Effect of Molecular Characteristics on Cellular Uptake, Subcellular Localization, and Phototoxicity of Zn(II) N-Alkylpyridylporphyrins

Received for publication, August 19, 2013, and in revised form, October 30, 2013. Published, JBC Papers in Press, November 8, 2013, DOI 10.1074/jbc.M113.511642

Rima Ezzeddine1, Anwar Al-Banaw2, Artak Tovmasyan3, James D. Craik2, Ines Batinic-Haberle2, and Ludmil T. Benov3

From the 1Department of Biochemistry, Faculty of Medicine, and 2Department of Medical Laboratory Sciences, Faculty of Allied Health Sciences, Kuwait University, Kuwait City 13110, Kuwait and the 3Department of Radiation Oncology, Duke University Medical Center, Durham, North Carolina 27708

Background: Delivering molecules to selected cellular compartments is important for analytical and practical purposes. Results: Varying the length and positions of alkyl substituents results in preferential uptake of zinc porphyrins by particular cellular structures. Conclusion: Uptake, distribution, and phototoxicity of porphyrins depend on charge, lipophilicity, and molecular shape. Significance: Systematic chemical modification provides the basis for rational design of molecules targeting specific cellular compartments.

Photodynamic therapy (PDT)4 is a relatively noninvasive therapeutic option for treating neoplastic and nonneoplastic diseases. Approved initially for the treatment of a small number of selected tumors (1), it has expanded to encompass a wide range of applications in dermatology (2, 3), ophthalmology (4, 5), dentistry (6, 7), cardiology (8, 9), cosmetics (10, 11), blood purification (12), and water disinfection (13, 14).

PDT is based on the preferential uptake of a photosensitizing dye, the photosensitizer (PS), by the targeted cells/tissue, followed by irradiation of the selected area with visible light. Upon absorption of light the PS reaches an excited state, which is followed by either transfer of electron or abstraction of hydrogen atom (type I reaction) to/from a neighboring organic molecule or transfer of energy or electron to oxygen (type II reaction) to generate singlet oxygen (15, 16). As a result of these reactions, biomolecules and cellular structures could be modified to an extent that causes cell death. Because most of the species generated by the excited PS have a short life in the cellular environment, the range of immediate damage is limited by the localization of the PS (16, 17). The nature of the damaged structures and the extent of damage determine the predominant mechanism of cell death (18), which in turn is a major predictor of PDT outcome. It has been shown that overall charge, charge distribution, and lipophilicity of the molecule are among the most important parameters that control cellular uptake and subcellular distribution of a PS (19–21). Our previous investigations with Zn porphyrin-based PSs and photoinactive Mn porphyrin analogs revealed that an additional factor influencing the uptake and localization of cationic molecules is the nature of the substituents linked at

---

* This work was supported by the College of Graduate Studies and by grants YM05/11 and SRUL02/13 from Kuwait University.
1 Part of an M.Sc. thesis, College of Graduate Studies, Kuwait University.
2 Supported by the Ines Batinic-Haberle General Research Fund.
3 To whom correspondence should be addressed: Dept. of Biochemistry, Faculty of Medicine, Kuwait University, P. O. Box 24923 Safat, 13110 Kuwait. Tel.: 965-531-9489; Fax: 965-533-8908; E-mail: lbenov@hsuc.edu.kw.

---

4 The abbreviations used are: PDT, photodynamic therapy; MTT, 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PS, photosensitizer; SRB, sulfonphthaldamine B; ZnP, Zn porphyrin, Zn(II) meso-tetraakis(N-alkylpyridinium-2- or -3 or -4-yl)porphyrin, alkyl being methyl (M, ZnTMPyP4+), ethyl (E, ZnTEPyP4+), n-butyl (nBu, ZnTnBuPyP4+), n-hexyl (nHex, ZnTnHexPyP4+); 2, 3, and 4 relate to ortho, meta, and para isomers, respectively. For simplicity, charges are omitted throughout the text.
the periphery of the porphyrin ring; these substituents determine the size, three-dimensional shape, and the bulkiness of the molecule (for review, see Ref. 22). Initial experiments showed that fluorescence of a number of Zn alkylpyridylporphyrin derivatives was sufficient for assessment of cellular and subcellular localization by fluorescence microscopy.

This study examines how structural modifications at the periphery of the porphyrin ring affect cellular uptake and subcellular distribution, and consequently photodynamic activity, of cationic Zn(II) meso-tetakis(N-alkylpyridinium-2(or -3 or -4)-yl)porphyrins.

**MATERIALS AND METHODS**

**Synthesis**

The porphyrinic ligands H2T-2-PyP, H2T-3-PyP, and H2T-4-PyP were purchased from Frontier Scientific. The *ortho* and *meta* N-hexylated porphyrins, H2TnHex-2-PyPCl4 and H2TnHex-3-PyPCl4, were synthesized according to the procedure published earlier (23). Synthesis of the *para* isomer is given below.

*Meso-tetakis(N-n-hexylpyridinium-4-yl)porphyrin Tetrachloride (ZnTnHex-4-PyPCl4)—* To the solution of the H2T-4-PyP (150 mg, 0.242 mmol) in N,N-dimethylformamide (15 ml, preheated for 15 min at 110 °C under nitrogen atmosphere), the n-hexyl p-toluenesulfonate (9.32 ml, 36.4 mmol) was added. The course of N-hexylation was followed by thin layer chromatography on silica gel TLC plates using 1:1:8 acetonitrile as a mobile phase. Upon the completion of reaction (4 h) the porphyrin was precipitated by diethyl ether. It was then dissolved in hot water, and precipitated as a chloride salt by the addition of 1M NaOH to pH 11. Then ZnCl2 (0.66 mmol, 90 mg, ~20-fold excess) was added at 25 °C and stirred, which resulted in a pH drop from 11 first to 6 and then to 3. The course of metallation was followed by thin layer chromatography on silica gel gel TLC plates using 1:1:8 KNO3(H2O):H2O:acetonitrile as a mobile phase as well as by UV-visible spectroscopy. After overnight stirring at room temperature, the reaction was completed. The porphyrin was precipitated from the solution as a PF6- salt by the addition of a concentrated aqueous solution of NH4PF6. The precipitate was thoroughly washed with diethyl ether (5 × 30 ml). The dried precipitate was then dissolved in acetone, filtered, and precipitated as a chloride salt by the addition of concentrated acetone solution of tetrabutylammonium chloride. The precipitate was washed thoroughly with acetone (5 × 30 ml). The product was dried in vacuo at room temperature. Yield 35 mg (95%). Elemental analysis: ZnTnHex-4-PyPCl4 × 9H2O: Anal. Calcd. for C64H93Cl4N8ZnO11.5: C, 56.04; H, 7.27; N, 8.17. Found: C, 55.96; H, 6.42; N, 8.60. UV-visible, λmax nm (log ε, M⁻¹ cm⁻¹): 262.5 (4.30), 321.0 (4.32), 429.0 (4.53), 558.5 (4.24), 597.0 (3.39). Elemental analysis: ZnTnHex-3-PyPCl4 × 11.5H2O: Anal. Calcd. for C64H95Cl4N8ZnO15.5: C, 56.04; H, 7.27; N, 8.17. Found: C, 55.76; H, 6.42; N, 8.60. UV-visible, λmax nm (log ε, M⁻¹ cm⁻¹): 264.0 (4.43), 328.0 (4.49), 427.0 (5.64), 557.5 (4.40), 594.0 (3.88). Fig. 1 depicts the structure of the *ortho*, *meta*, and *para* ZnPs.

**Lipophilicity**

Lipophilicity was determined from chromatographic retention factor *Rf* on TLC plates (Z122777-25EA; Sigma-Aldrich), and as log *P* ow as described earlier (23).
Cell Culture

The LS174T human colon adenocarcinoma cell line was used in this study. To check for cell line-specific effects, the MCF7 breast cancer cell line was used in parallel. Monolayer cultures were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin as an antibacterial agent and 0.1% amphotericin as an antifungal agent. Cultures were maintained at 37 °C and 5% CO2 and used for experiments at 80–90% confluence. The growth medium was removed, and the cells were washed with phosphate-buffered saline (PBS). Cells were then detached by trypsinization and incubation at 37 °C for 2–3 min. Overexposure to trypsin was avoided because excessive trypsinization results in leaky and nonviable cells. Fresh medium (10× trypsin volume) was added to inhibit trypsin action. The cell suspension was centrifuged at 500 × g for 3 min. The supernatant was discarded, and the pellet obtained was resuspended in fresh medium. Cells were then counted and used in experiments, and the remaining portion was subcultured and maintained. Cells were counted prior to seeding into the plates or use in experiments. Cell counting was performed with an improved Neubauer hemocytometer and trypan blue to differentiate between viable and nonviable cells.

Photosensitizers

Photosensitizers investigated in this study were isomeric methyl (ZnTMPyP), ethyl (ZnTEPyP), butyl (ZnBuPyP), and hexyl (ZnHxPyP) (Fig. 1) Zn(II) N-alkylpyridylporphyrins. Stock solutions were prepared in distilled water and filter-sterilized.

Light Source

Cell cultures were illuminated by two 5-W, white fluorescent light tubes mounted under a translucent screen providing a fluence of 2.0 mW/cm².

Phototoxicity of Zn(II) N-Alkylpyridylporphyrins

The photoefficacy of the Zn(II) N-alkylpyridylporphyrins was assessed by investigating effects on cell viability and cell proliferation.

Cell Viability

Cell viability was determined by the surrogate MTT assay based on metabolic reduction of the yellow tetrazolium dye MTT to an insoluble purple-colored formazan product that can be determined spectrophotometrically (24). The cells were counted, seeded into a flat-bottom 96-well microplate at a concentration of 5 × 10⁴ cells in 100 μl of medium/well and incubated overnight to permit cells to adhere. ZnP was added at the indicated concentrations in quadruplicate wells, and cells were incubated with the PSs in the dark for 6, 12, and 24 h. Control wells without PSs were incubated under the same conditions. After the incubation period, the medium was replaced with PBS, and plates were subjected to illumination (or dark incubation). Following the illumination, PBS was removed and replaced with culture medium. The MTT reagent (10 μl/well), prepared by dissolving 5 mg of MTT in 1 ml of PBS, was then added to each well. The cultures were incubated for 3 h at 37 °C, and then 10% SDS in 0.01 M HCl was added followed by incubation overnight. Absorbance was measured at 560 (formazan product) and 650 nm (background) using a microplate reader. Dark toxicity of the tested compounds was assessed by following the same protocol except that illumination was omitted.

Cell Proliferation

Cell proliferation was followed using the sulforhodamine B (SRB) assay (25), which is based on binding of SRB to proteins of cells fixed with trichloroacetic acid (TCA). The cells were counted and seeded into a flat-bottom 96-well microplate at 5 × 10³ cells/well and were left overnight to adhere. ZnP was added to quintuplet wells, and cells were incubated in the dark for 24 h. Control wells had no PS added. After the incubation, the medium was replaced with PBS, and the plates were illuminated. After illumination, PBS was replaced with medium. The SRB assay was performed at 0, 24, 48, and 72 h, with zero time being immediately after illumination. Cells were fixed with cold TCA to a final concentration of 10%. The plates were then incubated at 4 °C for 1 h, followed by five washes with deionized water. Fixed cells were stained with 0.4% SRB dissolved in 1% acetic acid for 30 min. Cells were then washed five times with 1% acetic acid to remove unbound stain and air-dried at room temperature. The bound dye was solubilized with 10 mM Tris solution with volume equal to the volume of the original culture medium, and the content of the wells was mixed and left for 5 min. The plates were analyzed on a microplate reader at 510 nm, and the background was measured at 690 nm.

Cellular Uptake of Zn(II) N-Alkylpyridylporphyrins

Cells were counted and seeded in a 6-well plate at 25 × 10⁴ cells/well and were incubated to ~80% confluence. ZnP isomers were added at a concentration of 20 μM, and the cells were incubated with the PSs in the dark for 24 h. The medium was then removed, and the cells were washed with PBS and solubilized with 0.25% (v/v) Brij-98. The intracellular accumulation of ZnP was assayed by measuring their fluorescence emission at an excitation wavelength (λex) matching their Soret band and emission wavelength range (λem) of 520–700 nm. Peak areas at emission were calculated, and the amount of ZnP in each sample was determined using peak areas generated by pure Zn(II)P isomers with known concentrations as standards. Quinine was used as a fluorescence standard.

Cellular Localization of ZnP Isomers

The subcellular distribution of ZnP isomers was studied using confocal fluorescence microscopy. Cells were counted and seeded on sterile glass coverslips at 5 × 10⁴ cells/slip and incubated overnight to adhere. ZnP were added, and the cells were incubated with the PSs in the dark for 24 h. Control cells without PS were incubated the same way. Cells were then fixed with ice-cold 60% ethanol, and the plate containing the slips was kept on ice for 10 min. The cells were washed twice with PBS, and the slips were transferred to microscopic slides and mounted with Vectashield mounting medium (Vector Laboratories). The slides were examined using a Zeiss LSM 510.
META confocal fluorescence microscope fitted with standard FITC, rhodamine, and DAPI filter sets.

For co-localization experiments, organelle-specific stains were obtained from Invitrogen, Molecular Probes. Mitochondria were visualized with MitoTracker Green FM at 200 nm, lysosomes were stained using LysoSensor Green DND-189 at 3 μM, DIOC6(3), iodide at 1.5 μM was used to visualize the endoplasmic reticulum, and Golgi were stained using BODIPY-Ceramide at 250 nm. The organelle tracers were first dissolved in dimethyl sulfoxide to obtain 1 mM stock solutions, except for lyso sensor which was already 1 mM in dimethyl sulfoxide and BODIPY-Ceramide that was dissolved in sterile deionized water to reconstitute the ready-made Ceramide-BSA (bovine serum albumin) complex. The tracers were then diluted to water before fixation, and tracers were added 30 min before fixing, washing, and subsequent to fixation, and Golgi were stained using BODIPY-Ceramide at 250 nm. The tracers were then dissolved in dimethyl sulfoxide to obtain 1 mM stock solutions, except for lysosensor which was already 1 mM in dimethyl sulfoxide and BODIPY-Ceramide that was dissolved in sterile deionized water to reconstitute the ready-made Ceramide-BSA (bovine serum albumin) complex. The tracers were then diluted to water before fixation, and tracers were added 30 min before fixing. Images were acquired with a Zeiss AxioCam HCc camera fitted to the microscope.

**Statistical Analysis**

Each experiment was repeated at least three times. Mean values ± S.E. were used for statistical analysis of the data with Student’s t test. A p value ≤ 0.05 was accepted as statistically significant.

**RESULTS**

**Lipophilicity**—The lipophilicity of the ortho, meta, and para isomeric Zn porphyrins was evaluated by measurement of a chromatographic retention factor, Rf (Fig. 2A) and a partition coefficient between n-octanol and water, log P_{ow} (Fig. 2B). As expected, both Rf and log P_{ow} showed that lipophilicity increases upon the relocation of the nitrogen atom from ortho to meta to para positions in the pyridyl rings; lipophilicity of the compounds follows the order: ZnTnHex-4-PyP > ZnTnHex-3-PyP > ZnTnHex-2-PyP. A similar order was established earlier for a series of Mn(III) N-alkylpyridylporphyrins (23). A linear relationship between Rf and log P_{ow}, shown for a series of metal-free N-alkylpyridylporphyrin and their Mn(III) complexes (23), is valid for the ortho, meta, and para isomeric Zn(II) meso-tetakis(N-hexylpyridyl)porphyrins (Fig. 2C). The small differences in Rf values translate into large differences in log P_{ow} values.

**Phototoxicity of Zn(II) N-Alkylpyridylporphyrins**—Fig. 3A compares the phototoxicity of the most hydrophilic (ZnTMPyP) and the most amphiphilic-lipophilic (ZnTnHex-PyP) PSs explored in this study. It is notable that all isomers of the amphiphilic ZnTnHexPyP were more efficient in suppressing MTT reduction than the hydrophilic ZnTMPyP isomers. The higher efficiency of the lipophilic PSs is most apparent at the lowest concentration of 5 μM. The figure also demonstrates differences among ortho, meta, and para isomers; for each of the PSs tested, efficiency followed the order ortho < meta < para.

Because illumination was performed after the medium containing exogenous PS was removed (and replaced by PBS), the phototoxicity efficiency will depend on PSs present within the cells. The more efficient the uptake, the shorter the time needed to accumulate the PS to a photocytotoxic concentration. Fig. 3B demonstrates that for LS174T cells, 6 h of preincubation was sufficient for all ZnTnHexPyP isomers to achieve their highest photodynamic effect whereas methyl derivatives showed increased effects with more time. This is particularly so when identical experiments were performed with MCF7 cells (Fig. 3C). These results suggest that cellular uptake of the amphiphilic Zn porphyrins is much faster than that of more hydrophilic analogs and that equilibrium is achieved within 6 h irrespective of the cell type. Under parallel experimental conditions, MCF7 cells loaded with ZnTMPyP or ZnTnHexPyP were more sensitive to PDT than LS174T cells; however, the different alkyl derivatives showed the same order of PS effectiveness.

**Cell Proliferation**—Reduction of MTT to formazan is an enzymatic process that depends on the availability of NAD(P)H. Because the MTT assay was performed immediately after illumination, it provided information only on the immediate effects of phototreatment on the metabolic activity of the cells. To investigate the delayed consequences of phototreatment, the ability of the PSs to suppress cell proliferation was studied. At 10 μM all tested compounds completely inhibited cell division (data not shown), a result that did not allow discrimination among different compounds. At 2 μM, clear differences among the ortho, meta, and para ZnTMPyP isomers were observed; their photocytotoxicity followed the order established by the MTT studies, para > meta > ortho (Fig. 3D). The amphiphilic hexyl analogs were much more efficient and completely prevented cell division.
Dark Toxicity—To determine whether the PSs exert light-independent toxicity, the cells were preincubated for 24 h with \(20 \mu M\) PSs, and then the MTT test was performed. None of the methyl isomers caused noticeable suppression of MTT reduction (data not shown). Among the hexyl derivatives, the ortho compound did not show any toxic effect in the dark, but ZnTnHex-3-PyP and ZnTnHex-4-PyP suppressed MTT reduction by approximately 15% compared with nontreated cells.

Cellular Uptake of Zn(II) N-Alkylpyridylporphyrins—Results presented in Fig. 3, B and C, indicate that the amphiphilic ZnTnHexPyP PSs are taken up by cells much more rapidly than their more hydrophilic methyl analogs, which may result in higher intracellular concentrations, particularly at shorter incubation times. This more efficient cellular uptake of the amphiphilic compounds would be an important factor contributing to higher PDT efficacy. Our experiments demonstrated that isomeric ZnTnHexPyP PSs are not only taken up faster by the cells, but also accumulate in cells to much higher concentrations than their methyl analogs. The hydrophilic methyl isomers accumulated intracellularly to 1.25 ± 0.80 \(\mu M\) (ortho), 3.15 ± 1.70 \(\mu M\) (meta), and 4.87 ± 1.90 \(\mu M\) (para); for the amphiphilic hexyl isomers these values were 13.90 ± 5.60 \(\mu M\), 33.40 ± 6.65 \(\mu M\), and 47.60 ± 7.25 \(\mu M\) for ortho, meta, and para isomers, respectively. For each group of compounds, cellular uptake followed the order para > meta > ortho. These results are only an approximate estimate of intracellular ZnP accumulation because of technical limitations of the method used. Cellular content of ZnP was determined from the area under the fluorescence emission peak of cell lysates obtained from cells preincubated for 24 h with the compounds. At lower concentrations of hydrophilic ZnP, the area under the peak was too small to be determined reliably. Therefore, results are shown only for cells preincubated with 20 \(\mu M\) ZnP. The established order for cellular uptake of isomeric Zn porphyrins is in good agreement with their respective lipophilicities, i.e. \(\log P\) and \(R_f\) (Fig. 2).

Subcellular Localization of the ZnP Photosensitizers—Data accumulated so far have pointed to more efficient cellular uptake as an explanation for the higher photoefficiency of the amphiphilic hexyl isomers. In addition, the ortho, meta, and para isomers may localize predominantly in different cellular compartments and thus target more or less sensitive cellular structures due to differences in the three-dimensional shape and lipophilicity of the molecules. To investigate this possibility, subcellular localization of the PSs was determined by confocal microscopy after staining cells with standard fluorescent markers. For clarity of presentation, only selected images demonstrating the most prominent differences in distribution are shown.

Fig. 4 shows fluorescence microscopy images of LS174T cells preincubated for 24 h with ZnP. The untreated controls (A) exhibited faint autofluorescence. All ortho isomers, ZnTM-2-PyP (B), ZnTE-2-PyP (data not shown), ZnTnBu-2-PyP (E), and ZnTnHex-2-PyP (F), demonstrated extranuclear distribution. Meta methyl isomer, ZnTM-3-PyP, was found in both nucleus
and cytoplasm/membranes (C), and accumulation of its para analog was predominantly nuclear (D). For the hydrophilic methyl-substituted isomers, shifting the substituent from ortho to para position strongly affected the subcellular distribution of the PS, shifting it from cytoplasmic to nuclear compartments. The effect of the position of the substituent on the molecule was confirmed by further analyses (Fig. 4I).

For the amphiphilic hexyl derivatives, however, moving the hexyl alkyl chain from ortho to para position did not greatly affect the distribution of the ZnP between cytoplasm and nucleus (Fig. 4, F–H). The subcellular localization of ZnTnHexPyP was independent of the PS concentration over the range 5–20 μM and remained essentially unchanged when the incubation time was varied from 30 min to 24 h (data not shown). These results confirm that cellular uptake of the amphiphilic hexyl derivatives is very fast.

To clarify the relationship between PS structure and subcellular localization, organelle-specific fluorescent probes were used in co-localization experiments. Mitochondria were stained with MitoTracker, lysosomes were visualized with Lysosensor Green, DIOC6(3) iodide was used to visualize the endoplasmic reticulum, and BODIPY-Ceramide was used to stain the Golgi apparatus.

Even though no substantial bleaching of the ZnP was detected under the illumination conditions effective for photocytotoxicity, under the intense light of the microscope the methyl-substituted ZnP bleached very rapidly; this made it difficult to obtain good quality images. To avoid this obstacle, results for similar, but more stable, ethyl analogs are presented. Fig. 5, images A and B, demonstrate that ZnTE-2-PyP localized mainly to the lysosomes and negligibly to endoplasmic reticulum and mitochondria (data not shown). Increasing the length of the alkyl chains at the ortho position with two carbon atoms increased the affinity of the molecule toward mitochondria. The more lipophilic ZnTnBu-2-PyP exhibited partial localization to mitochondria in addition to localizing in lysosomes and endoplasmic reticulum (data not shown).

A further increase in the PS lipophilicity by addition of two carbon atoms (n-butyl to n-hexyl) suppressed localization to lysosomes, but increased accumulation of PSs in mitochondria and PS association with the plasma membrane (Fig. 5C). This tendency is particularly apparent with ZnTnHex-3-PyP (Fig. 5D) and especially with ZnTnHex-4-PyP (Fig. 5, E and F). ZnTnHex-4-PyP was rapidly incorporated into membranous structures, including plasma membrane. These results demonstrate that the subcellular localization of a ZnP is affected not only by the length of the alkyl chains but also by the position of the chains relative to the porphyrin ring.

**DISCUSSION**

Singlet oxygen is regarded as the principal damaging species in photodynamic treatment (26). Its short lifetime (~4 μs in
Effect of Molecular Structure on Subcellular Localization

FIGURE 5. Subcellular localization of ZnPs. A, co-localization of ZnTE-2-PyP with lysosensor. B, co-localization of ZnTE-2-PyP with DIOC6(3) iodide. C, co-localization of ZnTnHex-2-PyP with MitoTracker. D, co-localization of ZnTnHex-3-PyP with MitoTracker. E, co-localization of ZnTnHex-4-PyP with MitoTracker. Cells were incubated with PS for 24 h. F, co-localization of ZnTnHex-4-PyP with MitoTracker. Cells were incubated with PS for 30 min. ZnPs display red fluorescence, and tracers fluoresce in green; overlay is yellow-orange in color.

pure water) (27) implies that the maximum distance it can travel (assuming no reaction with any biomolecule occurs) would be ~125 nm (17). Taking into account the average size of mammalian cells (~10–30 μm) or even of organelles such as mitochondria (~500 nm) (17), it becomes clear that the primary reaction targets of 1O2 would be determined by PS localization. Uptake and subcellular localization of PSs in turn are controlled mainly by the net charge and lipophilicity of the molecule (22). Studies on photo-insensitive MnP analogs have shown that desired lipophilicity can be achieved by adding suitable alkyl substituents at the meso pyridyl positions, without affecting the net positive charge of the molecule (23, 29). Assessment of partitioning between n-octanol and water revealed that each additional carbon atom added to the N-alkyl chains increases lipophilicity by 10-fold. An additional 10-fold increase of lipophilicity can be achieved by moving the N-alkyl groups from the ortho to meta N-pyridyl position (29). Here we demonstrate that a 50-fold increase in the lipophilicity of Zn N-alkylpyridylporphyrins produced by lengthening the side chains from 1 (methyl) to 6 carbons (hexyl), caused ~5-fold increase in the uptake of the PS by the cancer cells. As a result, the amphiphilic hexyl derivatives (ZnTnHexPyP) were more efficient than the more hydrophilic ZnTMPyP derivatives in suppressing cell metabolism (MTT assay) and cell proliferation. In addition, our study disclosed significant differences among the ortho, meta, and para isomers with respect to the photoefficiency. For each of the series, the phototoxicity of the ZnP isomers followed the order para > meta > ortho.

A factor that strongly affects PDT activity is the subcellular localization of the PS (20, 30). The location of the PS determines which cellular targets would be damaged and which cell-response mechanisms would be triggered (18, 30, 31). The effect of specific structural alterations of the Zn N-alkylpyridylporphyrins on subcellular localization was investigated by co-staining with organelle-specific fluorescent markers. These studies demonstrated that the increase in the length of the alkyl chains directed the distribution of the PSs from predominantly lysosomal to mitochondrial sites. For molecules of the same alkyl chain length, the distribution was dependent upon the position of the substituents. Whereas the hydrophilic ortho methyl isomer displayed predominantly cytoplasmic allocation, the para isomer was distributed primarily in the nucleus. A plausible reason for preferential ZnTM-4-PyP nuclear allocation is intercalation in DNA, as already reported for its Mn porphyrin analog (32). For the amphiphilic isomeric ZnTnHexPyP, shifting the location of the alkyl chains from ortho to para positions had quite a different effect. It facilitated accumulation in the mitochondria, and the overall fluorescence signal suggests preferential association with membranes in general. Such differences in the behavior of the ZnP molecules can be more easily understood if their tri-dimensional structure is considered (Fig. 6). The interactions of ZnP molecules with cellular components would be influenced by the spatial orientation of the substituents at the periphery of the porphyrin ring. The higher affinity for mitochondria and membranes, demonstrated by the para hexyl isomer, can be attributed to the particular shape of the PS molecule. The planar and more flexible para ZnTnHex-4-PyP (Fig. 6E) should fit more readily into the spaces among the fatty acid chains of a lipid bilayer than the more rigid and spherical ortho ZnTnHex-2-PyP molecule (Fig. 6). B, D, and C). These molecular properties would facilitate both diffusion across membranes and intercalation of the para isomer in the lipid bilayer (depicted in Fig. 7).

Initially, the positively charged PS will be electrostatically attracted by the predominantly negatively charged membrane...
surfaces. Although all isomers carry the same total tetracationic charge, this interaction may differ from one to another positional and rotational isomer, as the charge is differently distributed across the entire molecular scaffold. This in turn leads to differential solvation of isomers, a property also guided by distinctive hindrance of variously positioned alkyl chains with respect to the porphyrin plane. Changing the position of the alkyl chain, especially in the hexyl analogs, allows charges to be differently exposed and thus become more or less available for interactions with anionic surfaces. With para analogs, all charges are exposed, with likelihood of greater exposure with methyl than with hexyl substituents. Hydrophobicity of hexyl chains suppresses solvation/hydration of the cationic nitrogens (33). With ortho isomers, each of the four atropoisomers will be differently distributed based on the differences in bulkiness, lipophilicity, and exposure of charges (34). Thus, both total charge and charge exposure are critical parameters for ZnP biodistribution as these factors affect not only electrostatic interactions but are also key contributors to lipophilicity. Exposure of charges will coincide with planarity; directional differences for alkyl substituents in different atropoisomers (Fig. 6) will influence charge exposure and molecular shape. Synergistic effects between exposure of charges and bulkiness will modulate membrane interactions through electrostatic and packing constraints and thus strongly affect subcellular accumulation.

In the vicinity of the membrane, hydrophobic interactions will start to have increasing impact; the sum of these forces would depend upon the length and position of the alkyl chains attached at the periphery of the porphyrin. Increasing the length of the alkyl chains increases lipophilicity; and shifting the positions of the chains from ortho to meta, and to para, makes the molecule more planar with exposed positive charges, which could increase favorable electrostatic and van der Waals interactions and decrease packing constraints, consequently favoring binding and submersion of the ZnP into the membrane fluid lipid bilayer. Detailed investigations by Engelmann et al. have
shown that binding to membranes is proportional to the lipophilicity of the compounds, expressed as a partition coefficient between n-octanol and water, log $P_{ow}$ (35). Our study supports their conclusion. Investigations on asymmetric porphyrins revealed that the doubly charged cis-isomers bind more strongly than predicted from their log $P_{ow}$ (36). The authors concluded that the structure of the molecule controls the depth of penetration within lipid membranes and the strength of hydrophobic and hydrophilic interactions. The same authors suggested that PDT efficiency depends not only on the amount of bound porphyrin but is also affected by the PS location within the cell (36). Higher photodynamic efficacy of PSs which can intercalate into the lipid bilayer may be attributed to higher solubility and higher concentration of O$_2$ in lipids, where the excited state of the PS has higher chances to interact with oxygen to generate 1O$_2$ (37). In turn, singlet oxygen, generated within the hydrophobic interior, has a greater opportunity to react with unsaturated fatty acids thus initiating self-propagating lipid peroxidation chain reactions. In contrast, if the PS is weakly attracted by the membrane and stays at its surface, the excited state would be exposed to lower aqueous oxygen concentrations, and a larger fraction of the total 1O$_2$ will become neutralized before damaging membrane components (36).

Cellular components and organelles vary with respect to PDT sensitivity. As noted above, the hydrophilic ZnTM-2-PyP displayed predominantly lysosomal localization. Photodamage of lysosomes can release lysosomal hydrolytic enzymes thus causing cell death, but the relative efficacy of lysosomal PSs is lower than that of PSs localized in mitochondria or the plasma membrane (18, 38 – 41). A possible reason for lower activity of PS directed to liposome is photoinactivation of lysosomal enzymes and/or their inhibition by cytosolic inhibitors (28).

Knowledge obtained with fluorescent Zn porphyrin photosensitizers will help in the development of related families of alkylmetalloporphyrins with similar structures but different activities. The Zn porphyrins act as potent photosensitizers, but replacement of the chelated metal gives compounds capable of rapid redox cycling, superoxide dismutase-mimetic function, and compounds that show promise as fluorescent imaging agents. Compounds with very similar or identical molecular dimensions and very similar physical properties, e.g. lipophilicity, will show strong similarities in cellular and subcellular accumulation and distribution. Study of localized photochemical modification actions of these Zn analogs thus generates a body of knowledge of much broader significance with a wide potential range of applications for investigation and manipulation of cell responses and signal pathways, including induction of apoptotic and necrotic cytotoxic responses, in scientific investigations and future therapeutic applications.

CONCLUDING REMARKS

The main features of the metalloalkylpyridylporphyrin PS molecule, which determine its uptake and subcellular allocation and in turn the PDT efficiency, are: (i) total charge and exposure of charges to support electrostatic interactions with biomolecules and cell structures; (ii) lipophilicity; and (iii) tridimensional shape of the molecule. Such data could be utilized in design of metalloporphyrin molecules to specifically target particular cellular compartments.

Acknowledgments—We thank Professor C. Ford for providing the cell lines used in this study and Milini Thomas and Fatima Sequeira for excellent technical assistance.

REFERENCES

1. Dougherty, T. J., Gomer, C. J., Henderson, B. W., Jori, G., Kessel, D., Korbek, M., Moan, J., and Peng, Q. (1998) Photodynamic therapy. J. Natl. Cancer Inst. 90, 889–905
2. Babillas, P., Schreml, S., Landthaler, M., and Szeimies, R. (2010) Photodynamic therapy in dermatology: state-of-the-art. Photodermatol. Photoimmunol. Photomed. 26, 118–132
3. Lee, Y., and Baron, E. D. (2011) Photodynamic therapy: current evidence and applications in dermatology. Semin. Cutan. Med. Surg. 30, 199–209
4. Silva, J. N., Filipe, P., Morlière, P., Mazière, J. C., Freitas, J. P., Gomes, M. M., and Santos, R. (2008) Photodynamic therapy: dermatology and ophthalmology as main fields of current applications in clinic. BioMed. Mater. Eng. 18, 319–327
Effect of Molecular Structure on Subcellular Localization

5. Michels, S., and Schmidt-Erfurth, U. (2001) Photodynamic therapy with verteporfin: a new treatment in ophthalmology. *Semin. Ophthalmol.* 16, 201–206

6. Meisel, P., and Kocher, T. (2005) Photodynamic therapy for periodontal diseases: state of the art. *J. Photochem. Photobiol. B* 79, 159–170

7. Konopka, K., and Goslinski, T. (2007) Photodynamic therapy in dentistry. *J. Dental Res.* 86, 694–707

8. Waksman, R., McEwan, P. E., Moore, T. I., Pakala, R., Kolodgie, F. D., Hellinga, D. G., Seabron, R. C., Rychnovsky, S. J., Vasek, J., Scott, R. W., and Virmani, R. (2008) PhotoPoynt photodynamic therapy promotes stabilization of atherosclerotic plaques and inhibits plaque progression. *J. Am. Coll. Cardiol.* 52, 1024–1032

9. Woodburn, K. W., Fan, Q., Kessel, D., Wright, M., Mody, T. D., Hemmi, G., Magda, D., Sessler, J. L., Dow, W. C., Miller, R. A., and Young, S. W. (1996) Phototherapy of cancer and atheromatous plaque with tetracycline. *J. Clin. Laser Med. Surg.* 14, 343–348

10. Szeimies, R. M., Lischner, S., Philipp-Dormston, W., Walker, T., Heipe-Wegener, D., Feise, K., Poddà, M., Prager, W., Kohl, E., and Karrer, S. (2013) Photodynamic therapy for skin rejuvenation: treatment options. Results of a consensus conference of an expert group for aesthetic photodynamic therapy. *J. Germ. Soc. Dermatol.* 11, 632–637

11. Taub, A. F. (2012) Cosmetic clinical indications for photodynamic therapy. *Cosmetic Dermatol.* 25, 218–224

12. Wainwright, M. (2002) Pathogen inactivation in blood products. *Curr. Med. Chem.* 9, 127–143

13. Bonnett, R., Krysteva, M. A., Lalov, I. G., and Artarsky, S. V. (2006) Water disinfection using photosensitizers immobilized on chitosan. *Water Res.* 40, 1269–1275

14. Magaragga, M., Faccenda, F., Gandolfi, A., and Jori, G. (2006) Treatment of microbiologically polluted aquaculture waters by a novel photochemical technique of potentially low environmental impact. *J. Environ. Monit.* 8, 923–931

15. Foote, C. S. (1991) Definition of type I and type II photosensitized oxidations. *Photochem. Photobiol.* 54, 659

16. Ogilby, P. R. (2010) Singlet oxygen: there is indeed something new under the sun. *Chem. Soc. Rev.* 39, 3181–3209

17. Redmond, R. W., and Kocheven, I. E. (2006) Spatially resolved cellular responses to singlet oxygen. *Photochem. Photobiol.* 82, 1178–1186

18. Buntett, E., Dewaele, M., and Agostinis, P. (2007) Molecular effectors of multiple cell death pathways initiated by photodynamic therapy. *Biochim. Biophys. Acta* 1776, 86–107

19. Jensen, T. J., Vicente, M. G., Luguya, R., Norton, I., Franckez, F. R., and Smith, K. M. (2010) Effect of overall charge and charge distribution on cellular uptake, distribution and phototoxicity of cationic porphyrins in HEp2 cells. *J. Photochem. Photobiol. B* 100, 101–111

20. Lei, W., Xie, J., Hou, Y., Jiang, G., Zhang, H., Wang, P., Wang, X., and Zhang, B. (2010) Mitochondria-targeting properties and photodynamic activities of porphyrin derivatives bearing cationic pendant. *J. Photochem. Photobiol. B* 98, 167–171

21. Boyle, R. W., and Dolphin, D. (1996) Structure and biodistribution relationships of photodynamic sensitizers. *Photochem. Photobiol.* 64, 469–485

22. Benov, L., Craik, J., and Batinic-Haberle, I. (2011) The potential of Zn(II) N-alkylpyridylporphyrins for anticancer therapy. *Anticancer Agents Med. Chem.* 11, 233–241

23. Kos, I., Reboçaš, J. S., DeFreitas-Silva, G., Salvemini, D., Vujaskovic, Z., Dewhurst, M. W., Spasojevic, I., and Batinic-Haberle, I. (2009) Lipophilicity of potent porphyrin-based antioxidants: comparison of ortho and meta isomers of Mn(III) N-alkylpyridylporphyrins. *Free Radic. Biol. Med.* 47, 72–78

24. Berridge, M. V., Herst, P. M., and Tan, A. S. (2005) Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnol. Annu. Rev.* 11, 127–152

25. Skelan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S., and Boyd, M. R. (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.* 82, 1107–1112

26. Davies, M. J. (2004) Reactive species formed on proteins exposed to singlet oxygen. *Photochem. Photobiol. Sci.* 3, 17–25

27. Rodgers, M. A., and Snowden, P. T. (1982) Lifetime of O2(1Δg) in liquid water as determined by time-resolved infrared luminescence measurements. *J. Am. Chem. Soc.* 104, 5541–5543

28. Oleinick, N. L., and Evans, H. H. (1998) The photobiology of photodynamic therapy: cellular targets and mechanisms. *Rad. Res.* 150, S146–S156

29. Konopka, K., and Goslinski, T. (2007) Photodynamic therapy in dentistry. *J. Dental Res.* 86, 694–707

30. Castano, A. P., Demidova, T. N., and Hamblin, M. R. (2004) Mechanisms in photodynamic therapy. Part one: photosensitizers, photochemistry and cellular localization. *Photoimagery. Photodyn. Ther.* 1, 279–293

31. Kramer-Marek, G., Serpa, C., Szurko, A., Widel, M., Sochanik, A., Sinetura, M., Kus, M., Nunes, R. M., Arnaut, L. G., and Ratuszna, A. (2006) Spectroscopic properties and photodynamic effects of new lipophilic porphyrin derivatives: efficacy, localisation and cell death pathways. *J. Photochem. Photobiol. B* 84, 1–14

32. Faulkner, K. M., Liochev, S. I., and Fridovich, I. (1994) Stable Mn(III) porphyrin mimics superoxide dismutase in vitro and substitute for it in vivo. *J. Biol. Chem.* 269, 23471–23476

33. Batinic-Haberle, I., Spasojevic, I., Stevens, R. D., Hambricht, P., and Fridovich, I. (2002) Manganese(III) meso-tetakis(ortho-N-alkylpyridyl)porphyrins. synthesis, characterization, and catalysis of O2− dismutation. *Dalton Trans.* 2689–2696

34. Spasojevic, I., Menezelev, R., White, P. S., and Fridovich, I. (2002) Rotational isomers of N-alkylpyridylporphyrins and their metal complexes: HPLC separation, 1H NMR, and x-ray structural characterization, electrochemistry, and catalysis of O2− disproportionation. *Inorg. Chem.* 41, 5874–5881

35. Engelmann, F. M., Rocha, S. V., Toma, H. E., Araki, K., and Baptista, M. S. (2007) Determination of n-octanol/water partition and membrane binding of cationic porphyrins. *Int. J. Pharm.* 329, 12–18

36. Engelmann, F. M., Mayer, I., Gabrielli, D. S., Toma, H. E., Kowaltowski, A. J., Araki, K., and Baptista, M. S. (2007) Interaction of cationic meso- porphyrins with liposomes, mitochondria and erythrocytes. *J. Bioenerg. Biomembr.* 39, 175–185

37. Ehrenberg, B., Anderson, J. L., and Foote, C. S. (1998) Kinetics and yield of singlet oxygen photosensitized by hypericin in organic and biological media. *Photochem. Photobiol.* 68, 135–140

38. Castano, A. P., Demidova, T. N., and Hamblin, M. R. (2005) Mechanisms in photodynamic therapy. Part two: cellular signaling, cell metabolism and modes of cell death. *Photoimagery. Photodyn. Ther.* 2, 1–23

39. Ricchelli, F., Franchi, L. M., Miotti, G., Borsetto, L., Gobbo, S., Nikolov, P., Bommer, J. C., and Reddi, E. (2005) Meso-substituted tetra-cationic porphyrins photosensitize the death of human fibrosarcoma cells via lysosomal targeting. *Int. J. Biochem.* 37, 306–319

40. Agostinis, P., Buitaert, E., Breuysens, H., and Hendrickx, N. (2004) Regulatory pathways in photodynamic therapy-induced apoptosis. *Photochem. Photobiol. Sci.* 3, 721–729

41. Oleinick, N. L., Morrise, R. L., and Belichenko, I. (2002) The role of apopto- sis in response to photodynamic therapy: what, where, why, and how. *Photochem. Photobiol. Sci.* 1, 1–21