates in HpD are in every respect similar to derivatives obtained by covalently linking HP via ether bonds. The techniques developed to synthesize HE may lead to better clinical preparations of hematoporphyrin derivative.

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