Quantum yield measurements on some new hematoporphyrin mixtures, including zinc and HpD precursor

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Abstract

Evidence has been reported in the literature which suggests that one or more of the components of HpD may be photoactive in tissue. Despite the exhaustive investigation of the HpD drug, its biological and photophysiology, the fluorescence efficiency of this drug remains low. This report provides a brief review of the current situation, and reports an improved fluorescence quantum yield for the stage I HpD precursor loaded with 20% hematoporphyrin in PBS. We also provide some evidence which suggests that any heme-like porphyrin containing zinc is unsuitable for use as a photodetector of cancer cells as these metalloporphyrins are virtually non-fluorescent in PBS.

Introduction**

Hematoporphyrin derivative (HpD) is a drug currently undergoing extensive investigation for the treatment of neoplastic disease [1–9]. In the body it is used in association with photodynamic therapy (PDT), a process which uses light to induce fluorescence in HpD. Certain deactivation mechanisms then lead to the formation of singlet oxygen [10], a species which is toxic and lethal to the survival of cancer cells [11]. The exact processes which govern the uptake of, delivery to, and internalization of HpD in cancer cells is still being investigated, however mounting evidence seems to suggest that HpD is taken up in the body by lipoproteins and human serum albumin [12], internalized into cells, and ultimately localized in cytoplasmic regions [13]. Subsequent activation by PDT results in the creation of the lethal singlet oxygen agents which ultimately destroy the life of the cell.

HpD is composed of several components [14]: 20% hematoporphyrin (Hp), 20–30% hydroxyvinyldeuteroporphyrin (HVD), 3–5% protoporphyrin (PP) and 50% dihematoporphyrin ether/ester (DHE). The first three are monomers, but DHE is believed to be an oligomer composed primarily of dimers and trimers of HpD [9]. HpD is synthesized using a two step process; first, acid acetylation of hematoporphyrin-IX, followed by a base hydrolysis using 0.1 N NaOH. The first step produces diacetate monomers (Hp, PP and HVD), while step two of the synthesis produces DHE [15]. All of these components are apparently fluorescent *in vitro*. Hematoporphyrin has been reported to be the only component inactive in culture [16], however Bertoloni [17–19] has produced evidence which shows that although Hp does not bind cells initially, once light is introduced, the cells become photoactive and allow Hp units into cytoplasmic regions where cell damage ultimately results. Dougherty [20], however, has produced evidence which showed that some commercial batches of Hp contained a DHE-like impurity which produced the photoinactivation effects observed by some researchers using Hp-IX. Notwithstanding the findings reported by Dougherty, reports of cancer cell photoinactivation effects produced by solutions of protoporphyrin and hematoporphyrin continue. For example, Santus *et al.* [21] reported that protoporphyrin produced wide destruction of cells during PDT.

DHE is still considered to be the 'active' component of HpD and the component responsible for the destructive photoactivity of cells *in vivo* [22]. Because it is aggregated and hydrophobic, DHE is easily taken

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**Abbreviations: HpD, hematoporphyrin derivative; PP, protoporphyrin; Hp, hematoporphyrin; ZnPP, zinc(II) protoporphyrin; ZnHp, zinc(II) hematoporphyrin; ZnDHE, zinc(II) dihematoporphyrin ether; HpDp, hematoporphyrin derivative precursor; PDT, photodynamic therapy; LDL, low density lipoproteins; HSA, human serum albumin; TPP, tetraphenylporphyrin; ZnTPP, zinc(II) tetraphenylporphyrin.

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up by lipoproteins (the body's natural drug delivery system) and transported to cancerous sites. It is now believed that DHE undergoes a remarkable disaggregation or unfolding process mediated by biomolecular proteins before its photoactivity can be manifested on the cells [23]. All these findings focus new attention on the monomers and their roles in photosensitization processes using HpD.

Aqueous neutral solutions of HpD possess two fluorescence emission bands near 615 and 670 nm when excited near 400 nm. Several years ago, an emission band was observed at 580 nm in aqueous solutions of HpD [12]. This band has now been shown to originate from the presence of extraneous zinc in manufactured hematoporphyrin-IX, in its water solvent supplies, or in the body [12, 24]. Lamalo and Sassaroli [24] then proposed a mechanism for the uptake and localization of these zinc components in humans, which appeared to involve a competition for the available iron deficient heme sites often found in patients with neoplastic disease. The 580 nm band appears in the spectrum of HpD as early as 2 h after preparation and grows in intensity over a 24 h period [12]. Because of the possibility that zinc might be present in HpD, each zinc-metallated component of HpD except that of HVD was also studied in this report. This study examines the effects of zinc on the fluorescence efficiency of Hp-IX and other HpD components.

There have been long and extensive studies of both HpD and DHE, but not much emphasis has been placed on the importance of the stage one precursor formed during HpD synthesis, abbreviated here as HpDp. The best in-depth evaluation of the fluorescence of the components which make up the precursor was completed by Moan *et al.* [25]. HpDp contains the monomers, Hp, PP and HVD, but no DHE. Moan *et al.*, however, have reported that HpDp contains a polymeric component. In an effort to further identify fluorescent HpD-like porphyrins which may serve as drugs for use in conjunction with cancer cell photodetection techniques, e.g. laser probes, HpDp was featured in this report; both alone and mixed with 20% solutions of PP, Hp, DHE, and their zinc analogues. While there are many reports of quantum yield measurements for HpD and/or its constituents, few, if any, of these reports contain information about the fluorescence yields of HpDp mixed with other HpD components. This is the principal area of concern of this report.

HpD has previously been used as a cancer cell photoinactivator in conjunction with PDT. However, the use of increased doses of HpD often produced a high sensitivity to light, especially in the skin [26]. Dougherty and Gomer [26] have also observed significant amounts of HpD in the liver, spleen and kidney. It therefore appears that while HpD can easily be used to identify and locate tumors, some problems still exist when its use is expanded to include therapeutic techniques. With recent advances in laser and fiberoptic technology, smaller doses of HpD will likely be sufficient for localizing tumor cells.

Experimental

Hematoporphyrin derivative precursor (HpDp) was prepared using a method outlined by Lipson *et al.* [27], involving acid acetylation of hematoporphyrin-IX (Sigma Chemical, St. Louis, MO) with a 19:1 vol./vol. mixture of acetic and sulfuric acid, followed by titration with 3% sodium acetate to precipitate the product. The precipitate was filtered, washed five times with distilled water, and dried under vacuum for 24 h. It was then collected in an amber vial and refrigerated at 4 °C. There was no base hydrolysis of this precipitate with 0.1 N NaOH.

Photofrin II (DHE) was graciously supplied by Quadralogic, Inc. (Vancouver, BC, Canada). Protoporphyrin-IX and hematoporphyrin-IX were purchased from Sigma Chemical Company and used without further purification because it was not important for purposes of this experiment. Zinc was inserted into protoporphyrin and DHE using the method outlined by Adler *et al.* [28]. ZnHp was prepared by Porphyrin Products (Logan, UT). In a separate experiment solutions of PP, Hp and DHE were refluxed in DMF for 1 h without adding any zinc to check for any decomposition of the porphyrins. The electronic spectra of PP and Hp were essentially the same before and after heating; however, there were minor changes in the spectrum of DHE. The zinc complexes were recrystallized in methanol/benzene solutions and subsequently stored in amber

TABLE 1. Visible band positions (nm) for HpD components in DMF

Component	Soret band position[a]		Visible band position (nm)[b]			
HpDp	380	D	499	533	568	622
Hp	394	M	497	531	567	621
ZnHp	410	M		546	581	
DHE	374	A	502	535	581	
ZnDHE	410	M		548	584	
PP	389	D	505	539	575	630
ZnPP	415	M		546	581	

[a]In PBS (pH = 7.4) at 4 h; visible band positions were not all discernible. A = aggregate; M = monomer; D = dimer; HpDp = HpD precursor; Hp = hematoporphyrin-IX, PP = protoporphyrin-IX; DHE = dihematoporphyrin ether (as Photofrin II); and Zn = zinc(II). [b]Solvent is DMF. DMF was used for characterization because of the low solubility of the zinc components in PBS (pH = 7.4).

vials at 4 °C. A characterization of these complexes by UV–Vis spectrophotometry is shown in Table 1.

Stock solutions of HpDp, PP, Hp, DHE, ZnPP and ZnDHE were prepared in PBS at a concentration of 1.0 μg/ml. HpDp, Hp, DHE and ZnHp were soluble with vigorous stirring, while ZnPP, PP and ZnDHE required heating at approximately 80 °C for several hours in addition to the stirring. Stock solutions of HpDp and each of the six components listed above were prepared in PBS at concentrations of 1.0 μg/ml HpDP:0.20 μg/ml component. These concentrations were chosen in order to prepare samples of HpDp containing zinc at concentrations well above that which might be present in commercially available Hp-IX dichloride.

Fluorescence quantum yields were determined by the reference method on an SLM 8000 spectrofluorometer operated in the ratio mode [29]. The instrument was interfaced to a Macintosh IIcx computer for data collection and analysis. The computer used a program written with National Instrument's LabVIEW instrument control software developed in the laboratory of Dr Mary Barkley at Louisiana State University, Baton Rouge. The cell holder temperature was regulated at 25 °C with a Lauda circulating bath. Samples were excited at 400 nm. All spectra were collected with the single excitation and emission monochromators set at 8 and 4 nm bandpass, respectively. Polarizers were set to 55° on the excitation side and 0° on the emission side to avoid the Wood's anomaly of the emission grating. The spectra were corrected for the wavelength-dependent instrument response with correction factors determined with a standard lamp from Optronics and a solvent blank was subtracted. Fluorescence quantum yields were determined relative to H_2TPP in benzene using a value of 0.12 [30]. The concentration of all samples was adjusted such that their absorbance was less than 0.1 at the excitation wavelength.

Results and discussion

The effects of zinc on HpD-like components

Table 2 contains fluorescence quantum yields for all the hematoporphyrin derivative components studied, monitoring emission bands at 580, 613, 640 and 670 nm (see Fig. 1(a)). These values clearly point out the fact that most of the fluorescence of HpDp derives from the presence of Hp, even without including HVD in this study, since the quantum yields for HpDp and Hp are essentially the same (0.061 and 0.059, respectively). Moan *et al.* [25] reported a value of 0.066 for Hp solutions in PBS (pH = 7.3). Protoporphyrin (PP) is virtually non-fluorescent in PBS (0.002), DHE is only

TABLE 2. Quantum yields for HpD components[a]

Solutions	Quantum yield	Comparison with HpDp (%)
Reported 4–6 h after mixing		
HpDp	0.061	
Hp	0.059	−3.3
DHE	0.022	−63.9
PP	0.002	−96.7
ZnHp	0.0003	−99.5
ZnDHE	0.0041	−93.5
ZnPP	0.0008	−98.6
Reported 24 h after mixing		
HpDp	0.052	
HpDp + Hp	0.059	+13.5
HpDp + ZnHp	0.052	none
HpDp + DHE	0.054	+4.0
HpDp + ZnDHE	0.041	−26.8
HpDp + PP	0.048	−8.3
HpDp + ZnPP	0.052	none
Reported ten days after mixing[b]		
HpDp	0.00745	
HpDp + Hp	0.00930	+25.0
HpDp + ZnHp	0.00803	+8.0
HpDp + PP	0.00650	−13.0
HpDp + ZnPP	0.00475	−36.0
HpDp + DHE	0.00550	−26.0
HpDp + ZnDHE	0.01090	+46.0

[a]The value of 0.061 was used for HpDp when comparing unmixed HpDp components, while a value of 0.052 was used for the comparison of HpDp and HpDp mixtures of other components. The unmixed porphyrins were prepared at a concentration of 1.0 μg/ml in PBS (pH = 7.4). The mixtures were prepared at a concentration of 0.80 μg/ml HpDp and 0.20 μg/ml HpD component. [b]Average margin of error = 7%. Compared to the four hour quantum yields: average decrease in quantum yield at 24 h = 15%; average decrease in quantum yield at 10 days = 88%.

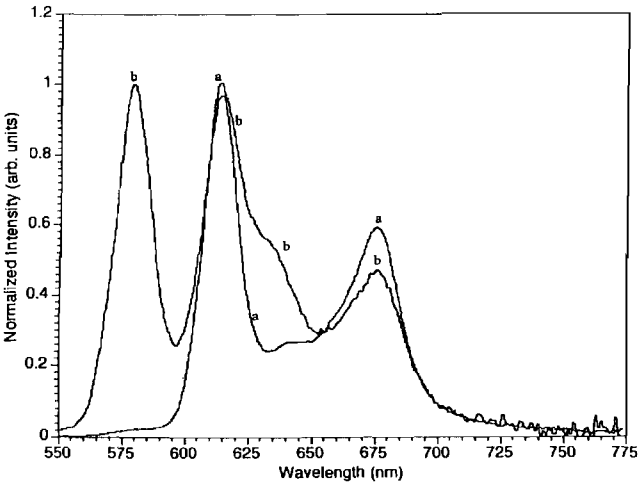

Fig. 1. A comparison of the fluorescence emission spectra for the 4 h solution containing only HpDp (a) and that of the 10 day old solution of this same mixture (b).

about 38% as fluorescent as HpDp (0.022) and all of the zinc components are virtually non-fluorescent. Low quantum yields for non-metallated HpD components have been shown to be associated with aggregation and dimerization, respectively [25]. The Soret bands for these two components occur near 370 (DHE) and 389 (PP) nm, as opposed to 395 nm for monomeric HP. According to Pottier *et al.* [31], Soret bands occurring near 393, 390 and 370 nm are indicative of monomeric, dimeric and aggregated porphyrins, respectively (see Table 1). Based on these criteria, DHE is clearly aggregated, PP is dimerized, HpDp is clearly aggregated, and Hp is monomeric.

Most striking is the very low fluorescence efficiency of the HpD components containing zinc, e.g. ZnHp, ZnPP and ZnDHE. There are apparently no reports in the literature of quantum yields for zinc metallated PP or Hp solutions in aqueous buffer (pH 7); presumably, because of a limited solubility. Feitelson and Barboy [32] have reported quantum yields for PP of 0.034 in EtOH and 0.033 in EtOH/H_2O mixtures (pH=8). Both these values are almost a hundred-fold greater than the values reported here. Because the presence of EtOH enhances the solubility and the fluorescence, it is likely that the low values obtained in this report for the zinc porphyrins can be attributed to a low solubility in water and an increased aggregation effect. Regardless of the reason for the magnitude of the quantum yield, the values are easily reproducible. Both of these latter effects are known to exert a negative influence on the fluorescence of porphyrins. A direct comparison of the fluorescence efficiencies of Hp, PP and DHE with their zinc-metallated analogues, ZnHp, ZnPP and ZnDHE, yields a dramatic ten-fold or hundred-fold decrease in fluorescence efficiency (Table 2). Although there have been reports in the literature claiming that HpD and its starting material, Hp-IX, may contain a zinc impurity [12], and others have presented evidence linking the presence of zinc to the 580 nm peak sometimes found in the fluorescence emission spectrum of aqueous solutions of HpD [12, 24], there have been no reports which have addressed the effects of zinc on the photodection efficiency of HpD toward cancer cells. The results presented here do allow for some general conclusions regarding the ability of zinc porphyrins to serve as fluorescing photodetection agents for cancer cells.

(1) The data presented in Table 3 indicate no significant difference in the quantum yields of HpDp mixtures containing zinc porphyrin and HpDp mixtures containing non-metallated porphyrins. The band located at 580 nm appears unaffected by the deliberate addition of zinc porphyrins even at 24 h. Therefore, the origin of this band does not originate from the presence of a metallated porphyrin impurity. The results in Table 3 do, however, suggest that bands prominent in HpDp components at 4 h (613, 670 nm) are markedly reduced in intensity after 10 days while bands located near 640 and 580 nm grow in intensity (see Fig. 1 and Table 3).

(2) The presence of minute concentrations of zinc porphyrin in commercially prepared Hp-IX or synthesized HpD is not likely to alter the otherwise 'pure' fluorescence quantum yield of either HpDp or HpD, since the results in Table 2 suggest that deliberately mixing HpDp with ZnHp, ZnPP or ZnDHE at concentrations well above those expected for any commercially prepared Hp-IX has little or no effect on the fluorescence yield of HpDP.

(3) Kessel and Chou [32] have reported that commercially prepared Hp-IX dichloride contains rather significant amounts of PP and HVD, both of which are apparently capable of photosensitizing cancer cells. Dougherty [20] has also reported that some samples of Hp-IX may contain a DHE impurity. Therefore, the quantum yields reported here for Hp and HpDp (Table 2) probably originate from the presence of several porphyrins. However, the quantum yield obtained for PP (0.002) is such that one would expect that very little of the fluorescence exhibited by solutions of Hp derive from the presence of PP, although the same cannot be said for the DHE impurity.

(4) It is unlikely that any porphyrin, including HpD, HpDp, HP, DHE or PP, containing large concentrations of coordinated zinc would be suitable for cancer cell photodetection since these metalloporphyrins are not very fluorescent in aqueous media. Some researchers have reported Q_t (quantum yield for triplet formation) of 0.90–0.93 [34–37] for some zinc porphyrins. Such large quantum yields leave relatively little energy for fluorescence.

(5) It is very likely that the HpD precursor, HpDp, would fulfil the role of a cancer cell detection agent since its fluorescence efficiency is high (particularly when enriched with Hp) and it is aggregated.

The effects of zinc on the photoinactivation efficiency of HpD toward cancer cells cannot be evaluated in this report. However, it does appear that the presence of zinc quenches the fluorescence efficiency of porphyrins. No further conclusions can be drawn regarding the photophysical relationship between the presence of zinc, the triplet state formation yield and singlet oxygen concentrations. These studies are currently underway.

Beneficial effects of Hp and HpDp

There is mounting evidence that Hp and PP (and presumably HpDp) are significantly internalized by cells once the photodynamic process begins. A drug accumulation study of Hp, HpD and Photofrin II (DHE) in L 1210 cells [35] showed that after intracellular uptake, Hp was released by the cells at a much faster rate than HpD or DHE, with very little of the Hp

TABLE 3. Emission peaks for HpD components

	Peak I	Peak II	Peak III	Peak IV
4 h				
HpDp	–	615(1.7×10^5)	640(sh)	670(1.0×10^5)
HpDp + ZnHp	580(0.1×10^5)	615(2.1×10^5)	640(sh)	670(1.2×10^5)
HpDp + Hp	580(0.2×10^5)	615(2.2×10^5)	640(sh)	670(1.2×10^5)
HpDp + DHE	580(0.1×10^5)	615(1.3×10^5)	640(sh)	670(0.7×10^5)
HpDp + ZnDHE	580(0.1×10^5)	615(1.5×10^5)	640(sh)	670(0.7×10^5)
HpDp + PP	580(0.1×10^5)	615(1.2×10^5)	640(sh)	670(0.7×10^5)
HpDp + ZnPP	580(0.3×10^5)	615(2.1×10^5)	640(sh)	670(1.0×10^5)
10 days				
HpDp	580(0.5×10^5)	615(0.4×10^5)	640(sh)	670(0.2×10^5)
HpDp + Hp	580(0.3×10^5)	615(0.6×10^5)	640(sh)	670(0.3×10^5)
HpDp + ZnHp	580(1.0×10^5)	615(0.4×10^5)	640(sh)	670(0.1×10^5)
HpDp + PP	580(0.9×10^5)	615(0.4×10^5)	640(sh)	670(0.1×10^5)
HpDp + ZnPP	580(0.5×10^5)	615(0.2×10^5)	640(sh)	670(0.05×10^5)
HpDp + DHE	580(0.3×10^5)	615(0.6×10^5)	640(sh)	670(0.3×10^5)
HpDp + ZnDHE	580(0.4×10^5)	615(1.4×10^5)	640(sh)	670(0.8×10^5)

drug retained by the cells after 2 h. This accelerated release could explain why Dougherty [38] found large amounts of DHE but very little Hp present in tissue extracted from human tumors.

The argument is now presented that the role of Hp and HpDP in the localization of cancer cells should be revisited. The fact that HpDp is aggregated suggests that its behavior *in vivo* might be similar to DHE. It is known that fluorescence quantum yields in aqueous media are not accurate indicators for *in vivo* fluorescence efficiency. For example, the physiologic cooperation between oligomeric HpD components and lipoproteins *in vivo* is known to produce disaggregation of the porphyrins, leading to a significant fluorescence enhancement. Regardless of this, the work presented here suggests that HpDp and HpDp/Hp mixtures be examined for possible use in cancer cell photodetection and/or phototherapy.

Conclusions

Results indicate that most of the fluorescence of the HpD precursor (and presumably, HpD) originates from the presence of hematoporphyrin. DHE and HpDp appear to be aggregated in PBS, and all other porphyrins are dimerized. ZnPP, ZnHp and ZnDHE are virtually non-fluorescent in PBS, and also are likely dimerized. Deliberately mixing HpDp with 20% ZnHp, ZnPP or ZnDHE had little effect on the fluorescence efficiency of HpDp, suggesting that minute concentrations of zinc present in commercially prepared Hp-IX should not affect the fluorescence efficiency of Hp-IX or HpD. When PP, HP, DHE, ZnPP, ZnHp or ZnDHE were mixed HpDp, only Hp and DHE enhanced the fluorescence yield of the HpD precursor, while mixing PP with HpDP significantly lowered the quantum yield of the precursor. Therefore, solutions containing mixtures of HpDp and Hp appear to be the logical drug for improving the photodetection efficiency of this type drug. A study of localization of these drugs in cancer cells is underway.

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