A Comparative Study of the Cellular Uptake and Photodynamic Efficacy of Three Novel Zinc Phthalocyanines of Differing Charge

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ABSTRACT

Three novel substituted zinc (II) phthalocyanines (one anionic, one cationic and one neutral) were compared to two clinically used photosensitizers, 5,10,15,20-tetra(m-hydroxyphenyl)chlorin (m-THPC) and polyhematoporphyrin (PHP), as potential agents for photodynamic therapy (PDT). Using the RIF-1 cell line, photodynamic efficacy was shown to be related to cellular uptake. The cationic phthalocyanine (PPC, pyridinium zinc [II] phthalocyanine) had improved activity over the other two phthalocyanines and slightly improved activity over PHP and m-THPC. The initial subcellular localization of each photosensitizer was dependent upon the hydrophobicity and plasma protein binding. The phthalocyanines had a punctate distribution indicative of lysosomes, whereas m-THPC and PHP had a more diffuse cytoplasmic localization. A relocalization of phthalocyanine fluorescence was observed in some cases following low-level light exposure, and this was charge dependent. The anionic phthalocyanine (TGly, tetracylglycine zinc [II] phthalocyanine) relocalized to the nuclear area, the localization of the hydrophobic phthalocyanine (TDOPC, tetraiodotyramine zinc [II] phthalocyanine) was unchanged, whereas the distribution of the cationic phthalocyanine (PPC) became more cytoplasmic. This suggests that relocalization following low-level irradiation is a critical factor governing efficacy, and a diffuse cytoplasmic distribution may be a determinant of good photodynamic activity.

INTRODUCTION

Photodynamic therapy (PDT) is a treatment for cancer and other diseases, involving the administration of a photosensitizing compound that, when activated by light, results in tumor destruction via the production of active oxygen species, particularly singlet oxygen (\(\text{O}_2\)) (1). The main clinical photosensitizer is a porphyrin-based compound referred to as Photofrin\(^\text{R}\), consisting of a mixture of porphyrin oligomers (2). Other photosensitizers that have been used experimentally are phthalocyanines (3) and chlorins (4). In homogeneous solution, the factors affecting the efficiency of photosensitization include: the concentration of both oxygen and sensitizer, the reaction rate of the excited sensitizer with oxygen and target molecules and the rate of sensitizer triplet decay (5). It is well established that the efficiency of photosensitizers is reduced upon aggregation (6) and we have recently shown this to be the case for 5,10,15,20-tetra(m-hydroxyphenyl) chlorin (m-THPC), polyhematoporphyrin (PHP, a compound identical to Photofrin\(^\text{R}\)) and the three novel zinc (II) phthalocyanines used in the current study (7).

In the more complex environment of a cell, these factors are further complicated by the subcellular localization of the sensitizer and by interactions with cellular material that can affect the aggregation state of photosensitizers (8). In vivo, further levels of complexity exist as damage may occur to tumor cells and/or the tumor vasculature depending on the sensitizer used (9).

The lifetime and diffusion of \(\text{O}_2\) in a cellular environment is limited by its reactivity with cellular components, and damage is reported to occur close to the site of generation (10,11). Therefore, many recent reports have concentrated on the subcellular localization of sensitizers in an attempt to identify potential cellular targets. In \(\text{in vitro}\) studies suggest that the more hydrophobic sensitizers target cellular membranes, implicating membrane damage as a primary mechanism of cell death by PDT (8), with internal membranes becoming more sensitive following prolonged incubation (12,13). Cationic compounds appear to have selective affinity for mitochondria (14), lysosomes (15) and membranes (16), whereas lysosomes appear to be the primary target for tetrasubstituted anionic sensitizers (14). However, light-induced relocalization of some sensitizers suggests that the initial site of localization therapy; PHP, polyhematoporphyrin; PPC, pyridinium zinc (II) phthalocyanine; RME, receptor-mediated endocytosis; TAB, cetyltrimethylammonium bromide; TDOPC, tetraiodotyramine zinc (II) phthalocyanine; TGly, tetracylglycine zinc (II) phthalocyanine.

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calization may not be as important to cell damage and/or repair as secondary sites (17,18).

A causal relationship between sensitizer structure, uptake, localization and hence cell death has been postulated (19-25). In vitro studies have largely been directed toward structure–function relationships between differentially substituted porphyrins and phthalocyanines. In general, it appears that lipophilic compounds are taken up more readily through the plasma membrane than hydrophilic sensitzers, and in most cases this is accompanied by an increased photocytotoxicity. However, additional factors are likely to be involved because the uptake of some phthalocyanines is reported to be greater than porphyrins of similar lipophilicity (26), and the arrangement of peripheral substituents has been shown to affect cellular uptake (24,27).

We have previously characterized the five photosensitizers with respect to aggregation and photoactivity using amino acid oxidation and red blood cell hemolysis as simple systems (7). Here hydrophobicity and charge were the major determinants of efficacy. The cationic phthalocyanine (PPC, pyridinium zinc [II] phthalocyanine), but not the anionic (TGly, tetraglycine zinc [II] phthalocyanine) or the neutral (TDOPc, tetracytiamine zinc [II] phthalocyanine) phthalocyanine, showed slightly improved activity over the clinical photosensitizers, m-THPC and PHP.

In this study, we have used fluorescence microscopy to study the subcellular localization of these novel phthalocyanines, together with that of m-THPC and PHP, in the RIF-1 cell line. These observations, before and after low light exposure, have been related to photodynamic activity in an attempt to determine whether efficiency can be predicted by a particular localization pattern.

MATERIALS AND METHODS

Photosensitizer.s. The three zinc (II) phthalocyanines, TGly, TDOPc and PPC, were prepared by Mr. J. Schofield, Department of Colour Chemistry, Centre for Photobiology and Photodynamic Therapy, University of Leeds. The TGly and TDOPc are tetrastituted (J. Schofield, personal communication). Hydroxyethylphosphonate zinc (TGly, tetraglycine zinc [II] phthalocyanine) or the neutral (TDOPc, tetracytiamine zinc [II] phthalocyanine) phthalocyanine, showed slightly improved activity over the clinical photosensitizers, m-THPC and PHP.

In this study, we have used fluorescence microscopy to study the subcellular localization of these novel phthalocyanines, together with that of m-THPC and PHP, in the RIF-1 cell line. These observations, before and after low light exposure, have been related to photodynamic activity in an attempt to determine whether efficiency can be predicted by a particular localization pattern.

MATERIALS AND METHODS

Photosensitzers. The three zinc (II) phthalocyanines, TGly, TDOPc and PPC, were prepared by Mr. J. Schofield, Department of Colour Chemistry, Centre for Photobiology and Photodynamic Therapy, University of Leeds. The TGly and TDOPc are tetrastituted (J. Schofield, personal communication) while PPC is reported to have an average tetrastituted configuration corresponding to the dication (28). The PPC was supplied by Scotia QuantaNova (Guildford, Surrey) and solutions were prepared fresh each day according to the manufacturer’s guidelines in polyethylene glycol/ethanol/water (2:3:5 vol/vol).

Plasma protein binding. Plasma binding of phthalocyanines (0.1 mg mL-1) was determined by density-gradient ultracentrifugation according to the method of Havel et al. (29) using a gradient of KBr/NaCl prepared according to Redgrave et al. (30). The protein and sensitizer content of each fraction was determined by absorbance at 280 nm and 680 nm, respectively.

Cell culture. The cell line used was a RIF-1 murine fibrosarcoma kindly donated by Dr. J. Bremner, MRC Radiobiology Unit, Harwell, Oxford), maintained in a culture medium of RPMI supplemented with 10% fetal calf serum, 1% glutamine (200 mM) and 0.1% gentamycin (10 mg mL-1).

Sensitizer uptake into RIF-1 cells. Cells were plated in 35 mm petri dishes at a density of 2 x 10^5 cells per dish. A period of 18–24 h was allowed for attachment and growth after which the culture medium was removed and replaced with fresh medium containing sensitiser. The cellular uptake of each sensitizer as a function of extracellular concentration was determined over a 2 h incubation period at 37°C in the dark following serial dilution in medium from a 1 mg mL-1 stock solution.

Following incubation, the medium containing sensitizer was removed and the monolayers were washed three times with cold PBS. The cells were then dissolved by the addition of 0.1 M sodium hydroxide. The protein content of each sample was determined by the method of Bradford (31) using the Bio-Rad protein assay. Quantification of sensitizer was achieved as described below, by comparison to known standards. The amount of cell-associated sensitizer was expressed as nmole per gram cellular protein.

TGly analysis. Sensitizer content was analyzed by fluorescence (λex = 614 nm, λem = 684 nm) following dilution in detergent (50 mM N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid] [HEPES], 10 mM cetyltrimethylammonium bromide [CTAB], pH 7.4).

TDOPc analysis. Cell homogenates were diluted with 50 mM HEPES, 10 mM CTAB, pH 7.4 (1:4). The TDOPc was extracted by the addition of ethyl acetate/acetone (4:1 vol/vol) and centrifugation (2000 g, 5 min). The volume of the upper organic layer was measured and the amount of cell-associated sensitizer determined by fluorescence (λex = 612 nm, λem = 682 nm).

PPC analysis. Cell homogenates were diluted 1:4 with the ion-pairing reagent, 1-hexane sulfonic acid (HSA) (50 mg mL-1). The PPC was extracted by the addition of butan-1-ol and centrifugation (2000 g, 5 min). The volume of the upper organic layer was measured and the amount of cell-associated sensitizer determined by fluorescence (λex = 418 nm, λem = 652 nm).

m-THPC analysis. Cell homogenates were diluted with 50 mM HEPES, 10 mM CTAB, pH 7.4 (1:4) and PPC was extracted by the addition of ethyl acetate/acetone (4:1 vol/vol) and centrifugation (2000 g, 5 min). The organic layer was removed into a clean centrifuge tube containing an equal volume of 1 M HCl. After mixing, the tubes were re-centrifuged (2000 g, 5 min). The upper organic layer was discarded and the lower acid layer was boiled for 30 min to allow hydrolysis of ester and other linkages, thereby increasing the amount of fluorescent monomeric material present (32). Following boiling the samples were allowed to cool, the volume measured and porphyrin content was determined by fluorescence (λex = 400 nm, λem = 595 nm).

Subcellular localization and relocalization of photosensitizers. Cells were seeded onto glass coverslips (22 x 22 mm) at a density of 1 x 10^5 cells per dish. Sensitizer (10 μM phthalocyanine, 1 μM PHP, 1 μM m-THPC) was added in normal growth medium for 2 h at 37°C. Following incubation, the medium containing sensitizer was removed and the monolayers washed three times with PBS (37°C). The cells on coverslips were then inverted onto a specialized chamber slide containing fresh medium (37°C). Sensitizer fluorescence was monitored using a Leitz Dialux 22 upright microscope equipped for epifluorescence illumination. The excitation source for PHP and the phthalocyanines was a HeNe laser (632.8 nm, 4 mW); the excitation light for m-THPC was a filtered compact arc lamp providing light centered on 540 ± 25 nm producing 300 mW at the end of a 3 mm liquid light guide. Emitted light was isolated using interference filters (580-750 nm). Images for PHP and the phthalocyanines were captured, following a 1 min exposure, on a liquid nitrogen-cooled charge-coupled device camera (AstroMed). For m-THPC an exposure of 5 ms was sufficient. The light dose at the cell surface was estimated to be in the region of 0.1-0.5 mW cm^-2 (6-30 mJ cm^-2). Relocalization of sensitizer following excitation was determined by a series of light exposures, with each exposure corresponding to the capture of one image. Captured images were processed using Visilog ImagePlus software.

Identification of cellular organelles. Organelles were identified using vital stains as described previously (18).

Photocytotoxicity experiments. Cells were plated in 96 well plates for 18-24 h at a density of 1 x 10^4 cells per well for attachment and growth. Sensitizer was then added at a range of concentrations for 2 h. Following incubation, the cells were washed three times with PBS and then fresh medium was added. Illumination was performed using a 10 W copper vapor–pumped tunable dye laser (Oxford lasers, UK), the light from which was focused into a 600 μm diameter core optical fiber. The distal output was collected and collimated into a 36 mm diameter beam. The power experienced by...
Table 1. Hydrophobicity, plasma protein binding and intracellular levels (nmol sensitizer/g cell protein) of photosensitizer in RIF-1 cells

<table>
<thead>
<tr>
<th>Sensitizer</th>
<th>Hydrophobicity</th>
<th>Major plasma protein binding</th>
<th>Intracellular concentration (nmol g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGly</td>
<td>Hydrophilic</td>
<td>Albumin</td>
<td>56 ± 2</td>
</tr>
<tr>
<td>TDOc</td>
<td>Hydrophilic</td>
<td>LDL</td>
<td>237 ± 21</td>
</tr>
<tr>
<td>PPC</td>
<td>Hydrophilic</td>
<td>Albumin</td>
<td>1127 ± 35</td>
</tr>
<tr>
<td>m-THPC</td>
<td>Hydrophilic</td>
<td>Lipoproteins (36)</td>
<td>469 ± 12</td>
</tr>
<tr>
<td>PHP</td>
<td>Amphphilic</td>
<td>Lipoproteins (37)</td>
<td>583 ± 19</td>
</tr>
</tbody>
</table>

*Extinction coefficients were calculated in methanol at 680 nm (phthalocyanines), 652 nm (m-THPC) and 630 nm (PHP). Hydrophobicity was determined by octanol buffer partition experiments as previously described (7). Intracellular concentration was determined as described in the text following a 2 h incubation with sensitizer (10 μM). Experiments were performed at least three times with triplicate points per experiment.

The hydrophobicity of each sensitizer was determined by octanol buffer partition experiments as described previously (7). The hydrophilic sensitizers were found exclusively in the octanol phase while the hydrophobic sensitizers were found in the octanol phase (Table 1). The distribution of PHP was dependent upon pH, but at pH 7 there was an approximately equal amount in the octanol and buffer phase. Extinction coefficients for each sensitizer are also given in Table 1.

Plasma protein binding

The binding of each photosensitizer to the components in human plasma correlated to the hydrophobicity (Table 1). The hydrophilic sensitizers bound to albumin, the hydrophobic neutral phthalocyanine bound exclusively to low-density lipoproteins (LDL), and both m-THPC and PHP have been reported to distribute among the high-density lipoproteins, LDL and very low-density lipoproteins (36,37).

Cellular uptake studies

The time dependence of uptake was studied for each sensitizer and the results are given in Table 2. The relative amounts of each sensitizer taken up by the cells showed considerable variation. For this set of data, the extracellular concentrations are expressed in molar terms for comparative purposes and, although this is not consistent for each sensitizer (because experiments were performed with the concentration in μM and because of variation in molecular weights), it is nevertheless clear that the intracellular concentration continues to increase at least up to the 24 h incubation time. For further studies, a 2 h incubation was chosen. This proved to give good discrimination between the sensitizers in terms of intracellular concentration, localization and PDT effect. Cellular uptake at 2 h, as a function of extracellular concentration, was dose dependent for each sensitizer, as shown in Fig. 1. In order to determine the relative uptake on an equimolar basis, the sensitizers were each studied at an extracellular concentration of 10 μM. The order of intracellular concentration was in the sequence PPC > PHP > m-THPC > TDOc > TGly (Table 1).

Cytotoxicity and photocytotoxicity in the RIF-1 cell line

No dark toxicity was observed with any of the photosensitizers at concentrations used. To correlate cell uptake studies with PDT effect a 2 h incubation was chosen. For each sensitizer cells were incubated with sensitizer at a range of concentrations and illuminated using laser light (Fig. 2). The extracellular concentration required to produce 50% cell kill, as determined by a 50% decrease in MTT reduction (LD₅₀ex, value), was thus calculated, and the corresponding intracellular concentrations at each LD₅₀ex (LD₅₀int values) were determined from separate cell uptake curves constructed over the relevant concentration range (Table 3). Generally, an increase in the concentration of extracellular sensitizer was accompanied by an increase in PDT effect at a given light dose. From a plot of the LD₅₀int versus light dose

Table 2. Intracellular sensitizer levels (nmol g⁻¹) in RIF-1 cells following different incubation times

<table>
<thead>
<tr>
<th>Sensitizer</th>
<th>μM</th>
<th>2 h</th>
<th>8 h</th>
<th>12 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGly</td>
<td>7.2</td>
<td>60 ± 8</td>
<td>88.5 ± 4</td>
<td>119 ± 9</td>
<td>159 ± 11</td>
</tr>
<tr>
<td>TDOc</td>
<td>5.6</td>
<td>163 ± 8</td>
<td>251 ± 23</td>
<td>353 ± 39</td>
<td>501 ± 80</td>
</tr>
<tr>
<td>PPC</td>
<td>10</td>
<td>1127 ± 35</td>
<td>1858 ± 62</td>
<td>2918 ± 312</td>
<td>3348 ± 213</td>
</tr>
<tr>
<td>m-THPC</td>
<td>5.87</td>
<td>290 ± 24</td>
<td>547 ± 16</td>
<td>871 ± 183</td>
<td>1766 ± 63</td>
</tr>
<tr>
<td>PHP</td>
<td>16.66</td>
<td>687 ± 45</td>
<td>820 ± 49</td>
<td>983 ± 69</td>
<td>1364 ± 49</td>
</tr>
</tbody>
</table>

*Each value represents the mean of three experiments (± standard deviation).
Figure 1. The RIF-1 cells were incubated with (a) TGly, (b) TDOPc, (c) PPC, (d) m-THPC and (e) PHP for 2 h in the dark at 37°C. Intracellular concentration was determined by extraction procedures as described in the Materials and Methods. Results are expressed as nmol sensitizer per gram cell protein. Points represent the mean of three experiment (± standard deviation).

(Fig. 3), it can be seen that the intracellular concentration of PHP was much higher than that of the other photosensitizers, whereas relatively less PPC or m-THPC was required to give the same effect.

Subcellular localization and relocalization of sensitizers

The localization of each sensitizer is given in Fig. 4a–e. Cells incubated with the phthalocyanines gave a punctate staining pattern similar to that of the lysotrophic dye, acridine orange (18). The PHP and m-THPC did not appear to target any particular organelle, with a diffuse fluorescence observed throughout the cell. The similar staining pattern to that of dihexaaxacarbocyanine iodide (18) suggests that these sensitizers have a general affinity for lipophilic membranes. There was also a greater intensity of staining in the perinuclear region suggestive of a more specific localization, possibly the Golgi.

Light exposure of cells at a light dose 1000-fold lower than that used for photocytotoxicity experiments resulted in a relocalization of fluorescence that was sensitizer dependent (Fig. 4a–e'). The most pronounced effect was observed with

Figure 2. The RIF-1 cells were incubated with (a) TGly, (b) TDOPc, (c) PPC, (d) m-THPC and (e) PHP for 2 h and then illuminated using laser light (10–40 J cm⁻²). Loss in mitochondrial dehydrogenase activity was determined 24 h later by the MTI assay. Light doses were 0 (●), 10 (●), 20 (●) and 40 (□) J cm⁻².

Table 3. The extracellular concentration of sensitizer required to give 50% cell kill (LD₅₀ values) for each sensitizer following 20 J cm⁻² laser light illumination*

<table>
<thead>
<tr>
<th>Sensitizer</th>
<th>LD₅₀ex (µM)</th>
<th>LD₅₀ext (nmol g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGly</td>
<td>2.1 ± 0.05</td>
<td>15.6 ± 1.2</td>
</tr>
<tr>
<td>TDOPc</td>
<td>2.6 ± 0.04</td>
<td>59 ± 2.5</td>
</tr>
<tr>
<td>PPC</td>
<td>0.028 ± 0.001</td>
<td>2.6 ± 0.45</td>
</tr>
<tr>
<td>m-THPC</td>
<td>0.12 ± 0.002</td>
<td>3.6 ± 0.26</td>
</tr>
<tr>
<td>PHP</td>
<td>2.5 ± 0.1</td>
<td>141 ± 11</td>
</tr>
</tbody>
</table>

*Intracellular concentrations corresponding to each extracellular concentration (LD₅₀ values) were calculated from cell uptake curves prepared over the relevant concentration range. Values are given ± standard deviation where n = 3–6.
The RIF-1 cells were incubated with TGly (Δ), TDOPc (■), PPC (●), m-THPC (+) and PHP (○) for 2 h and then illuminated using laser light (10–40 J/cm²). The concentration of sensitizer resulting in 50% cell kill was calculated for each light dose and the corresponding intracellular concentration (LD₅₀) calculated from cell uptake experiments. Each experiment was performed at least three times. Insert shows the data for PPC and m-THPC on an expanded scale.

TGly: the initial punctate fluorescence disappeared following three 1 min exposures producing a much more diffuse staining pattern that was most intense in the nuclear area. This relocalization of sensitizer was accompanied by a dramatic increase in fluorescence intensity. At this time the cells were still flat in appearance and did not show any morphological changes until exposed to a further 9 min of light, when some blebbing of the plasma membrane started to develop (Fig. 4a'). Despite the similar initial localization pattern, light exposure of cells incubated with TDOPc was not accompanied by a relocalization of fluorescence until morphological changes occurred (Fig. 4b'). Light exposure of cells incubated with PPC resulted in a more diffuse fluorescence distribution and a slight increase in fluorescence intensity (Fig. 4c'). The PHP and m-THPC gave a diffuse fluorescence throughout the cell with membranes appearing the major target (Fig. 4d, e). Successive light exposure resulted in a decrease in the fluorescence intensity (photobleaching) rather than any relocalization (Fig. 4d', e').

**DISCUSSION**

Following the initial use of Photofrin® and PHP, a number of second-generation photosensitizers have been developed that absorb further in the red (3,4). Many of these new compounds have been peripherally substituted to change the overall charge and hydrophobicity, which will in turn affect cell uptake and localization (19,21,23–25). Studies of these compounds are important for understanding possible mech-
nisms of action and for the development of potentially useful PDT agents.

It is generally accepted that non-ionized species can cross the plasma membrane more easily than charged compounds (38,39). Therefore, it would be expected that the charged photosensitizers will be less readily taken up into cells than the neutral and hydrophobic ones. Uptake experiments (Fig. 1) confirmed this to be the case for TGLy and are in agreement with that reported for sulfonated zinc (22,25), aluminum (20,26) and gallium (19) phthalocyanines, in which the tetrasubstituted derivatives had relatively poor uptake compared to the more hydrophobic, lower substituted derivatives. However, PPC had improved uptake over the more hydrophobic sensitizers (Fig. 1, Tables 1 and 2). Three possible mechanisms of photosensitizer uptake have been postulated: simple diffusion of lipophilic sensitizers, fluid phase endocytosis of water-soluble sensitizers and receptor-mediated endocytosis (RME). The high uptake of PPC may be attributed to the position of the pyridinium groups on the phthalocyanine skeleton (20). The synthesis of PPC gives a mixed product with an average degree of substitution corresponding to the dication (28). Although the actual position of the pyridinium groups has not been elucidated, it has been reported that disubstituted sensitizers with adjacent, rather than opposite, groups have improved cellular uptake compared to the tetrasubstituted derivatives (20).

The punctate fluorescence of PPC, TDOPc and TGLy is suggestive of lysosomal localization and is consistent with results that have been previously reported for PPC (18). Plasma protein binding indicated that TDOPc binds to LDL (Table 1) and therefore RME would be a possible mechanism of uptake that would result in a specific localization in the lysosomes. The PPC and TGLy did not bind to lipoproteins but delivery to lysosomes would also result from pinocytosis. The m-THPC and PHP have also been reported to distribute among the lipoproteins (36,37), suggesting that localization may be lysosomal. However, the diffuse localization of both these compounds suggests that the lysosomes are not the only target (Fig. 4). It is possible that by using different mechanisms of uptake, such as simple diffusion through the lipid bilayer and endocytosis, multiple cellular targets are accessible.

It is generally accepted that photochemical efficiency of metallophthalocyanines is not affected by peripheral substituents as long as the dye is monomeric (6). In solution, a low quantum yield of singlet oxygen produced with lower sulfonated derivatives correlated well with the tendency of these sensitizers to aggregate (6). At the cellular level, association of sensitizers with cellular proteins and lipids can affect the aggregation state and hence the photodynamic activity (7,13,16,17). Using laser illumination, the efficiency of photoinactivation, based on the extracellular concentration of sensitizer to give 50% MTT reduction (LD50ex), was PPC > m-THPC > PHP > TGLy > TDOPc. Comparisons of the intracellular concentrations at these LD50ex values (LD50in) was in the order PPC < mTHPC < TGLy < TDOPc < PHP (Table 3). This suggests that the activity of PHP was improved by the high uptake of this sensitizer, as confirmed by a plot of LD50in against light dose (Fig. 3). However, the efficiency of PHP is underestimated by these criteria alone, because when comparing photodynamic activity other factors must also be considered such as the extinction coefficient at the treatment wavelength (Table 1), the relative efficiency of photoinactivation per quantum of light absorbed, singlet oxygen yields, aggregation state and localization (7). The phthalocyanines all have similar extinction coefficients and their singlet oxygen yields are comparable to methylene blue so these factors do not account for the improved activity of PPC over TGLy and TDOPc.

Localization patterns have been shown to be an important determinant in the mode of cell death (38-41). Comparisons of localization suggested that all three phthalocyanines had a similar distribution (Fig. 4). However, when cells were exposed to light, at a much lower level than that used to produce a PDT effect, differences in localization patterns became apparent. The TDOPc fluorescence remained punctate, TGLy relocalized to the nuclear area with a concomitant increase in fluorescence intensity and the distribution of PPC became more cytoplasmic (Fig. 4). It appears that relocalization to the nucleus is common to tetrasubstituted, anionic photosensitizers, and it has been reported that this is not accompanied by an increased PDT effect (17). Using NHK 3025 cells, Berg et al. (42) showed that incubation with differentially substituted meso-tetraphenylporphines resulted in a punctate distribution indicative of lysosomes. Following exposure to light, the tetrasubstituted derivative relocalized to the nucleus, whereas the disubstituted derivative gave a more diffuse cytoplasmic distribution. In agreement with the work presented here, it was shown that the disubstituted derivative was more efficient photosensitizer after relocalization, whereas the tetrasubstituted derivative was less efficient. The two clinically used photosensitizers, PHP and m-THPC had a diffuse distribution pattern that did not change following light exposure, although some photobleaching occurred.

In conclusion, we have demonstrated that following a 2 h incubation there is a relationship between the uptake and photocytotoxicity of these compounds. The PPC has comparable, if not slightly improved, activity over the two clinically used photosensitizers, m-THPC and PHP and is much better than the anionic (TGLy) and neutral (TDOPc) phthalocyanines. This can be explained by the increased extinction coefficient at the treatment wavelength compared to m-THPC and PHP (7) and improved cellular uptake over TGLy and TDOPc (Fig. 1, Table 1). However, comparisons of the intracellular levels of PPC required to give a PDT effect were much lower than TGLy and TDOPc. The differences in localization of these phthalocyanines following low-level light exposure suggests that the diffuse localization common to PHP, m-THPC and PPC (following relocalization) may be an important determinant of good photodynamic activity.

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