Tolyporphin: A Natural Product from Cyanobacteria with Potent Photosensitizing Activity against Tumor Cells in Vitro and in Vivo

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ABSTRACT

Tolyporphin (TP), a porphyrin extracted from cyanobacteria, was found to be a very potent photosensitizer of EMT-6 tumor cells grown both in vitro as suspensions or monolayers and in vivo in tumors implanted on the backs of C.B17/Icr severe combined immunodeficient mice. Thus, during photodynamic treatment (PDT) of EMT-6 tumor cells in vitro, the photokilling effectiveness of TP measured as the product of the reciprocal of D50 (the light dose necessary to kill 50% of cells) and the concentration of TP is ~5000 times higher than that of Photofrin II (PII), the only PDT photosensitizer thus far approved for clinical trials. TP almost exclusively localizes in the perinuclear region and specifically in the endoplasmic reticulum (ER), as shown by microspectrofluorometry on single living EMT-6 cells costained with the ER and/or Golgi fluorescent vital probes, 3,3’-dihexyloxacarbocyanine iodide and AR-[4,4-difluoro-(5,7-dimethyl-BODIPY®)-1-pentanoyl]-6-erythro-sphingosine (Molecular Probes, Eugene, OR). As a result, the singlet oxygen-mediated photodynamic activity of TP induces an effective inactivation of the acyl CoA:cholesterol-O-acyl transferase, a sensitive marker of ER membrane integrity and alterations of the nuclear membrane. In vivo, with the EMT-6 mouse tumor model, an exceptional effectiveness is also observed as compared to that of PII and other second generation photosensitizers of the phorbale class, which are all themselves much more potent than PII. The outstanding PDT activity of TP observed in vivo may be due to its unique biodistribution properties, in particular much less extraction by the liver, resulting in a higher delivery to other tissues, including tumor.

INTRODUCTION

For more than two decades, mechanisms associated with the PDT3 of tumors have been aggressively investigated at the molecular and cellular levels. Several photosensitizing compounds were found to be effective in treating rodent tumors, and a few have progressed to Phase I and II clinical trials (1). At this time, PII, a mixture of porphyrin derivatives made from hematoporphyrin, has been approved in the United States, Canada, Europe, and Japan for clinical use in the treatment of some esophageal, bladder, and lung cancers. PII is characterized by light absorption bands at wavelengths similar to those of hemoglobin. Consequently, blood produces a strong screening effect for PII-activating light, limiting its penetration into tissue so that PDT with this drug is largely limited to superficial tumors (2). In addition, a major side effect of PII is its long retention in dermal tissue, which can result in serious superficial burns for up to 6 weeks after PDT treatment in patients not protected from exposure to normal sunlight. Because of the above limitations to the clinical use of PII, considerable research efforts have been expended on identifying second-generation photosensitizers that are activated by longer-wavelength (>650 nm) light and are metabolized and excreted more rapidly from normal tissues (3). New photosensitizing drugs that incorporate phthalocyanine, chlorin, phorbale, and benzoporphyrin chromophores have been identified and proposed for new medical applications. Some of them are undergoing clinical trials (4, 5).

There has been recent research interest in porphyrin compounds extracted from blue-green algae for their ability to reverse the multidrug resistance phenotype of various tumor cells (6). TP, a leading compound in these studies, possesses several chemical properties that could be advantageous for second-generation photosensitizers. In solution, it exhibits a monomer chemical structure with high molar absorbance at 676 nm (68,600 M⁻¹ cm⁻¹ in ethanol) and a relatively high water solubility (7). Preliminary testing indicated strong photokilling activity when TP was illuminated with red light. We, therefore, performed a more detailed study of its photosensitizing properties with EMT-6 tumor cells under well-defined in vitro conditions and as solid tumors growing subcutaneously on the backs of scid mice. When the in vitro and in vivo photosensitizing effectiveness of TP was compared to that of MPPH (8) or Ph4-OH (9), exceptional activity was seen. In earlier studies, with this same tumor model, Ph4-OH was shown to be a much more effective photosensitizer than PII (10). The intracellular localization and the sites of photosensitizing of TP in EMT-6 cells were investigated by microspectrofluorometry techniques (11). TP was found to selectively associate with the ER and nuclear membrane, which might also be the primary sites for its potent photosensitizing effects. Preliminary studies with tumor-bearing mice indicated exceptional PDT activity and a very different pattern of biodistribution than observed for Ph4-OH and MPPH. Its potent photosensitizing effectiveness both in vivo and in vitro, along with its unique intracellular distribution and in vivo biodistribution, suggests that this novel PDT photosensitizer could play a useful role in both mechanistic and clinical studies of PDT.

MATERIALS AND METHODS

Chemicals

All of the chemicals for routine use (buffer components, ethanol, and DMSO) were purchased at the purest commercial grade and used as received. The fluorescent probes for the Golgi apparatus (bodipy-ceramide; BPC), and ER (carbocyanine: DiOCj) were purchased from Molecular Probes-Europe (Leiden, The Netherlands) and used as received.

Photosensitizers

TP: Preparation and Properties. [7,17 di(2-O-acetyl-3,6-dideoxy-xylo-hexopyranosyl)-7,8,18,19-tetrahydro-8,18-dioxo-2,7,13,17-tetramethyl-21H,2H-porphine] was extracted from Tolypothrix nodosa and purified for use
by described procedures (7). In ethanol, the molar absorbances at 402 nm (Soret band) and at 676 nm were 148,000 and 68,500 M⁻¹ cm⁻¹, respectively, whereas fluorescence maxima were observed at 677.5 and 714 nm, with a shoulder at 758 nm. The yield of singlet oxygen (¹O₂) formation from TP was estimated to be 0.25 in 0.1 M phosphate-buffered aqueous solution (pH 7.4) using a comparison method with hematoporphyrin as a reference photosensitizer (12) and tryptophan as a singlet oxygen substrate.

Other Photosensitizers. MPPH was a gift from K. M. Smith (Department of Chemistry, University of California at Davis, Davis, CA). Ph₄-OH was prepared and purified by procedures previously described (9).

Cells

Our murine mammary tumor cell line (EMT-6) was originally obtained from the laboratory of Dr. J. M. Brown (Stanford, CA). These mouse tumor cells are anaplastic and aneuploid and display an in vitro doubling time of ~10 h. They were cultured as monolayers in plastic tissue culture flasks in Waymouth's medium supplemented with 12.5% FBS and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) or in Ham's F-10 medium supplemented with 5% FBS and antibiotics as above. Stock cultures were transferred twice weekly.

Photosensitizing Effectiveness in Tumor Cells in Vitro

Cell Suspensions. EMT-6 tumor cells were trypsinized from exponentially growing cultures and suspended in calcium-free minimal essential medium (spinner MEM), which contained 5% FBS and antibiotics (as above). Photosensitizers were dissolved in absolute ethanol, diluted to required concentrations in complete spinner MEM, and mixed with cells at room temperature (23°C) for 1 h prior to and during their illumination with graded doses of laser light of specific wavelength. During illumination, cells were maintained in homogeneous suspensions with a magnetic stirrer (13). Aliquots of cell suspensions were removed before and after fractions of light dose, diluted in spinner MEM, plated into 10 × 60-mm Petri dishes in complete Waymouth's medium, and incubated in a humidified 5% CO₂ incubator at 37°C. After 6–7 days, colonies were fixed, stained with méthylène blue, and counted with an automatic colony counter (Minicount; Imaging Products International, Inc.). Survival fractions were computed relative to the plating efficiencies of control cells (drug-treated with no illumination), which were usually ~80%. The light dose required to inactivate 50% of each cell population (D₅₀) was interpolated from the survival curves, and its reciprocal, 1/D₅₀, was used as an inactivation rate to describe the biological effectiveness of each concentration of photosensitizer (13).

Cell Monolayers. Forty-eight h after seeding at a density of 5 × 10³ · cm⁻² in 35-mm Nunc Petri dishes, cells were incubated for 1 h with 1 µM TP in Ham's F-10 medium supplemented with 10% FBS and then washed three times with PH 7.4 PBS. Irradiation with desired red light doses was carried out in 1 ml of HBSS. Immediately after irradiation, the irradiation medium was discarded and replaced by 1 ml of fresh supplemented Ham's F-10 medium. Then, the irradiated cells were further incubated for 24 h at 37°C under a 5% CO₂ humidified atmosphere before the viability assay. Cell viability was determined by the Neutral Red assay. This assay (14) has been proven quite reliable as compared to [³H]thymidine incorporation or to the thiazolyl blue (the so-called MTT) test for the evaluation of phototoxicity of drugs toward skin cells (15). It was slightly modified as follows: at the end of the 3-h incubation at 37°C with the dye (0.005% in 10% FCS-supplemented Ham's F-10 medium), cells were washed three times with PBS and incubated for 10 min at 37°C under gentle shaking in 1 ml of 10% SDS. The absorbance of the resulting solution was read at 535 nm with a UVIKON 922 spectrophotometer (Kontron Instruments). Experiments were performed at least in triplicate, and data are expressed as percentage of controls (sham-irradiated cells).

ACAT Activity in EMT-6 Cells

For ACAT activity measurements, EMT-6 cells were seeded at a density of 5 × 10³ · cm⁻² in 100-mm Nunc Petri dishes, cultivated, and then incubated with 1 µM TP as described above for the measurement of the photosensitizing effectiveness. Twenty-four h after illumination, cells were harvested with a rubber policeman, and ACAT activity in cell homogenates was measured by the method of Brown et al. (16), with modifications described previously (17).

Each assay was performed with 0.2 mg/ml cell protein, 0.1 M phosphate buffer (pH 7.4), 5 mM MgCl₂, 0.2 mg/ml BSA, 0.2 µCi [¹⁴C]oleoyl CoA, and 100 µM unlabeled oleoyl CoA in a final incubation volume of 100 µl. The reaction mixture was heated at 37°C for 15 min and then stopped on ice. A 30-µl aliquot of the reaction mixture was applied to a Schleicher & Schuell (Dassel, Germany) silica gel plate. Radioactive lipids were separated by chromatography using hexane/diethylether/acetic acid 70:30:2 (v/v/v; Ref. 18). The cholesterol ester spots were cut out, and the radioactivity was measured by liquid scintillation using a Beckman LS 6000C counter. The activity was expressed as nmol/h/mg cell protein.

Photosensitizing Effectiveness on EMT-6 Tumors in Vivo

EMT-6 tumor cells (5 × 10⁶) were injected in 0.02 ml of sterile physiological saline s.c. on the backs of C.B17/1cr scid mice bred at the Fox Chase Cancer Center. When tumors reached volumes of ~0.1 cm³, photosensitizing drugs were injected via a tail vein in 15% DMSO and 85% FBS 1 h prior to illumination of the tumor. Light at 664, 673, or 681 nm (specific to each photosensitizer) was transported from the laser via an optical fiber and focused onto the tumor surface through a microtens that produced a relatively flat field of slowly diverging light. On average, these tumors had a depth of 0.4 cm, and light dose to tumor cells at this depth would be >60% of that delivered to the tumor surface. Tumor response was measured by the growth delay assay (10). Approximate tumor volumes were computed from caliper measures of three mutually perpendicular diameters minus appropriate skin flap dimensions with the equation $V = \pi/6(D₁ × D₂ × D₃)$ (Ref. 10).

Measurements of Photosensitizer Biodistribution in Tumor-Bearing Mice

EMT-6 tumors were grown in C.B17/1cr scid mice by procedures described above. TP, MPPH, and Ph₄-OH were administered at a dose of 2 µg/g via a tail vein in 15% DMSO and 85% FBS to tumor-bearing animals. One h after photosensitizer injection, the time at which light was delivered to evaluate in vivo photosensitizing effects, the animals were sacrificed, selected tissues were necropsied for extraction, and photosensitizer levels were quantified by microspectrofluorometry. Briefly, tissue extracts were illuminated with excitation light, the wavelength of which was ~410 nm and the fluorescence emissions of which were quantified at 664, 673, and 681 nm for MPPH, Ph₄-OH, and TP, respectively. After a correction for any endogenous fluorescence from the different animal tissues, absolute levels of photosensitizer were obtained from standard curves generated with known quantities of each drug. Photosensitizer levels are reported in units of µg/g in the respective animal tissues.

Light Sources and Irradiation Procedures

Experiments were performed in different laboratories that were equipped with different light sources. Laser light was generated by a coherent 899 TYE/DYE laser pumped by a Coherent INNOVA 200 Argon-Ion laser. Light at wavelengths of 600–700 nm was generated with the laser operating in the DYE mode with DCM SPECIAL/LC 6501 dye and transported via optical fiber to a diffusing lens. Light intensity was measured with an optical power meter (Newport, model 835) with a silicon photodetector (Newport, model 818-SL). For in vitro studies of photosensitizer effectiveness, light exposure is reported in units of J/cm² at the surface of the glass irradiation chamber. For mouse tumors, light exposure is reported as J delivered to a 1 cm diameter circular field at the tumor surface.

For irradiation of cell monolayers using broad-band red light, a custom-built table with two 150-W tungsten-halogen lamps was used, the light of which was filtered with Balzers R 65 and Callflex 3000 optical filters. The lamps were placed below the 30 × 25-cm table thermostated at 37°C. Under these conditions, 99% of the delivered light arose from wavelengths in the range 645–730 nm. An average light intensity of 1.2 × 10⁻⁸ mol · s⁻¹ · cm⁻² · nm⁻¹ was determined by chemical actinometry based on the measurement of the photodegradation rate of hydroxyvinyldeuteroporphyrin chlorin in the presence of metronidazole (19). In the case of weakly absorbing TP solutions, this is equivalent to a monochromatic absorbable light intensity at 677 nm of 1.3 × 10⁻⁸ mol · s⁻¹ · cm⁻² (2.2 mW · cm⁻²).
Lucifer Yellow and the mitochondrial probe rhodamine 123 were also used.

Staining of EMT-6 cells with these probes was carried out as previously described (11, 20).

RESULTS

Photostimulating Effectiveness in Vitro. Fig. 1 shows survival curves generated after a 1-h exposure of suspensions of EMT-6 tumor cells to selected concentrations of TP and irradiated with 681 nm light. These cell inactivation curves have broad shoulders with little or no evidence of cell killing at low light doses. After a threshold dose specific for each concentration of photosensitizer, cell killing is approximately exponential, with a slope characteristic of the photosensitizer concentration. This response is similar to that reported for PII (21) and Ph4-OH (9) and is indicative of an intracellular buildup of a specific toxin (presumably \(^{1}O_2\)). The relative photostimulating effectiveness of each concentration of TP was expressed as \(1/D_{50}\). These "inactivation coefficients" are shown in Fig. 2 along with those for Ph4-OH and MPPH. All of these data, as well as those that defined photoeffectiveness of PII (21), were performed with the same target cells using the same irradiation chambers with light delivered 1 h after photosensitizer addition to the tissue culture medium. It is evident that TP is approximately six times more potent a photosensitizer in this tumor cell assay than is Ph4-OH, and both are significantly more effective than is MPPH. The linearity of photoinactivation rate vs. [TP] suggests that, over this concentration range, TP localizes to nonsaturable sites within the cells, i.e., \(1/D_{50} \times [TP]\) is constant. This hypothesis was tested in subsequent microspectrofluorometric studies. TP is the most effective photosensitizer tested to date with our EMT-6 tumor cells in vitro. These and previously published data indicate that TP is \(\sim 6\) times, \(\sim 70\) times, and \(\sim 5000\) times more effective than is Ph4-OH, MPPH, and PII, respectively (9, 21). Whereas in vitro phototolerance assays of novel compounds yield information about their intrinsic photosensitizing potential, their localization within and their delivery to tumor target cells in vivo can significantly modulate their ultimate effectiveness against tumors. In that regard, the intracellular localization of TP...
Fig. 3. Bright-field and fluorescence micrographs of EMT-6 cells after incubation with TP in supplemented culture medium. After incubation with TP, cells were first washed three times with supplemented culture medium and then twice with culture medium. Bright-field images were obtained with green light, and fluorescence images were recorded beyond 630 nm under excitation with 405 nm-wavelength light. Exposure time, 4 s. Bars, 10 μm. A, bright-field (A1) and fluorescence (A2) micrographs of a cell incubated for 2.5 h with 1 μg/ml TP. Objective magnification, ×63 (NA, 1.3). Light fluence rate at 405 nm, 1.5 J/cm². B, bright-field (B1) and fluorescence (B2) micrographs of a cell incubated for 1 h with 2 μg/ml TP. Objective magnification, ×40 (NA, 0.7). Light fluence rate at 405 nm, 100 mW/cm². C, bright-field micrographs of a cell incubated for 15 min with 1 μg/ml TP, before (C1) and after (C2) a 10-s exposure to 405 nm-wavelength light (fluence rate, 100 mW/cm²). Objective magnification, ×63 (NA, 1.3).

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Fig. 4. Fluorescence spectrum (A) and fluorescence topographic profile (B) of an EMT-6 cell incubated for 15 min with 0.4 μg/ml TP. Incubation and washing conditions are the same as in Fig. 3. Excitation wavelength, 405 nm (fluence rate, 25 mW/cm²). Objective magnification, ×63 (NA, 1.3). Exposure time, 4 s. The spectrum was recorded at position 23 μm shown on the X axis. The topographic profile of the emission was recorded at position 23 μm shown on the Y axis.
Fig. 5. Bright-field and fluorescence micrographs of an EMT-6 cell incubated with 1 µg/ml TP and 4 µg/ml DiOC₆(A) or with 1 µg/ml TP and 4 mM BPC (B). Incubation with TP and washings were carried out as in Fig. 3. Objective magnification, X63 (NA, 1.3). Excitation wavelength, 405 nm (fluence rate, 100 mW/cm²). Exposure time, 4 s/image. The bright-field micrographs (A1 and B1) were obtained with green light. BPC and DiOC₆ fluorescence (A2 and B2, respectively) were first recorded with a band-pass filter centered at 536 nm (half height bandwidth, 90 nm), and then the TP fluorescence (A3 and B3) was recorded beyond 630 nm using a cutoff filter.

quenching of the DiOC₆ fluorescence (to ~60% of normal) is induced by the presence of TP (Fig. 6A). Considering the relative electronic energy of the first excited singlet states of DiOC₆ and TP, this quenching is most probably explained by a Forster-type resonance energy transfer from DiOC₆ (the donor) to TP (the acceptor). In cells, Forster-type resonance energy transfer requires close proximity (typically 1–10 nm) of interacting molecules (22, 24). Thus, this effect suggests that some proportion of intracellular TP is localized in the ER.

As are most PDT photosensitizers, TP is readily photobleached upon illumination with UV, visible, or red light when incorporated into cells. Porphyrin photobleaching also occurs during clinical PDT (25). This photobleaching has been attributed to secondary reactions of the photosensitizer with lipid peroxidation products or oxidized protein residues produced by membrane photosensitization (25, 26). A light dose rate-dependent photobleaching of TP can be induced by irradiation of EMT-6 tumor cells with 405 nm light in the microspectrofluorometer and is accompanied by intense photochemistry at the nuclear membrane (compare micrographs in Fig. 3, C1 and C2). Photobleaching experiments carried out with DiOC₆ or BPC colocalized with TP also confirm that both probes are in the vicinity of TP in the Golgi-ER complex. Thus, DiOC₆ and BPC are quite stable when irradiated alone with 405 nm light, but both probes are readily photobleached after costaining of EMT-6 tumor cells with TP (data not shown) as a result of its photodynamic action (¹O₂ production). All of these results strongly support the ER and the nuclear membrane as major sites of TP localization and primary photochemical targets in EMT-6 tumor cells.

Photosensitized ACAT Inactivation by TP. ACAT is a typical membrane-bound enzyme localized in the ER and is a key enzyme for cholesterol metabolism. It is very sensitive to alterations of its membrane microenvironment, and we have shown that it was inactivated
Thus, ACAT inactivation induced by the photodynamic action of TP regrowth time in days are shown ± SE. The administration of various concentrations of photosensilizers. number of mice: mean doses of red light (A. >645 nm). Data are means (bars, SD) of six measurements. demonstrates that pharmacokinetic and other factors associated with where drug delivery to tumor cells was assured. This result clearly should be noted that Ph4-OH and MPPH have nearly equal effective killing of 80-90% of the tumor cells by the PDT treatments (10). It was induced. This amount of growth delay is consistent with the administered at 2 /xg/g and the tumors were illuminated with 300 J of approximately 3.5 days (see Table 1). When Ph4-OH and MPPH were tumor response was expected to be primarily via a vascular damage mechanism. EMT-6 tumors at this volume exhibit a doubling time of photosensitizer concentrations in the blood were relatively high and tumors were irradiated 1 h later, when the photosensitizer levels in blood, liver, muscle, and tumor 1 h after i.v. administration of 2 /xg/g were measured. Fig. 8 shows similar biodistribution patterns for the two pheophorbide compounds (Ph4-OH and MPPH) and a strikingly different pattern for TP. At this time, the absolute levels in unflushed liver tissue of the pheophorbide compounds were approximately 100 times that found in blood, muscle, and tumor. The higher level of MPPH in the blood relative to Ph4-OH could account for its increased photosensitizing effect in vivo at this early time. In contrast to these data, TP levels in blood were ~10 times less than the other photosensitzers and was equally distributed at a much higher concentration (note the logarithmic scale in Fig. 8) to the three solid tissues that were sampled. Consequently, its potent in vivo photosensitizing effectiveness cannot be correlated with its level in the blood. In addition, there has been significantly less extraction of TP by the liver, resulting in a higher delivery to other tissues, including tumor. Complete pharmacokinetic measurements over 24 h are required to better understand the in vivo biodistribution of TP.

These preliminary studies indicate that TP can have potent in vivo photosensitizing effectiveness against tumors and has biodistribution properties quite different from some second-generation photosensitzers of the pheophorbide class. Additional studies are in progress to measure TP specificity for tumor tissue, its clearance from normal tissues associated with long-term toxicity, and its mechanism(s) of tumor response.

by photosensitization with PII in transformed human fibroblasts (27). Large variations in ACAT activity among animal species are not unusual. As a result, in EMT-6 cells, the ACAT activity level is about an order of magnitude lower than that observed in the human fibroblasts. Fig. 7A demonstrates that about 50% of ACAT activity is lost from cells treated for 1 h with 1 /xM TP and then irradiated with red light at doses corresponding to about 50% cell survival (Fig. 7B). Thus, ACAT inactivation induced by the photodynamic action of TP is in agreement with the microspectrofluorometry results, suggesting the ER as a main site of intracellular TP localization. Incidentally, Fig. 7B demonstrates nearly a 10-fold difference in TP effectiveness as photosensitizer of monolayer and suspension cultures. Part of this difference could result from the dosimetry of the different light sources and/or differential uptake of the drug into monolayer versus suspended cells.

**Photosensitizing Effectiveness and Biodistribution of TP in Vivo.** The in vivo photosensitizing effectiveness of TP was measured with EMT-6 tumors growing s.c. on the backs of C.B17/Scid mice. Photosensitzers were administered through a tail vein in 15% DMSO + 85% FBS, and tumors were irradiated 1 h later, when the photosensitizer concentrations in the blood were relatively high and tumor response was expected to be primarily via a vascular damage mechanism. EMT-6 tumors at this volume exhibit a doubling time of approximately 3.5 days (see Table 1). When Ph4-OH and MPPH were administered at 2 /xg/g and the tumors were illuminated with 300 J of light of specific wavelength, an additional 8–10 days of growth delay was induced. This amount of growth delay is consistent with the killing of 80–90% of the tumor cells by the PDT treatments (10). It should be noted that Ph4-OH and MPPH have nearly equal effectiveness in vivo, whereas Ph4-OH was significantly more potent in vitro, where drug delivery to tumor cells was assured. This result clearly demonstrates that pharmacokinetic and other factors associated with drug transport can significantly modulate photosensitizer effectiveness in vivo. Preliminary studies with TP indicated that it was more potent in vivo than were Ph4-OH and MPPH. Table 1 shows tumor growth delays achieved with 0.5, 1.0, and 2.0 /xg/g TP administered i.v. 1 h prior to illumination with only 30 J of 681 nm light. Tumor responses equal to those observed with Ph4-OH and MPPH were obtained with 1.0–2.0 /xg/g TP and 30 J of light. These preliminary data indicate that TP is 10–20 times more potent a photosensitizer against EMT-6 tumors in vivo than is Ph4-OH or MPPH. Additional studies will determine the optimal time for light delivery after TP administration to obtain maximal tumor response. In addition, it is not yet known whether or not TP is selectively retained within tumor relative to normal tissues at longer times.

The photosensitizer levels in blood, liver, muscle, and tumor 1 h after i.v. administration of 2 /xg/g were measured. Fig. 8 shows similar biodistribution patterns for the two pheophorbide compounds (Ph4-OH and MPPH) and a strikingly different pattern for TP. At this time, the absolute levels in unflushed liver tissue of the pheophorbide compounds were approximately 100 times that found in blood, muscle, and tumor. The higher level of MPPH in the blood relative to Ph4-OH could account for its increased photosensitizing effect in vivo at this early time. In contrast to these data, TP levels in blood were ~10 times less than the other photosensitzers and was equally distributed at a much higher concentration (note the logarithmic scale in Fig. 8) to the three solid tissues that were sampled. Consequently, its potent in vivo photosensitizing effectiveness cannot be correlated with its level in the blood. In addition, there has been significantly less extraction of TP by the liver, resulting in a higher delivery to other tissues, including tumor. Complete pharmacokinetic measurements over 24 h are required to better understand the in vivo biodistribution of TP.

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### Table 1 Times after various PDT treatments for EMT-6 tumors in scid mice to regrow to twice their treatment volumes

<table>
<thead>
<tr>
<th>n</th>
<th>Drug</th>
<th>Dosage (µg/g)</th>
<th>Wavelength (nm)</th>
<th>Light dose (J)</th>
<th>Regrowth time (days)</th>
</tr>
</thead>
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<tr>
<td>20</td>
<td>Control</td>
<td>2.0</td>
<td>673</td>
<td>300</td>
<td>3.5 ± 0.4</td>
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<tr>
<td>5</td>
<td>Ph4-OH</td>
<td>2.0</td>
<td>664</td>
<td>300</td>
<td>11.6 ± 1.8</td>
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<td>5</td>
<td>MPPH</td>
<td>2.0</td>
<td>681</td>
<td>30</td>
<td>14 ± 1.1</td>
</tr>
<tr>
<td>5</td>
<td>TP</td>
<td>0.5</td>
<td>681</td>
<td>30</td>
<td>7 ± 1.1</td>
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<tr>
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<td>TP</td>
<td>1.0</td>
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<tr>
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<td>TP</td>
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Mice were treated with 300 J or 30 J of light of appropriate wavelength 1 h after i.v. administration of various concentrations of photosensitzers. n, number of mice; mean regrowth time in days are shown ± SE.
DISCUSSION

The extremely high phototoxicity of TP, which was first shown to be an effective revertant of P-glycoprotein-mediated multiple drug resistance in human ovarian and breast cell lines, is strikingly highlighted in this study by the fact that this porphyrin compound is 5000 times more potent in killing EMT-6 cells in vitro than is PII. PII can be used as a reference PDT photosensitizer because it is the only porphyrin derivative thus far officially approved for the PDT of some human tumors. TP is also much more potent in vitro than other second-generation PDT photosensitizers, such as the phophorhobides Ph4-OH and MPPH, for which antitumor efficacy in mice has been previously reported (10, 28, 29).

The high photocytotoxicity of TP compared to Ph4-OH, MPPH, and PII cannot be ascribed to a much higher quantum yield of $^{1}O_2$ formation, the cytotoxic product produced by the photodynamic action of porphyrins. Thus, TP, MPPH, Ph4-OH, and PII have molar absorbances and/or $^{1}O_2$ formation quantum yields of the same order of magnitude. Consequently, other factors than differences in the $^{1}O_2$ production must be sought to explain the large variation in the in vitro photokilling effectiveness found for the three photosensitizers under study.

Another important factor involved in a phototoxic response is, of course, the subcellular localization of the photosensitizer. In tumor or transformed cells in vitro, TP has been shown by biochemical and microspectrofluorometric methods to localize mainly at the plasma membrane, lysosomes, and mitochondria (for review, see Ref. 30). Furthermore, we showed that some PII was also incorporated into the ER (27). With regard to MPPH, the lysosomes and the plasma membrane are its main sites of localization (11, 31), whereas Ph4-OH is bound mainly to the plasma membrane (11). This review of the sites of localization for PII, Ph4-OH, and MPPH clearly illustrates the unique intracellular specificity of PII, Ph4-OH, MPPH, and TP, because the latter specifically binds to the ER membranes. Several noncationic moderately hydrophobic porphyrin photosensitizers have been shown to distribute diffusely to all internal membranes, suggesting that ER could be one of their photochemical targets (32), but, to our knowledge, this is the first study that clearly demonstrates the importance of photochemical damage to the ER for inactivation of tumor cells in culture.

Singlet oxygen formed upon illumination of TP-stained ER membranes cannot diffuse far from its site of production, because its range of action has been estimated to be at most 10–20 nm (33). Thus, the nuclear membrane damage observed after PDT with TP in Fig. 3C2 can be attributed either to direct photosensitization by TP bound to the nuclear membrane or to transfer to the nuclear membrane of the photo-oxidative damage from photoactivated TP associated with rough ER.

Although the specific localization of TP in the ER is probably responsible for its strong photocytotoxicity in vitro, the many factors associated with the PDT response in vivo make it difficult to predict a direct relationship between in vitro cell mechanisms and the in vivo tumor response. Ph4-OH is about 10 times more effective a photosensitizer in vitro than is MPPH, but both photosensitizers have similar effectiveness when delivered i.v. to EMT-6 tumors in C.B17/Ic scid mice. MPPH is believed to be one of the most potent PDT photosensitizers thus far tested (28). It is the methyl ester of the hexyl ether of pyrophorphorbide(a) and is readily hydrolyzed by plasma esterases both in vitro and in vivo (28). Thus, it can be expected to behave in vivo like the hexyl ether of pyrophorphorbide(a), a member of a class of PDT photosensitizers for which a structure-activity relationship has been recently established (34). For these derivatives, it was shown that high levels of photosensitizer in the tumor tissue were not sufficient for optimal PDT efficiency, but the lipophilicity of the six-carbon alkyl ether chain was optimal to produce the antitumor and vascular effects of PDT. Here, TP is by far the most potent photosensitizer in vivo, because, relative to MPPH and Ph4-OH, 10 times less light is sufficient to produce increased tumor growth delays. But in contrast to MPPH and Ph4-OH, TP is considerably more hydrophilic because of its two hexopyranosyl groups linked to the tetapyrrole ring. Its partition coefficient is 1.3 (6) as compared to >100 and 100,000 for Ph4-OH (9) and unesterified MPPH (34), respectively.

Because lipophilicity does not seem to be a requirement for the antitumor activity of TP, the striking difference in its pharmacokinetic behavior and biodistribution may contribute to its enhanced effectiveness against the EMT-6 tumors. The pattern of distribution of TP in blood, liver, and tumor 1 h after i.v. administration is characterized by a very low TP level in blood and a relatively uniform distribution in tumor, muscle, and liver. This pattern of distribution is quite different from that of Ph4-OH and MPPH for which liver-bound photosensitizer is two orders of magnitude higher than that in tumor. As optimization of the PDT response was not the purpose of this work, drug-light intervals other than 1 h were not investigated. It is thought that this 1-h interval may favor the predominance of vascular damage mechanisms of tumor response. However, the concentration ratio [TP]tumor/[TP]blood is ~100 as compared to ~3 and ~1.5 for Ph4-OH and MPPH, respectively, and absolute levels of TP in EMT-6 tumors are much greater than those of Ph4-OH and MPPH. Consequently, direct tumor cell photokilling by TP may be an important mechanism for tumor cure. It is therefore conceivable that both direct effects on the tumor cells and indirect effects from vascular damage contribute to the overwhelming PDT response induced by TP. In that regard, TP and related compounds deserve further evaluation as potential candidates for the presently established clinical applications of PDT. In addition, potential interactions between its use in reversing drug-resistant phenotypes and its photosensitizing activity warrant further investigation.

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