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POTENCY AND SELECTIVE TOXICITY OF TETRA(HYDROXYPHENYL)- AND TETRAKIS(DIHYDROXYPHENYL)PORPHYRINS IN HUMAN MELANOMA CELLS, WITH AND WITHOUT EXPOSURE TO RED LIGHT

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Abstract—A series of tetra(hydroxyphenyl)-(2-, 3- and 4-hydroxy; THPP) and tetrakis(dihydroxyphenyl)porphyrins (2,3-, 2,4-, 2,5-, 3,4-, and 3.5-dihydroxy; TDHPP) was synthesized and tested for toxicity in HeLa cells and human melanoma cell lines. Irradiation of drug-treated cells with >600 nm light greatly increased the toxicity of all drugs except the 2,5- and 3,5-TDHPP. The THPP were more toxic than TDHPP in all cell lines, with or without irradiation; of the dihydroxy derivatives, the 3,4- and 2,4-isomers were the most toxic and the 2,5-isomer was the least toxic. The MM96E melanoma cell line, shown previously to be sensitive to hydrogen peroxide and superoxide ion, was not hypersensitive to killing by any of the above agents. HeLa cells, which lacked glutathione-*S*-transferase activity, were sensitive to the 4- and 2,3-isomers after irradiation; similar amounts of all drugs were taken up by HeLa cells. The pigmented melanoma cell line MM418, resistant to UV-B and *in situ*-generated hydrogen peroxide but sensitive to glutathione (GSH) depletion, was found to be resistant to the 2,3-isomer (no irradiation) and sensitive to the 3,4-isomer. The results indicate that (1) phototoxicity in these phenylporphyrins is not mediated by superoxide ions or hydroxyl radicals, (2) toxicity is dependent on the orientation of the hydroxy groups, (3) GSH transferase and possibly GSH itself offer protection from the 4- and 3,4-derivatives, respectively, and (4) the 3,4-derivative and analogues of similar selectivity should be evaluated further for the treatment of primary melanoma.

INTRODUCTION

In pursuit of more effective and selective photosensitizers for cancer photodynamic therapy, Berenbaum *et al.*[1] reported that some of the most promising second-generation photosensitizers include the 2-, 3- and 4-isomers of tetra(hydroxyphenyl)porphyrin (THPP).† Absorption maxima occurred at 656 nm for 4-THPP and 648 nm for 2- and 3-THPP in fetal calf serum (FCS) containing dimethylsulfoxide (DMSO) (2% vol/vol). Under similar conditions, Photofrin II® and hematoporphyrin derivative (HpD) absorbed at 20–30 nm toward the blue end of the spectrum. The *in vivo* phototoxicities of 2-, 3- and 4-THPP, Photofrin II and HpD were compared. It was found that Photofrin II was 1.5 times as toxic as HpD, 4-THPP 4–6 times as toxic, 2-THPP 12–16 times as toxic and 3-THPP 25–30 times as toxic as HpD.[1] When comparing photosensitizers, low toxicity is not the only consideration, as this can be overcome by increasing the dosage. The limits of increasing the dosage depend upon the toxicity toward normal tissues. A photosensitizer will therefore be more effective if it shows increased toxicity for tumor cells and decreased toxicity for normal cells. The THPP also possessed a high selectivity for tumors with less photosensitization of brain and skin compared to Photofrin II and HpD.[1]

Peng *et al.*[2] compared the *in vivo* tumor, skin and brain uptake of nine potential photosensitizers, including Photofrin II, tetraphenylporphyrin tetrasulfonate (TPPS4), and 3-THPP. The most efficient tumor localizer was 3-THPP. Moan *et al.*[3] also compared the effectiveness and relevant physical properties of several promising photosensitizers, including 3-THPP, which was shown to be the best tumor localizer *in vivo* and to possess one of the lowest skin/tumor ratios (*i.e.* high tumor selectivity). The results were in agreement with those of Berenbaum *et al.*[1] It was suggested that because a high lipophilicity favors binding to cells, a drug whose lipophilicity increases with decreasing pH should show greater selectivity for tumor cells. This suggestion was made on the basis of the fact that tumors have a significantly lower pH than normal tissues. The 3-THPP showed a marked increase in lipophilicity (measured as the Triton X-114/water partition coefficient) with decreasing pH, which may have contributed to the high cellular uptake and selectivity. However, TPPS4, which is also a good tumor localizer, did not show an increase. Therefore, this property may not be a major determining factor for increased cell uptake or selectivity in tumor cells. The above studies on THPP suggest that further investigation of hydroxylated tetraphenylporphyrins as cell-type-specific photosensitizers is warranted.

We previously found that certain human melanoma cell lines are very sensitive to killing by active oxygen species

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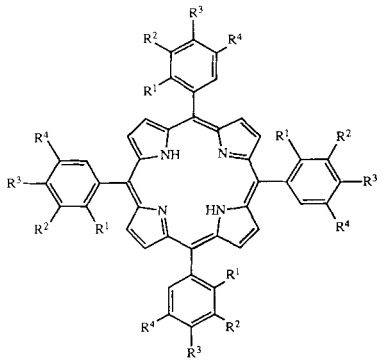
†*Abbreviations:* DMSO, dimethylsulfoxide; FAB-MS, fast atom bombardment mass spectrometry; FCS, fetal calf serum; GSH, glutathione; HpD, hematoporphyrin derivative; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline, pH 7.2; RPMI, Roswell Park Memorial Institute; SDS, sodium dodecylsulfate; TDHPP, tetrakis(dihydroxyphenyl)porphyrin; 3,4-TDMPP, tetrakis(3,4-dimethoxyphenyl)porphyrin; THPP, tetra(hydroxyphenyl)porphyrin; TLC, thin-layer chromatography; TPPS4, tetraphenylporphyrin tetrasulfonate.



Compound	R^1	R^2	R^3	R^4
2-THPP	OH	H	H	H
3-THPP	H	OH	H	H
4-THPP	H	H	OH	H
2,3-TDHPP	OH	OH	H	H
2,4-TDHPP	OH	H	OH	H
2,5-TDHPP	OH	H	H	OH
3,4-TDHPP	H	OH	OH	H
3,5-TDHPP	H	OH	H	OH

Figure 1. Structures of photosensitizers used in this study.

(presumably superoxide and hydroxyl radicals) generated in culture by the autooxidation of catechols.[4] A redox-resistant pigmented cell line established from a primary melanoma and a HeLa subline lacking in glutathione (GSH) transferase were also available. A series of THPP and tetrakis(dihydroxyphenyl)porphyrin (TDHPP) was therefore synthesized and tested on this panel of cell lines, for the purpose of identifying mechanisms of drug action and cell selectivities that could eventually be exploited *in vivo*. The structures of the compounds are described in Fig. 1.

MATERIALS AND METHODS

Chemical synthesis. Tetra(2-hydroxyphenyl)- and tetra(4-hydroxyphenyl)porphyrins were obtained from Mid Century (Posen, IL). Pyrrole (Aldrich) and proprionic acid (AJAX) were distilled before use. Boron tribromide (Aldrich) was used as a 1.15 *M* solution in dichloromethane (stored at −20°C). Dichloromethane, methanol, acetone, hexane, chloroform and ethyl acetate were analytical grade. All other chemicals and solvents were laboratory grade and were used as received. Column chromatography was performed on alumina (Merck, neutral, activity I). Thin-layer chromatography (TLC) was performed on Merck silica gel precoated aluminum sheets (0.2 mm thickness). Proton nuclear magnetic resonance (^{1}H-NMR) spectra were obtained on a Varian Unity 300 MHz spectrometer. Ultraviolet-visible spectra were recorded on a Varian DMS-100 or Cary 3 spectrophotometer.

Methoxy-substituted porphyrins. Propionic acid (150 mL) was heated to boiling and the heat was removed. The appropriate methoxy- or dimethoxybenzaldehyde (26 mmol) was added, then pyrrole (26 mmol) was added dropwise. The mixture was magnetically stirred and refluxed for 1.5 h, then left to cool and crystallize over 4 days. In all cases except the tetrakis(3,4-dimethoxyphenyl)porphyrin (3,4-TDMPP), the product was isolated by filtration of the mixture. Removal of tarry impurities was achieved by repeated washing of the crude solid with methanol. This solid was recrystallized by layering hexane over a concentrated chloroform solution to yield purple crystals. The 3,4-TDMPP did not precipitate, so the propionic acid was removed by distillation, leaving a black tarry residue. This residue was dissolved in dichloromethane, applied to an alumina column, and the porphyrin was eluted with dichloromethane. The residue from evaporation of the solvent was recrystallized as above. The crude 2,3-TDMPP isomer also required purification by column chromatography. The purity and identity of the compounds were confirmed by TLC and ^{1}H-NMR ($CDCl_3$) spectroscopy. Yields were as follows: 3-TMPP, 17%; 2,3-TDMPP, 11%; 2,4-TDMPP, 13%; 2,5-TDMPP, 5%; 3,4-TDMPP, 4%; 3,5-TDMPP, 25%. The 3,5-isomer has not been previously reported, and its characterization data were as follows: ^{1}H-NMR ($CDCl_3$) δ 8.93 (s, 8H, β-pyrrole), 7.4 (d, J = 2.5 Hz, 8H, 2,6-phenyl H), 6.90 (t, J = 2.5 Hz, 4H, 4-phenyl H), 3.96 (s, 18H, OCH_3), −2.85 (br s, 2H, NH); UV-visible ($CHCl_3$) λ_{max}/nm ($\epsilon/10^3 M^{-1}$ cm^{-1}) 421 (518), 515 (21.2), 549 (5.9), 588 (6.3), 646 (3.7); fast atom bombardment mass spectrometry (FAB-MS) (3-nitrobenzyl alcohol matrix) 855.2 (M + 1). Calculated for $C_{52}H_{46}N_4O_8$: C, 73.05; H, 5.4; N, 6.55. Found: C, 73.2; H, 5.5; N, 6.4%.

Hydroxy-substituted porphyrins. Boron tribromide solution (8 mL, 1.15 *M*, 9.2 mmol) was cooled in an acetone–CO_2 bath to −80°C. The appropriate methoxyphenylporphyrin (0.24 mmol) was dissolved in the minimum volume of dichloromethane (distilled from P_4O_{10}) and added dropwise with stirring. The mixture was protected from atmospheric moisture with a $CaCl_2$ drying tube. The mixture was stirred for 1 h at −80°C, then allowed to come to room temperature, with stirring, over 24 h. Two procedures were used for work-up. In procedure 1 (3-THPP, 2,4-TDHPP, 3,4-TDHPP and 3,5-TDHPP), the mixture was cooled in an ice bath, then methanol (7 mL) was added slowly. The green solution was neutralized to a red-purple color with triethylamine, then evaporated to dryness to produce a brown-purple residue. This was extracted with ethyl acetate and the organic layer was washed repeatedly with water to remove amine salts. After drying over anhydrous $MgSO_4$, evaporation left a solid, which was washed with dichloromethane to remove any unreacted starting material, then recrystallized from acetone/hexane to give purple solids. In procedure 2(2,3-TDHPP and 2,5-TDHPP), water (20 mL) was added to quench the ice-cold reaction mixture. Neutralization with triethylamine produced a brown-purple precipitate, which was collected by filtration, washed with water and dichloromethane and recrystallized from acetone/hexane. Products were identified by TLC and ^{1}H-NMR (acetone-d_6). Yields were as follows: 3-THPP, 82%; 2,3-TDHPP, 71%; 2,4-TDHPP, 87%; 2,5-TDHPP, 63%; 3,4-TDHPP, 82%; 3,5-TDHPP, 88%. The 2,4- and 3,5-isomers have not been previously reported, and their characterization data were as follows: 2,4-TDHPP ^{1}H-NMR (acetone-d_6) δ 8.91 (s, 8H, β-pyrrole), 8.78 (br s, 4H, 4-OH), 8.04–8.27 (five s, 4H, 2-OH), 7.7–7.8 (overlapping d, 4H, 6-phenyl H), 6.89 (br s, 4H, 3-phenyl H), 6.8 (d, 4H, 5-phenyl H), −2.7 (br s, 2H, NH); UV-visible (DMSO) λ_{max}/nm ($\epsilon/10^3 M^{-1}$ cm^{-1}) 426 (270), 518 (15.4), 555 (8.8), 594 (5.1), 650 (5.8); FAB-MS (3-nitrobenzyl alcohol/acetone matrix) 743 (M + 1) (this sample showed the presence of a small amount of monomethoxy compound, see text); 3,5-TDHPP ^{1}H-NMR (acetone-d_6) δ 9.03 (s, 8H, β-pyrrole), 8.74 (br s, 8H, OH), 7.25 (d, J = 2.1 Hz, 8H, 2,6-phenyl H), 6.85 (t, J = 2.1 Hz, 4H, 4-phenyl H), −2.8 (br s, 2H, NH); UV-visible (DMSO) λ_{max}/nm ($\epsilon/10^3 M^{-1}$ cm^{-1}) 425 (317), 516 (18.7), 551 (6.4), 590 (6.0), 648 (4.5); FAB-MS (glycerol/thioglycerol/1% trifluoroacetic acid matrix) 743.1 (M + 1). Calculated for $C_{44}H_{30}N_4O_8 \cdot 4H_2O$: C, 64.9; H, 4.7; N, 6.9. Found: C, 64.4; H, 4.4; N, 6.7.

UV-visible spectroscopic experiments. Stock solutions (*ca* 10^{-3} *M*) were prepared by dissolving the porphyrins in DMSO. Solutions of known concentration (*ca* 3×10^{-5} *M* for the visible bands and 6×10^{-6} *M* for the Soret band) were prepared by diluting the stock with phosphate-buffered saline, pH 7.2 (PBS). Spectra were recorded for each solution with a PBS-corrected baseline. The spectrum of 5% FCS in PBS was recorded, and it showed negligible absorption in the visible region. For spectra in DMSO only, the stock solutions were diluted with DMSO to *ca* 4.5×10^{-5} *M* and 3.0×10^{-6} *M*, and recorded with a DMSO-corrected baseline.

Cells. The origins of the human melanoma cell lines MM96E and MM418 and the cervical carcinoma line HeLa have been described.[5] Cells were cultured at 37°C in 5% CO_2/air in Roswell Park Memorial Institute medium-1640 (RPMI-1640) supplemented with 5% FCS, 100 μg/mL streptomycin, 100 IU/mL penicillin, 1 m*M* pyruvate, 50 μ*M* nicotinamide and 3 m*M* HEPES unless otherwise stated.

Cell survival assay. Cells were seeded in microtiter plates (1000–3000 cells/6 mm well). Stock solutions of drug were made by dis-

solving initially in DMSO, then diluting with RPMI-1640 culture medium. Final concentrations of DMSO were maintained below the cytotoxic concentration (<0.5%). Working solutions of drug were made from the stock solution, diluting with RPMI-1640 medium. Aliquots were taken directly from the working solutions and added to quadruplicate wells. The final dilutions of drug were determined from initial screening of a wide range of concentrations (*ca* 10^{-8}–10^{-4} *M*). Experiments were repeated until the concentrations were narrowed down to a range that contained, in most instances, at least three concentrations that gave evenly spaced levels of survival between 1% and 90% of the controls. Plates were set up for assays both without irradiation with red light (dark toxicity) and after irradiation (light toxicity). Cells were incubated with drugs for 18 h at 37°C in 5% CO_2/air, prior to irradiation with red light for 1 h. The irradiation source consisted of a fluorescent light box with a red photographic filter placed between the plate and the light source. The UV-visible absorption spectrum of the filter showed that only light above 600 nm was transmitted. The light intensity reaching the plates through the filter was 1.57×10^{-4} W cm^{-2} (IL-1700 radiometer). During irradiation, plates were placed in a candle box in order to maintain a 5% CO_2 atmosphere. After irradiation, the culture medium was replaced with fresh medium. The culture medium of the dark toxicity plates was also replaced. All plates were handled in subdued laboratory lighting. Plates were incubated for 5–7 days, until the controls were nearly confluent. Cell growth and morphology were scored visually at each dose (inverted microscope).

Medium was then replaced with fresh medium containing 2 μCi/mL (methyl-^{3}H)-thymidine and incubated for 2–4 h. Cells were washed with PBS, detached with 100 μL of PBS containing 0.2 mg/mL trypsin and 10 m*M* ethylenediaminetetraacetic acid and harvested onto glass fiber filter mats using a cell harvester. Radioactivity was determined in an LKB Beta Plate counter and the results plotted as log % control cpm *versus* dose. Each experiment was repeated and the mean of the combined data was used for the construction of cell survival curves.

Drug uptake experiments. HeLa cells (10^6) were incubated with 1.4×10^{-5} *M* drug in Eppendorf tubes at 37°C in 5% CO_2/air. A control tube containing cells without drug was included. After incubation, the cells were pelleted in a microfuge, medium was removed and cells were washed by resuspension in 1 mL ice-cold PBS, then centrifuged. After two further washes, sodium dodecylsulfate (SDS) (0.1 mL, 1 %) was then added and diluted with water (0.9 mL). Cells were sonicated and the fluorescence of the lysate was determined. Autofluorescence of the control cell lysate (no added drug) was subtracted. Emission wavelengths were determined from the emission spectrum using the Soret band absorption maximum as the excitation wavelength. Stock solutions (1.4×10^{-3} *M*) of each drug were obtained by dissolving it in DMSO, then diluting with 5% FCS/PBS to obtain a standard curve over the concentration range 0–1.4×10^{-5} *M*.

RESULTS

Porphyrin synthesis

The methoxyphenylporphyrins were prepared by the condensation of the appropriate methoxy- or dimethoxybenzaldehyde with pyrrole in refluxing propionic acid.[6] Yields for these were typical of this reaction (10–20%), with the exception of the 2,5- and 3,4-isomers. In the latter case, this was because the compound did not precipitate from the cooled propionic acid, necessitating chromatographic separation from the large quantity of tar. The low yield of the 2,4-isomer was probably also due to a higher solubility in propionic acid, but we did not investigate the mother liquors. Apart from 3,5-TDMPP, the methoxyphenylporphyrins have been previously reported.[7–9] The hydroxyphenylporphyrins were prepared by boron tribromide demethylation of the corresponding methyl ethers, according to the method of Milgrom.[10] The only compounds in this series not previously reported[8,9] are 2,4-TDHPP and 3,5-TDHPP. All compounds exhibited the expected ^{1}H-NMR resonances, especially the absence of peaks due to methoxy substituents in the spectra of the hydroxy compounds, with the exception of 2,4-TDHPP. In this case, the NMR and mass spectra indicated the presence of a small amount of a compound containing one remaining *ortho*-methoxy group, even after column chromatography and repeated recrystallization. The ^{1}H-NMR spectra of the compounds with methoxy or hydroxy groups *ortho* to the porphyrin showed the presence of atropisomers due to restricted rotation of the aromatic ring about the *meso*-carbon to aryl bond.[7,11]

Cell survival

Aqueous solutions of the THPP series were obtained by dissolving the porphyrins initially in DMSO followed by dilution with an aqueous solution. It was observed that relatively concentrated (*ca* 10^{-1} *M*) aqueous solutions of the porphyrins could only be obtained when the compound was dissolved in DMSO then diluted with 5% FCS in PBS or RPMI-1640 culture medium containing 5% FCS. Whenever the porphyrins were dissolved in DMSO then diluted with PBS or water, precipitation of the porphyrin occurred. Even at 10% DMSO, 4-THPP precipitated after addition of water or PBS (see below).

The series of THPP and TDHPP was screened for toxicity toward human cancer cell lines *in vitro* using the cell survival assay. The aim of the experiments was to determine the cell survival after treatment with the porphyrins for 24 h at various drug doses. In some experiments, cells were irradiated with red light (>600 nm). Cells were then allowed to recover for 5–6 days, at which time proliferation was measured by the level of DNA synthesis (incorporation of ^{3}H-thymidine). This assay gives similar results to clonogenic assays[12] and is more sensitive than the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, presumably because of the longer time allowed for cell replication to occur after treatment (Parsons, unpublished results). Preliminary results showed that similar levels of phototoxicity were achieved whether or not the drug was present in the culture medium during irradiation. Irradiation of untreated cells in culture medium did not generate toxicity.

Survival curves for HeLa cells treated with THPP or TDHPP are shown in Figs. 2 and 3, respectively. All of the monohydroxy compounds were toxic in the absence of light, in the 1–10 μ*M* range, but cell kill was enhanced up to 100-fold by red light (Fig. 2). The ratios of the D_{37} values in the dark and with light were calculated in order to compare the level of light activation of different drugs and cell lines (Table 1). Light activation was considerably greater in HeLa cells than for the two melanoma cell lines, the 4-THPP isomer giving a ratio of 155 for HeLa but only 7.4 for MM418 cells. The 2,3-, 2,4- and 3,4-TDHPP were an order of magnitude less toxic than the monohydroxy series and exhibited lower levels of light activation (Fig. 3, and Table 1). The 2,5-isomer was the least toxic and responded poorly to irradiation. Other differences between the responses of the three cell lines were found. Figure 4 shows that HeLa cells were particularly photosensitive to 4-THPP and 2,3-TDHPP. In the absence of light, MM418 cells were sensitive to 3,4-TDHPP relative to

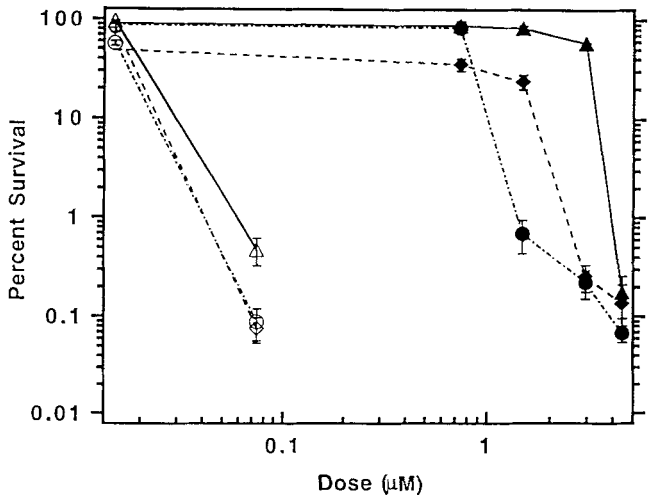


Figure 2. Toxicity of THPP in HeLa cells. Cells were incubated with drug for 24 h, irradiated with red light and survival determined after 6 days by incorporation of ^{3}H-thymidine. Closed symbols, dark toxicity; open symbols, phototoxicity. ▲, △: 4-THPP; ◆, ◇: 3-THPP; ●, ○: 2-THPP. Points are means ± SD of quadruplicates from two separate experiments.

the other two cell lines but highly resistant to killing by 2,3-TDHPP (Fig. 5).

Binding of drugs to cells and FCS

To compare the amount of drug taken up by cells, HeLa cells were incubated with the porphyrin in culture medium at 37°C for 1 h, washed, lysed in SDS and the amount of drug measured in a spectrofluorometer. The results showed an uptake of 0.4–1.3 $\mu g/10^6$ cells, an insufficient difference to account for the differences seen in cell survival. The increased solubility of THPP in 5% FCS prompted a study of the absorption spectra in the presence and absence of serum. Under the former conditions, the Soret bands of the THPP

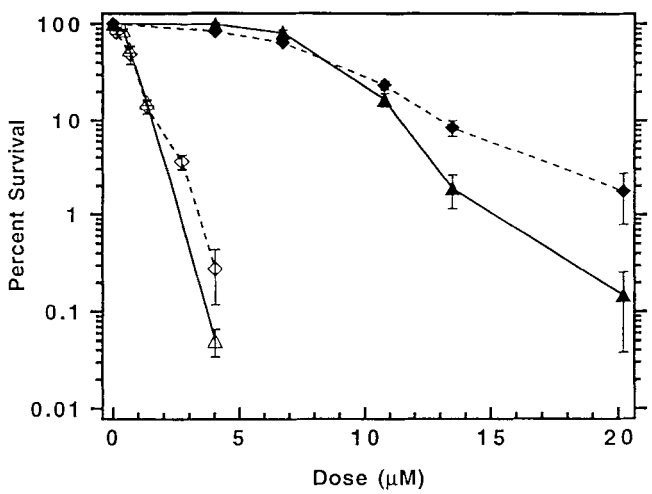


Figure 3. Toxicity of TDHPP in HeLa cells. Closed symbols, dark toxicity; open symbols, phototoxicity. ▲, △: 2,4-TDHPP; ◆, ◇: 3,4-TDHPP. Points are means ± SD of quadruplicates from two separate experiments.

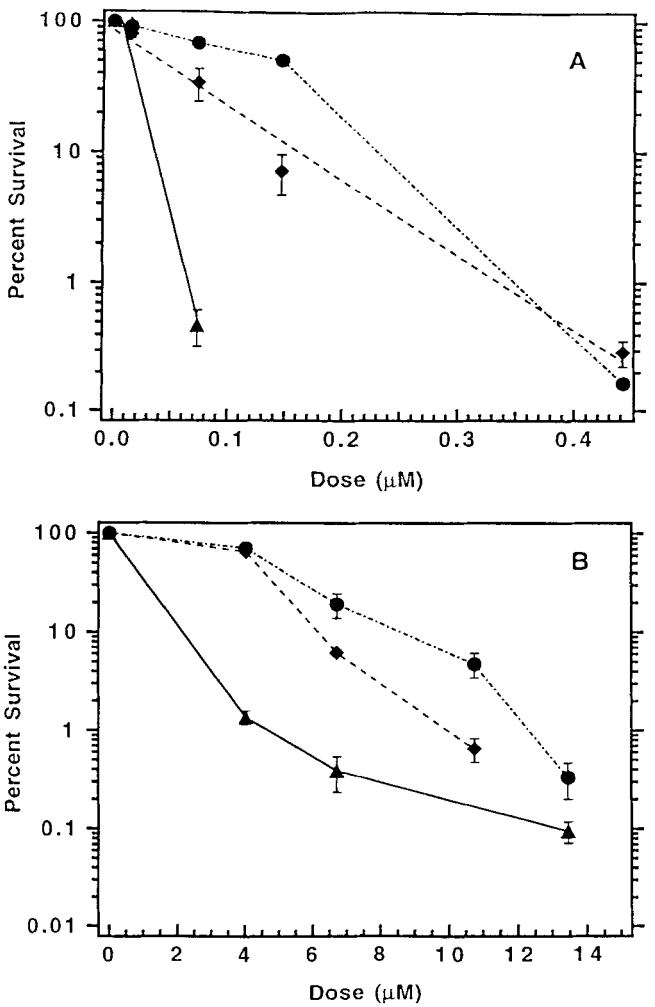


Figure 4. Selective phototoxicity of 4-THPP (A) and 2,3-TDHPP (B) in human tumor cells lines. ▲, HeLa; ◆, MM96E; ●, MM418. Points are means ± SD of quadruplicates from two separate experiments.

were exceptionally broad (width at half-height *ca* 3000 cm^{-1}). The TDHPP showed variable behavior, and in particular, the 2,5-isomer showed a broad band in DMSO only, and there was little difference in the presence of serum. Table 2 shows data summarizing the spectral changes observed in the Soret region. In the region of interest for our irradiation experiments ($\lambda > 600$ nm), the major changes were observed for the THPP, especially 4-THPP, and for 3,4-TDHPP. Broadening and merging of bands II and III were apparent for the latter two species, and a broad tail developed to low energy of the maximum of the longest wavelength band. Figure 6 shows a comparison of the UV-visible spectra of 4-THPP in the presence and absence of serum. Because the variations in the light toxicity of different compounds could depend on the amount of light absorbed at wavelengths greater than 600 nm, correlations were sought between the integrated absorbed dose (area under the absorption curve from 600 to 700 nm, corrected for concentrations, see Table 2) and light toxicity (ratio of D_{37} in the light to D_{37} in the dark). A linear correlation with the eight drugs was found for MM96E ($r = 0.933$) and HeLa ($r = 0.837$), but not for MM418 ($r = 0.486$).

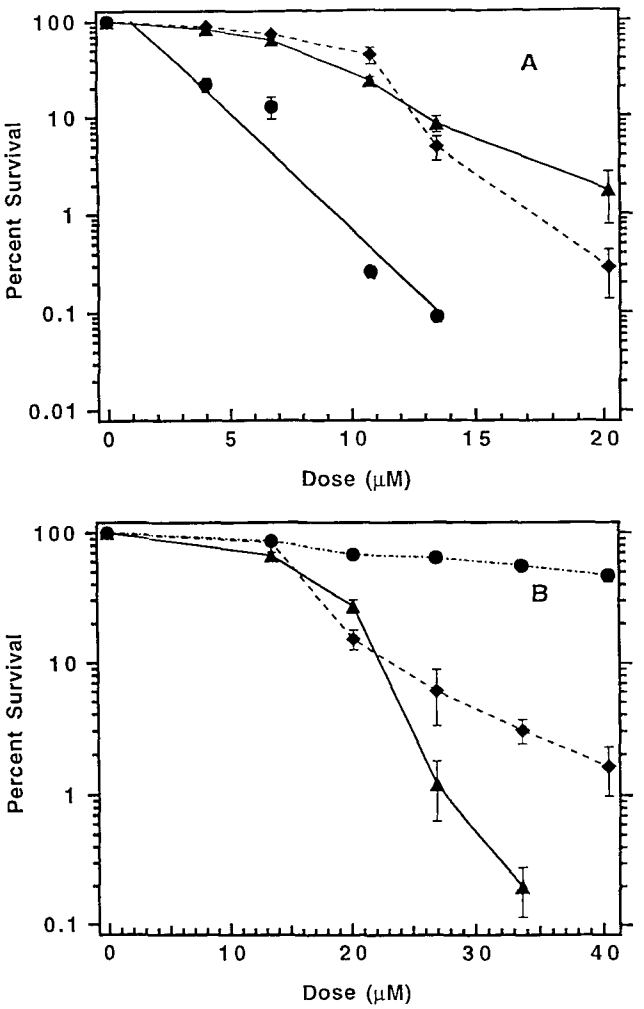


Figure 5. Selective dark toxicity of 3,4-TDHPP (A) and 2,3-TDHPP (B) in human tumor cell lines, ▲, HeLa; ◆, MM96E; ●, MM418. Points are means ± SD of quadruplicates from two separate experiments.

DISCUSSION

This study revealed major differences between closely related hydroxylated tetraphenylporphyrins in their dark- and light-induced toxicities *in vitro*, as well as selective killing of certain types of human tumor cells. The following questions therefore require discussion: (1) the nature of the phototoxic species and the variation in phototoxicity of different derivatives, (2) the differences in mechanism between the dark and light reactions and (3) the reasons for selectivity against particular human tumor cell lines.

A type I photosensitization reaction involving production of superoxide ion, H_2O_2 and hydroxyl radicals was ruled out in these experiments by the lack of sensitivity of the MM96E cell line. These cells were previously found, in contrast to HeLa and MM418, to be highly sensitive to killing by reactive oxygen intermediates generated by the autoxidation of catechols.[4] Thus singlet oxygen (generated by the type II reaction) is assumed to be the agent responsible for cell death. The differences in toxicity between the THPP and TDHPP after irradiation with red light could be attributed to differences in the fates of the generated singlet oxygen. Singlet oxygen has a limited lifetime and diffusion distance,[13] therefore any damage will occur close to its site of generation. Catechol and a range of catechol derivatives are oxidized by singlet oxygen generated by anthrapyrazoles to produce *o*-semiquinones and ultimately superoxide radicals.[14] Different absorbed light dose is an alternative explanation for the varying drug potencies in HeLa and MM96E cells. However, the poor correlation between absorbed dose and toxicity in MM418 suggests that other variables need to be considered when melanin is present.

Table 2. Wavelengths of Soret maxima and bandwidths at half-height for THPP and TDHPP, measured in DMSO and in 5% FCS/PBS (containing 0.3% DMSO)

Porphyrin	DMSO λ_{max} (nm)	DMSO $\nu_{1/2}$ (cm^{-1})	FCS/PBS λ_{max} (nm)	FCS/PBS $\nu_{1/2}$ (cm^{-1})	Relative area*	R†
2-THPP	420	795	427	3340	6.83	4.20
3-THPP	421	663	424	2920	6.32	4.40
4-THPP	425	764	426	3270	7.46	4.28
2,3-TDHPP	420	1280	421	2270	1.52	1.77
2,4-TDHPP	425	1060	421	1820	1.72	1.72
2,5-TDHPP	416	1630	416	1760	1.00	1.08
3,4-TDHPP	429	1320	427	2770	5.35	2.10
3,5-TDHPP	425	851	421	1670	1.65	1.96

*Area under the absorption curve (600–700 nm, in FCS/PBS, corrected for concentration) relative to 2,5-TDHPP = 1.00.
†$R = \nu_{1/2}(FCS/PBS)/\nu_{1/2}(DMSO)$.

Table 1. Comparison of the dark and light toxicity of THPP and TDHPP in human tumor cell lines.

Porphyrin	D_{37} (μM)* HeLa Dark	HeLa Light	HeLa D/L	MM96E Dark	MM96E Light	MM96E D/L	MM418 Dark	MM418 Light	MM418 D/L
2-THPP	0.88	0.01	88	0.50	0.03	17	0.79	0.07	11
3-THPP	0.70	0.01	70	0.76	0.03	25	0.97	0.05	19
4-THPP	3.1	0.02	160	1.8	0.06	30	1.2	0.16	7.5
2,3-TDHPP	18	0.92	20	17	4.6	3.7	55	5.4	10
2,4-TDHPP	8.7	0.77	11	5.2	0.59	8.8	3.6	0.71	5.1
2,5-TDHPP	49	32	1.5	34	20	1.7	54	19	2.8
3,4-TDHPP	9.1	0.75	12	11	0.68	16	3.1	0.85	3.6
3,5-TDHPP	15	7.2	2.1	29	4.1	7.1	19	3.8	5.0

*Dose required to reduce survival to 37%, interpolated from the combined dose–response of two experiments.

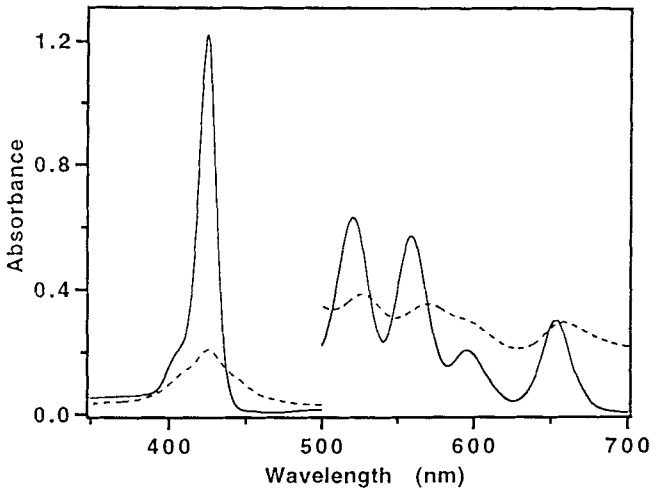
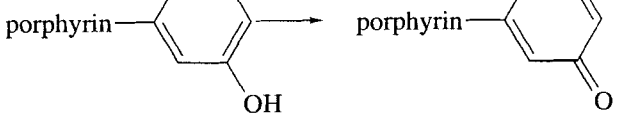


Figure 6. Ultraviolet-visible spectra of 4-THPP in DMSO (solid line) and in FCS/PBS (dashed line). Data are normalized to concentrations of 3×10^{-6} *M* (350–500 nm) and $4.5 \triangle 10^{-5}$ *M* (500–700 nm).

Singlet oxygen generated by a TDHPP could therefore react with the hydroxy groups within the same TDHPP or a nearby TDHPP molecule. Effectively, the TDHPP would act as scavengers of singlet oxygen resulting in decreased damage to cells compared to the THPP. The UV-visible spectroscopic results suggest that the number and disposition of the hydroxy groups on the phenyl rings lead to differences in binding to proteins or to other porphyrin molecules. Such pronounced broadening of Soret bands is indicative of extensive, nonspecific porphyrin aggregation, possibly involving protein binding, rather than of simple face-to-face dimerization, or of specific protein–porphyrin interaction.[15–17] Binding in different geometries may cause differences in the accessibility of the hydroxy groups for oxidation by singlet oxygen.

The observation that 2,5-TDHPP (the only isomer that has four hydroxy groups on each side of the porphyrin plane in all atropisomers) was significantly less toxic than the rest of the series and that irradiation with red resulted in only a minor increase in toxicity may also be a consequence of the photooxidation of hydroxy groups. A pathway available to this compound due to the orientation of the hydroxy groups is the formation of a stable *para*-quinone as shown below.



Tetrakis (*p*-benzoquinonyl)porphyrin was prepared from 2,5-TDHPP and found not to fluoresce.[8] In addition, replacement of even one phenyl substituent with a quinone was shown to result in a significant decrease in fluorescence quantum yield.[8] The fluorescence quenching has been suggested to occur *via* an electron transfer intermediate $(porphyrin)^{+\cdot} \cdots (quinone)^{-\cdot}$, which resembles the primary light-induced change separation product in photosynthesis. The 2,3- and 3,4-isomers can form less stable *ortho*-quinones, but the observation that the latter is one of the most toxic of the TDHPP weakens the argument that the formation of quinones by these reactions is important.

Mechanisms for the dark toxicity of porphyrins have received little attention. The dark toxicity of most of the present drugs arose from a different mechanism than phototoxicity because the toxicity spectrum for the three cells lines changed markedly. For example, the THPP and 2,3-TDHPP were five- to eight-fold more phototoxic to HeLa cells than to MM418, yet the dark toxicity and thus presumably drug uptake was similar in both cell lines. This would be consistent with the lack of GSH transferase in the HeLa cells preventing the detoxification of cellular oxidation products.

Further deductions can be made from the responses of MM418, which is a pigmented melanoma cell line established from a primary tumor of the skin and found to be resistant to UV-B and peroxide generated *in situ*,[18] but very sensitive to killing by the GSH-depleting agent buthionine sulfoximine.[5] MM418 was highly resistant to unirradiated 2,3-TDHPP, implicating the generation of hydrogen peroxide perhaps at a particular site within the cell. The possibility of 3,4-TDHPP lowering intracellular GSH was raised by the enhanced sensitivity to this drug in the dark of MM418 *versus* MM96E cells. Cell kill extended over more than two logs of survival, despite the fact that only 5–10% of the cells were heavily pigmented. This raises the possibility that 3,4-TDHPP or drugs with similar selectivity may find application in the treatment of primary melanoma, even where the cells are heterogeneous in their state of differentiation. Further *in vitro* and *in vivo* studies of cell type specificity, intracellular localization and related analogues are required to evaluate fully the potential of hydroxylated tetraphenylporphyrins for the treatment of melanoma.

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REFERENCES

1. Berenbaum, M. C., S. L. Akande, R. Bonnett, H. Kaur, S. Ioannou, R. D. White and U.-J. Winfield (1986) *Meso*-tetra(hydroxyphenyl)porphyrins, a new class of potent tumour photosensitisers with favourable selectivity. *Br. J. Cancer* **54**, 717–725.
2. Peng, Q., J. F. Evensen, C. Rimington and J. Moan (1987) A comparison of different photosensitizing dyes with respect to uptake by C3H-tumors and tissues of mice. *Cancer Lett.* **36**, 1–10.
3. Moan, J., Q. Peng, J. F. Evensen, K. Berg, A. Western and C. Rimington (1987) Photosensitizing efficiencies, tumor- and cellular uptake of different photosensitizing drugs relevant for photodynamic therapy of cancer. *Photochem. Photobiol.* **46**, 713–721.
4. Parsons, P. G. (1985) Modification of dopa toxicity in human tumour cell lines. *Biochem. Pharmacol.* **34**, 1801–1807.
5. Kable, E. P. W., D. Favier and P. G. Parsons (1989) Sensitivity of human melanoma cells to L-dopa and DL-buthionine-(S,R)-sulfoximine. *Cancer Res.* **49**, 2327–2331.
6. Adler, A. D., F. R. Longo, J. D. Finarelli, J. Goldmacher, J. Assour and L. Korsakoff (1967) A simplified synthesis for *meso*-tetraphenylporphin. *J. Org. Chem.* **32**, 476.
7. Dirks, J. W., G. Underwood, J. C. Matheson and D. Gust (1979) Conformational dynamics of $\alpha,\beta,\gamma,\delta$-tetraphenylporphyrins and their dications. *J. Org. Chem.* **44**, 2551–2555.
8. Chan, A. C., J. Dalton and L. R. Milgrom (1982) Tetrapyrroles.

Part 2. Synthesis and electronic spectra of some quinone-porphyrins. *J. Chem. Soc. Perkin Trans. II*, pp. 707–710.
9. Semeikin, A. S., O. I. Koifman, B. D. Berezin and S. A. Syrbu (1983) Synthesis of tetraphenylporphines with active groups in phenyl rings. 2. Preparations of tetrakis(hydroxyphenyl)porphines. *Khim. Geterosikl. Soedin.* **10**, 1359–1361.
10. Milgrom. L. R. (1983) Synthesis of some new tetra-arylporphyrins for studies in solar energy conversion. *J. Chem. Soc. Perkin Trans. I*, pp. 2535–2539.
11. Crossley, M. J., L. D. Field, A. J. Forster, M. M. Harding and S. Sternhell (1987) Steric effects on atropisomerism in tetraarylporphyrins. *J. Am. Chem. Soc.* **109**, 341–348.
12. Goss, P. and P. G. Parsons (1977) The effect of hyperthermia and melphalan on survival of human fibroblast strains and melanoma cell lines. *Cancer Res.* **37**, 152–156.
13. Kanofsky, J. R. (1990) Quenching of singlet oxygen by human plasma. *Photochem. Photobiol.* **51**, 299–303.
14. Reszka, K., J. W. Lown and C. F. Chignell (1992) Photosensitization by anticancer agents—10. *Ortho*-semiquinone and superoxide radicals produced during anthrapyrazole-sensitized oxidation of catechols. *Photochem. Photobiol.* **55**, 359–366.
15. Davila, J. and A. Harriman (1990) Photoreactions of macrocyclic dyes bound to human serum albumin. *Photochem. Photobiol.* **51**, 9–19.
16. Davila, J. and A. Harriman (1990) Photochemical and radiolytic oxidation of a zinc porphyrin bound to human serum albumin. *J. Am. Chem. Soc.* **112**, 2686–2690.
17. Oenbrink, G., P. Jürgenlimke and D. Gabel (1988) Accumulation of porphyrins in cells: influence of hydrophobicity aggregation and protein binding. *Photochem. Photobiol.* **48**, 451–456.
18. Musk, P. and P. G. Parsons (1987) Resistance of pigmented human cells to killing by sunlight and oxygen radicals. *Photochem. Photobiol.* **46**, 489–494.