A Photobiological and Photophysical-based Study of Phototoxicity of Two Chlorins

Brian W. Pogue, Bernhard Ortel, Norah Chen, Robert W. Redmond, and Tayyaba Hasan

ABSTRACT

To understand the fundamental determinants of phototoxic efficacy and absorbed photodynamic dose, the triplet state and photobleaching quantum yields in living cells, cellular uptake, intracellular localization, and correlation with cell viability were studied for the two porphyrins tin ethyl etiopurpurin I (SnET2) and tin octaethylbenzochlorin (SnOEBE) in ovarian cancer cells (OVCA5). Although the triplet yields of these two photosensitizers were not significantly affected by cellular incorporation, the photobleaching yields were shown to be 3 orders of magnitude higher for cellular-bound sensitizer than for free or albumin-bound photosensitizer and higher for SnET2 than for SnOEBE for all of the cases. The intracellular concentration of SnOEBE was half that of SnET2 after 3 h and 24 h incubation periods for both 0.1 μM and 1.0 μM incubation concentrations. Despite the lower concentrations of SnOEBE, the phototoxicity of the two photosensitizers was comparable at 1-μM incubation concentration and was up to 10-fold higher for SnET2 at the lower concentration. The subcellular localization established using confocal microscopy and molecular probes showed that both photosensitizers were primarily lysosomally localized, SnOEBE, however, had an extra-lysosomal, mitochondrial localization component. The photophysical measurements allowed calculation of the intracellular singlet oxygen production, which indicated that the photosensitizer-light dose reciprocity was limited by photobleaching for SnET2, but only minimally for SnOEBE, and this was confirmed through cell-survival studies. Taken together, these data indicate that the critical determinant of differences in phototoxicity between the two molecules was their relative rates of photobleaching and their subcellular localization. The study points to the importance of combining photosensitizer uptake and localization with photophysical measurements in the relevant biological milieu to reasonably interpret and/or predict photosensitization efficacies.

INTRODUCTION

PDT for treatment of malignant tissues has been investigated for an increasing number of applications in recent years. There have been significant advances in PDT-based treatments both in vivo and in vitro, and elegant models of photosensitized cell death have been suggested (1–7). Still, the basic understanding of tissue toxicity, as it relates to fundamental molecular properties of the PS, remains elusive. The development and planning of treatment protocols are difficult and have not been as well established as with chemotherapy or radiation therapies. This is attributable in part to the higher complexity of PDT with interactions between PS, light and tissue (13–15). Most clinical applications of PDT have been successful after empirical evaluation of the optimal treatment regimes. This has proven sufficient and will likely continue to be the main means of developing treatment plans in the near future. However, dosimetric planning could be aided significantly by developing a fundamental understanding of the complex interactions that underlie the phototoxicity. In this vein, it is important to assess the photophysics and photochemistry of the PS as it behaves in cells or tissue rather than how it behaves in solution (16–21), as has been shown in recent studies (20, 21). Combining these types of photophysical measurements with direct subcellular localization studies and intracellular quantification may be key to understanding how particular molecular PS behavior and environment affect the biological efficacy in vitro and in vivo (21). In this study, the pertinent photophysical and photobiological properties of two second-generation PSs, SnET2 and SnOEBE (Fig. 1), which are similar in chemical structure, are measured in cells in culture and used to compare their relative phototoxicities. Of these, SnET2 has been used in clinical studies for the treatment of cutaneous malignancies and is currently in Phase III clinical trials for the treatment of age-related macular degeneration, the leading cause of blindness in the elderly in the Western world. Other compounds from the same family of chemicals are in development for a variety of other PDT applications. Our interest in the current report was to establish whether or not results from simple photophysical and photobiological experiments can be used in the prediction of absorbed photodynamic dose and thus be a measure of photodynamic efficiency (21). In homogeneous media, the two compounds have similar photophysical parameters as shown by our previous studies (22) and might be expected to be comparably phototoxic to cells for equivalent cellular uptake.

The biological mechanisms of cellular toxicity have been extensively studied; however, the connection between photodynamic efficiency and specific photochemical or photophysical processes are not as well developed. Current clinical PSs are thought to cause cell death through production of O2 (1) by the quenching of the triplet state of the excited PS by ground state oxygen. The general pathway for this process (Type II photosensitization) is shown in Fig. 2, which illustrates excitation of the ground state by light, followed by intersystem crossing to the triplet state and then quenching by oxygen to produce O2 (1). If the relative efficiencies of some of the components of these pathways could be established in an appropriate environment, we could understand and perhaps predict the photodynamic dose absorbed and the resulting phototoxic efficacy of the sensitizer. Methods for measuring yields and rate constants are well established for PSs in homogeneous medium, but the ability to measure some of these parameters in heterogeneous environments such as cells or tissue is more complicated. Detection of O2 (1) produced by the PS would permit a better interpretation of cell survival data, but this is a difficult task in vitro (23, 24) and perhaps impossible in vivo because of the expected short lifetime of O2 (1) in biological environments (25). Accepting this, the most informative parameter to measure is the behavior of the triplet state of the sensitizer. The triplet state information would also be relevant to the Type II pathways components of the overall phototoxicity mechanisms. In this study, the relative triplet...
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state yields of two PSs are measured in cells using diffuse reflectance-based flash photolysis (26). These parameters were used to interpret the relative photosensitization potential by calculating the singlet oxygen yield.

Photobleaching of sensitizers is often overlooked when planning PDT treatment protocols both in vitro and in vivo. Studies have demonstrated that the product of the incident light and drug doses in cells/tissue determines cellular toxicity (25, 27). This basic observation provides a good first-order understanding of the photosensitization process. More recently, the lack of this PS-light dose reciprocity has been demonstrated when factors such as photobleaching are included (28, 29). Preliminary studies of the photophysical properties of SnET2 and SnOEBC demonstrated that the most significant difference between the two sensitizers lay in the quantum yield of photobleaching, which was nearly 100 times lower for SnOEBC than for SnET2 (22). For this reason, in this study the bleaching quantum yield of these two sensitizers was measured in OVCAR5 cells.

Cellular uptake of PSs is generally believed to be a major factor in determining their cytotoxic ability; therefore, quantification of cellular content of both PSs was performed. Studies have clearly demonstrated that different cellular localization sites can affect the efficacy of photosensitization. Therefore, confocal localization studies were carried out to characterize the spatial location of the two PSs examined here. Using a combination of the above information, calculations of singlet oxygen production as a function of photobleaching were extended to directly interpret the phototoxicity observed for SnET2 and SnOEBC in OVCAR5 cell line.

MATERIALS AND METHODS

Chemicals. The spectroscopic grade solvents DMSO and methanol were purchased from Fisher Scientific and used without further purification. PBS (×10) was purchased from Fisher Scientific and diluted to normal saline solution using deionized water.

SnET2 and SnOEBC were a generous gift from Miravant Inc. (Santa Barbara, CA). Stock solutions were prepared in DMSO at 10 and 1 mM and stored at 4°C throughout these studies. Samples were prepared immediately before use by dilution of stock solutions with PBS with <0.1% DMSO in the final sample.

Cell Culture. The NIH human ovarian cancer cell line, OVCAR5, was used in all of the experiments and was purchased from Dr. Thomas Hamilton, Fox Chase Cancer Center, Philadelphia, PA. The cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS (Life Technologies, Inc.) in a 5% CO2 atmosphere at 37°C. The PS stock solutions in DMSO were diluted in media before use. The concentrations were determined spectrophotometrically, using molar absorption coefficients (ε) for SnET2 of εmax = 26,400 M−1 cm−1 and for SnOEBC of εmax = 32,200 M−1 cm−1, as measured previously (22).

Triplet Yields in Cells. Triplet state behavior was measured in scattering samples using diffuse reflectance laser flash photolysis as described in the literature (19, 20, 30). Briefly, the remitted light from the front face of a 10 × 40 × 1 mm quartz cuvette was monitored at 470 nm using a monochromator with a photomultiplier tube for detection, which used probe light generated from a focused 75-W xenon arc lamp. Laser excitation was provided at 666 nm for SnET2 or at 690 nm for SnOEBC from a Nd:YAG (GCR 230; Spectra Physics, Mountain View, CA) pumped optical parametric oscillator (Spectra Physics MOPO 710) that caused conversion of the sensitizer to the excited triplet state. The resulting change in reflectance of the probe light because of the difference in absorption coefficient between the ground and triplet states was monitored before and after the laser pulse using a high numerical aperture lens to image the front of the cuvette onto the monochromator entrance slit before the detector. The incident probe light and the pump beam were oriented at an off angle (near 45 degrees) relative to the direction of captured reflectance light, which avoided specular reflection from entering with the analytically relevant diffuse reflectance signal.

The triplet state lifetimes were estimated by a nonlinear Marquardt fitting routine to match the transient reflectance curve to a single exponential decay, with the zero time point fixed to the incident laser pulse. All of the measurements of lifetime were carried out in nitrogen-purged solutions to minimize the quenching of the triplet states by molecular oxygen; however, it is well known that this purging will not completely remove the oxygen, so these lifetimes represent a lower limit for triplet lifetimes. The transient curve reflects the lifetime of the triplet state decay only, unaffected by the singlet state decay, because the singlet state lifetimes are three orders of magnitude faster, and the singlet decay occurs on the same time scale as the laser pulse duration (26). The relative triplet yields of the two sensitizers were measured in OVCAR5 cells as well as in tissue-simulating phantoms consisting of lipid emulsion to determine whether there was a difference in relative triplet yield between solution values (22) and when the sensitizers are incorporated into more complex systems such as lipids or cells (19, 20, 30). These measurements were carried out by recording the changes in reflectance when the PS was excited to the triplet state, in a manner similar to that used in flash photolysis measurements with triplet yields in solution. For the phantom measurements, serial dilutions of SnET2 and SnOEBC were prepared in 0.2% Intralipid in distilled water.

Fig. 2. Energy level diagram of a PS molecule in the photosensitization process. The molecules are excited from the ground state, S0, to the excited singlet state, S1, with a rate proportional to the product of the concentration, c, molar absorption coefficient, ε, and optical fluence rate, Φ. Once in the S1 state, the molecule can relax by fluorescence emission (with quantum yield, ΦF) or intersystem cross to the first triplet state, T1 (quantum yield, ΦT1) or be directly photobleached. From triplet state, the molecule can either relax by reverse intersystem crossing (ΦR1), phosphorescence emission (quantum yield, ΦP), or be quenched by interaction with a ground state oxygen molecule, O2, to produce O1 (ΦQ). The quantum yield for singlet oxygen generation, ΦO, is the ratio of O1/ΦQ. The singlet (ΦO) and triplet (ΦQ) molecules produced in the photoexcited state could come from many paths in this process including directly from S1 or T1 or from S0, S1, T1 in combination with O2. Some of these interactions are shown in the following pathways for the formation of the singlet oxygen: photobleached product, photobleached product, O2.
water (measured by volume) as the emulsion medium. This concentration of Intralipid was specifically chosen to match the absolute reflectance and transmittance values from OVCAR5 cellular slurries used in the next phase of these measurements, with only 10% difference between the two reflectances. Steady state reflectance and transmittance values were measured on a diffuse reflectance spectrophotometer with reference- and sample-integrating spheres (Model 5270; Beckman Instruments).

The comparative triplet yields in cells were measured in the same manner as in Intralipid, except that cells were incubated with either SnET 2 or SnOEBC for 3 h with 1 µM concentration in the cell medium, washed, trypsinized, and resuspended as dense slurries of cells in PBS. The final concentrations of cells were 116 ± 2 million cells/ml of solution. The cells were excited with laser pulses at the Q-band wavelengths given above, and the change in reflectance was monitored at 470 nm. The specific uptake values of the PSs by an aliquot of the same cells were measured simultaneously as described above.

**Photobleaching Yields in Cells.** The photobleaching yields of SnET 2 and SnOEBC were measured in situ in OVCAR5 cells. OVCAR5 monolayers were incubated with either SnET 2 or SnOEBC at 1.0 µM for 3 h and then trypsinized and resuspended in solution. The suspensions were irradiated at the Q-band absorption peaks for the two PSs (656 nm for SnET 2 and 690 nm for SnOEBC) using a dye laser (model Innova 100 argon laser pumping a CR-599 dye laser; Coherent Inc., Palo Alto, CA) while being stirred continuously to keep the cells in suspension. The samples were analyzed for changes in absorbance and fluorescence at varying times after irradiation. All of the samples were 2 ml in volume, contained in 1 × 1 cm path length quartz cuvettes, and irradiated by an expanded beam to homogeneously irradiate all of the solution. Incident fluence rates ranged between 20 mW/cm² to 50 mW/cm² depending upon the sample bleaching rate. Four to five measurements of absorption and fluorescence versus cumulative irradiation dose were plotted for each time point, and the photobleaching quantum yield for each sample was calculated from the ratio of the number of molecules photobleached to the number of photons absorbed, as described in a previous study (22). Briefly, the quantum yield was calculated as the ratio of the slopes of n exp (the number of molecules photobleached) divided by n ino (the number of photons absorbed) as a function of the number of irradiation intervals. The photobleaching rates of SnET 2 and SnOEBC dissolved in methanol at 1.0 µM were also measured to directly compare the cellular rate with these previously characterized values.

**Cellular Uptake of PS.** To evaluate the relative change between phototoxic efficacy of the PSs with their intracellular content at the time of irradiation, cellular uptake for both the PSs was established at two concentrations and at two time points. The incubations for cell phototoxicity and uptake were performed simultaneously with the same solutions for both parts of the experiment. For quantification of cellular PS uptake after incubation for 3 and 24 h, the cells were trypsinized and solubilized in 1% SDS in 0.1 N NaOH, as described in detail previously (31). The intensity of the emission peak of SnET 2 at 660 nm was determined after excitation at 436 nm and of SnOEBC at 684 nm after excitation at 426 nm using a SPEX-Fluorolog 2 Spectrophotometer equipped with PM 3000 analysis software (SPEX Industries, Inc., Edison, NJ). Calculations of concentration were based upon comparison of integrated spectral emission from these samples with the spectra from cell lysates mixed with known PS concentration. Cell numbers in aliquots were established by hemocytometer to allow calculation of the absolute PS concentration/cell.

**PS Localization in Cells.** OVCAR5 cells were plated at a density of 2 × 10⁵ on 20 × 20-mm microscope coverslips in 35-mm cell culture dishes and incubated for 24 h. The resulting confluent cell layers were incubated for 3 h in 5 or 1 µM of either SnET 2 or SnOEBC. For the final 20 to 30 min, 9 or 25 nm, respectively, of either rhodamine 123 (R123; Eastman Kodak, Rochester, NY) or Lysotracker (LT; Molecular Probes, Eugene, OR) were added for coinclusion. The coverslips were rinsed with PBS, mounted with PBS on a microscope slide, and imaged without delay.

A Leica confocal laser scanning microscope consisting of a Leica TCS 4D scanner attached to a Leitz DM B/E microscope was operated using the TCS-NT software. This instrument allows simultaneous recording of three channels. The 488-nm line of an argon ion laser was used for excitation. A 100× oil immersion objective with a numerical aperture of 1.40 was used to image a square (100 × 100 µm) field at a 1024 × 1024-pixel resolution. Assuming an index of refraction of 1.33, we calculated a lateral resolution of 0.12 µm and an axial resolution of 0.46 µm when using a 1-resel pinhole size. However, because of the low PS fluorescence intensity, the pinhole had to be opened, and thus the resolution values have to be assumed somewhat larger. One photomultiplier tube detector was used for acquiring a transmission differential interference contrast image that was displayed in gray scale. The fluorescent signal emitted from the sample is separated into two detection channels by a 580-nm dichroic mirror. The reflected portion in the green range (below 580 nm) is passed through a bandpass filter (525–550 nm) before it is collected by the first photomultiplier tube detector. The light that is transmitted by the dichroic mirror is in the red range (above 580 nm) and is passed through a 590-nm longpass filter and collected by the second detector. The fluorescence images were displayed in green and red “false” color output and electronically combined for visualizing colocalization.

**Photosemitization of OVCAR Cells.** OVCAR5 cells were plated in 35-mm tissue culture dishes at about 70% confluency at the time of laser irradiation. The cells were incubated with 1.0 µM PS in RPMI media for 3 h. Immediately before irradiation, the culture medium was removed, and the cells were washed with PBS containing calcium and magnesium and covered with 0.5 ml PBS. Irradiations were performed through the bottom of the cell culture dish, using 666 nm for SnET 2 and 696 nm for SnOEBC, with radiation from an argon ion-pumped dye laser (Coherent, Palo Alto, CA) that was expanded into a large homogeneous (top-hat profile) beam using a fiber optic collimator. Irradiations of typically 0.03 to 0.035 W/cm² were used to deliver fluences of 0.5, 1.0, 2.0, and 3.0 J/cm². A second set of cells was incubated with 0.1 µM PS and exposed to irradiation fluences of 5, 10, 20, and 30 J/cm². These two conditions were chosen such that the same number of photons would be expected to be absorbed in each case, i.e., the product of the intracellular PS content and the light dose (J/cm²) is the same for the two conditions. At 20 h after light treatment, the cells were incubated with 0.5 mg/ml MTT for 1 h to quantify its reduction by mitochondrial dehydrogenases (32). The dehydrogenase activity at 20 h after PDT provides a sensitive method of assessing the number of surviving cells and has been shown to correlate well with other established measures of cytotoxicity such as clonogenic assay (32). The reaction product, formazan, was quantified photometrically at 570 nm, and the data were normalized to a control set of cells where no light or PS was applied.

**RESULTS**

**Triplet Yields in Cells.** An example of the transient reflectance changes obtained on diffuse reflectance laser flash photolysis of these PSs is shown in the inset in Fig. 3a, from which the peak height of the change in reflectance is determined. These measurements were repeated for several pulse energies, and the initial value of the saturation change in reflectance (∆R/R) was measured as a function of pulse energy, as plotted for both SnET 2 and SnOEBC in Fig. 3a. This plot of change in reflectance versus pulse energy was measured from solutions of 0.2% Intralipid for both SnET 2 and SnOEBC. From these curves (see Fig. 3a) the ΔR/R was measured by fitting to a model of ∆R/R = S[1 − exp(−k E)], where S is the saturated value, and E is the pulse energy. The ratio of S values for SnET 2:SnOEBC should be related to the quantum yields of triplet formation and the ratio of triplet molar difference absorption coefficients at 470 nm.

The triplet difference spectra of SnET 2 and SnOEBC in organic solvent were measured in a previous study and exhibit triplet difference absorbance coefficients of 8,900 M⁻¹ cm⁻¹ and 39,000 M⁻¹ cm⁻¹ at 470 nm, respectively (22). The quantum yields of intersystem crossing were 0.94 and 0.86, with singlet lifetimes of 1.0 and 3.4 ns, respectively. Therefore, it can be assumed that during the time of the laser pulse (~10 ns) there is effectively complete conversion to the triplet state, because the molecules have time to cycle through the singlet state more than once during the laser pulse. In Intralipid and cells, the change in reflectance after the saturating laser pulse is because of this conversion to the triplet state. The ratio of triplet difference absorption coefficients at 470 nm in DMSO is 4.3, whereas the reflectance change ratio within Intralipid is 3.3.

The cellular measurements were very similar to the Intralipid
Photobleaching in Cells. The quantum yields of photobleaching for SnET₂ and SnOEBC within OVCAR5 cells were measured by comparison with the bleaching rate of SnET₂ in air-saturated methanol. For both sensitizers, the bleaching rates were measured in both aerated and deaerated (N₂-flushed) cell suspensions. The values from these measurements are shown in Table 1, indicating that SnET₂ had an aerated solution photobleaching yield of 3.7%, whereas all of the other yields were below 1%. Interestingly, the photobleaching yield of SnOEBC is higher in nitrogen-purged environment than when aerated, indicating that in non-aerated conditions photobleaching is dominated by a pathway other than singlet oxygen.

Cellular Uptake. The uptake of SnET₂ and SnOEBC in OVCAR5 cells was measured for incubation concentrations of 1.0 and 0.1 μM and, for 3-h and 24-h periods of incubation, are presented as fmol/cell in Table 2. For SnET₂, the intracellular concentrations for both 3-h time points were directly proportional to the initial incubation concentrations (i.e., as the incubated concentration increases by a factor of 10, the uptake concentration increases by a factor of 10 (first two rows of Table 2). This was not true for SnOEBC where a 10-fold increase in the incubation concentrations gave only a 7.5-fold enhancement of the intracellular concentrations. There was also a lack of linearity as the incubation time was increased from 3 to 24 h, possibly suggesting a saturation phenomenon.

Subcellular Localization. Cellular localization of purpurins was established in living cells using confocal laser scanning fluorescence microscopy (see Fig. 4). When imaging cells at low PS concentrations, the cellular autofluorescence signal needs to be considered. In untreated cells, there was a weak green fluorescence with a mitochondrial pattern and some yellow fluorescence that was apparently lysosomally localized. When cells were incubated for 3 h with 1 μM of either PS, a lysosomal localization was indicated by a color change of the lysosomes from yellow to orange or red in the combined fluorescence images. Co-staining experiments with the markers rhodamine 123 and Lysotracker confirmed predominant localization of the two PSs to the lysosomes. In addition, a weak but clearly distinguishable mitochondrial component of the red fluorescence distribution was visible for SnOEBC but not for SnET₂.

Cellular Photosensitization. Phototoxicity assays of the OVCAR5 cells incubated with SnET₂ or SnOEBC were carried out as a function of incubation time, and the result is shown in Fig. 4. When imaging cells at low PS concentrations, the cellular autofluorescence signal needs to be considered. In untreated cells, there was a weak green fluorescence with a mitochondrial pattern and some yellow fluorescence that was apparently lysosomally localized. When cells were incubated for 3 h with 1 μM of either PS, a lysosomal localization was indicated by a color change of the lysosomes from yellow to orange or red in the combined fluorescence images. Co-staining experiments with the markers rhodamine 123 and Lysotracker confirmed predominant localization of the two PSs to the lysosomes. In addition, a weak but clearly distinguishable mitochondrial component of the red fluorescence distribution was visible for SnOEBC but not for SnET₂.

Table 1 Measured photobleaching quantum yield for PS localized in OVCAR5 cells after incubation at 1.0 μM for 3 h.

<table>
<thead>
<tr>
<th>PS</th>
<th>Saturation condition</th>
<th>Photobleaching quantum yield, Φ_PB</th>
<th>Triplet state lifetime (μs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SnET₂</td>
<td>Air</td>
<td>0.037</td>
<td>61 ± 1</td>
</tr>
<tr>
<td>SnET₂</td>
<td>Nitrogen</td>
<td>0.0046</td>
<td>52 ± 1</td>
</tr>
<tr>
<td>SnOEBC</td>
<td>Air</td>
<td>0.0021</td>
<td></td>
</tr>
<tr>
<td>SnOEBC</td>
<td>Nitrogen</td>
<td>0.0071</td>
<td></td>
</tr>
</tbody>
</table>

Table 2 PS uptake of OVCAR5 cells incubated with SnET₂ and SnOEBC for the same sets of conditions.

<table>
<thead>
<tr>
<th>PS</th>
<th>Incubation concentration (μM)</th>
<th>Incubation time (hr)</th>
<th>Uptake (fmol/cell) (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SnET₂</td>
<td>0.1</td>
<td>3</td>
<td>0.014 (0.004)</td>
</tr>
<tr>
<td>SnET₂</td>
<td>1.0</td>
<td>3</td>
<td>0.147 (0.004)</td>
</tr>
<tr>
<td>SnET₂</td>
<td>0.1</td>
<td>24</td>
<td>0.024 (0.004)</td>
</tr>
<tr>
<td>SnET₂</td>
<td>1.0</td>
<td>24</td>
<td>0.267 (0.02)</td>
</tr>
<tr>
<td>SnOEBC</td>
<td>0.1</td>
<td>3</td>
<td>0.0081 (0.0004)</td>
</tr>
<tr>
<td>SnOEBC</td>
<td>1.0</td>
<td>3</td>
<td>0.058 (0.004)</td>
</tr>
<tr>
<td>SnOEBC</td>
<td>0.1</td>
<td>24</td>
<td>0.0146 (0.0004)</td>
</tr>
<tr>
<td>SnOEBC</td>
<td>1.0</td>
<td>24</td>
<td>0.104 (0.003)</td>
</tr>
</tbody>
</table>
the percentage of cell survival was measured by normalizing the results of the MTT assay with cells that where incubated with PS but were not irradiated. All of the experimental points are an average of three plates, and each experiment was performed at least twice on separate days. The results of these assays are plotted in Fig. 5.

With sensitizer incubations of 0.1 μM, irradiation fluences of 5, 10, 20, and 30 J/cm² were used. These light doses were specifically chosen to maintain a constant product of PS and light dose for the two drug dose conditions. The PS uptake measurements, shown in Table 2, had indicated that for 3-h incubations the amount of drug taken up within these concentrations is near linearly dependent upon the incubation dose (less linear for SnOEBC than for SnET2), so that cells incubated at 1.0 μM would take up 10 times the amount of the PS of cells incubated at 0.1 μM. Thus, if complete PS-light dose reciprocity holds, the cell toxicity should be identical for both Fig. 5a and b. The fact that the cell survival curve for SnET2 in Fig. 5b is significantly different from SnOEBC and from SnET2 in Fig. 5a demonstrates that reciprocity does not hold and other factor(s) are influencing the cellular toxicity.

**Prediction of Singlet Oxygen Production.** The measurement of photobleaching of the two sensitzers directly in the cells along with a known PS uptake, intersystem crossing quantum yields, and singlet oxygen yields allows calculation of the singlet oxygen concentration/cell that should have been produced. Ideally, the singlet oxygen generation would be directly measured; however, the short lifetime of this species in biological systems precludes this measurement. Using the equation derived in previous studies of these two PSs (22),

\[
[O_{2}^{\cdot}(\Delta_{g})] = \frac{\Phi_{isc} S_{A} [PS]_{0}}{\Phi_{PB}} \left[ 1 - \exp(\Phi_{PB} \epsilon \psi t) \right]
\]  

where \(\Phi_{isc}\) is the singlet to triplet intersystem crossing quantum yield, \(S_{A}\) is the probability that a singlet oxygen molecule will be produced in the quenching of the triplet state molecule by oxygen (i.e., the singlet oxygen quantum yield of the molecule is the product of these two ratios, \(\Phi_{isc} = S_{A} [PS]_{0}\), \([PS]_{0}\) is the initial PS concentration, \(\Phi_{PB}\) is the photobleaching quantum yield of the molecule, \(\epsilon\) is the molar absorption coefficient, \(\psi\) is the light fluence rate, \(t\) is the time of irradiation, and \([O_{2}^{\cdot}(\Delta_{g})]\) is the cumulative concentration of singlet oxygen produced (see Fig. 2 for illustration). This equation is derived with the assumption that there is abundant oxygen supply in the surrounding medium and that the oxygen consumption rate does not significantly affect the ambient supply, which is a valid assumption for in vitro conditions, as justified in the next section. The calculation of singlet oxygen generated/cell was carried out for the conditions used in the photosensitization experiments. For SnET2, the cellular uptake was 0.014 and 0.147 fmol/cell at the two incubation conditions, and for SnOEBC, the uptake was 0.0081 and 0.058 fmol/cell. The singlet oxygen quantum yields were taken as measured when bound to BSA, because direct measurement in the cells has not been possible. This latter assumption is somewhat validated by the fact that of light fluence after incubation with two PS concentrations, 1.0 μM and 0.1 μM. With the 1.0 μM incubations, light doses of 0, 0.5, 1.0, 2.0, and 3.0 J/cm² were delivered. The percentage of cell survival was measured by normalizing the results of the MTT assay with cells that where incubated with PS but were not irradiated. All of the experimental points are an average of three plates, and each experiment was performed at least twice on separate days. The results of these assays are plotted in Fig. 5.

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ably. In Fig. 5, where there is less PS and more light delivered, the calculated singlet oxygen curves overlap consider-
time, the cells showed similar toxicity levels to both PSs at the higher PS dose as measured when bound to BSA in aqueous solution. The calculated triplet state is not greatly influenced by the binding status in this data in Fig. 5 of high and low PS dose, respectively, to correspond directly with the relative intersystem crossing yields did not change when bound to a and b of cell survival. Interestingly, in Fig. 6a, where the cells showed similar toxicity levels to both PSs at the higher PS concentration, the calculated singlet oxygen curves overlap considerably. In Fig. 5b, where there is less PS and more light delivered, the cell survival curves clearly separate, with SnET2 having little phototoxic effect. In this case (Fig. 6b), the singlet oxygen produced is limited by photobleaching, whereas SnOEBC exhibits an increased toxicity and singlet oxygen generation with increasing light dose.

**DISCUSSION**

In general, there are many factors that can affect the photosensitization of cells. In this study, we attempted to directly measure the parameters for SnET2 and SnOEBC pertinent to sensitization of OVCAR5 cells including triplet state yields, photobleaching quantum yield, PS uptake, site of localization, and cell survival for two different sets of light-PS dose conditions. Together, these parameters can be used to calculate the singlet oxygen yield/cell and allow a quantitative interpretation of the cell survival data.

It has been suggested that the molecules that localize predominantly in the mitochondria are more potent PSs, possibly because they initiate cell death via the apoptotic pathway as compared with those that localize in lysosomes (33–37). The unexpected observations in this study that the PS SnET2 with a greater accumulation in OVCAR cells was less phototoxic than SnOEBC may be, to some extent, attributed to a mitochondrial localization component of the SnOEBC in contrast to SnET2. In addition to the slight difference in the subcellular distribution of the two molecules, there was a more striking difference in their photobleaching rates.

The measurement of photobleaching is most important here, because there is a significant change in the quantum yield of photobleaching for both sensitizers when taken up in the cells, as compared with solution. The bleaching quantum yields bound to albumin were previously measured to be $8 \times 10^{-4}$ and $3 \times 10^{-6}$ for SnET2 and SnOEBC, respectively, in air-saturated solution. The measurements in air-saturated cells are 0.037 and 0.0021, respectively, which indicates an increase of more than two orders of magnitude bleaching yield in cells, when compared with solution measurements. Additionally, the photobleaching yield of SnET2 is increased in the presence of oxygen, whereas that of SnOEBC is lowered in the presence of oxygen. This latter observation suggests that SnOEBC photobleaching results directly from the $T_1$ state and is a competitive pathway with singlet oxygen production. The increased triplet lifetime, when incorporated in cells, would effectively increase the photobleaching yield. In contrast, the SnET2 photobleaching decreases in the absence of oxygen, which suggests that this bleaching mechanism is from the interaction of $O_2(\Delta g)$ with the sensitizer. There is also considerable bleaching in the absence of oxygen, which suggests that there is more than one pathway for the degradation of the molecule, where one pathway is mediated by singlet oxygen and the other pathway involves direct photobleaching from the excited states $S_1$ and $T_1$ (38, 39).

The measurement of triplet yield in living cells was important to assess whether binding to the lysosomes affected the intersystem crossing rate to the triplet state. The measurements here indicate that, to first order, the intersystem crossing yields of SnET2 and SnOEBC are in the same ratio as in dilute solution. The absolute yields of intersystem crossing are also similar to those measured in Intralipid emulsion, where presumably the PS molecules may be bound to the lipid molecules. These measurements would indicate that the PS is not highly aggregated in cells under the conditions of low PS concentration examined here and that localization in the lysosomes did not significantly affect the triplet yields. This is in contrast to similar recent experiments (20, 21) on plasma membrane-localizing PSs where a correlation of photophysics, measured in vitro using laser flash photolysis, with phototoxicity demonstrated concentration-dependent photosensitization mechanisms and efficacy, but the concentration range studied was much higher than in the present work.

From the calculations presented in Fig. 6 and cell survival observations in Fig. 5, a minimum threshold singlet oxygen dose can be predicted for these sensitzers in OVCAR5 cells. Because the incubation of SnET2 at 0.1 $\mu$M produced no appreciable phototoxicity (Fig. 5b, ●) and the calculated singlet oxygen produced at this dose was limited to $5 \times 10^9$ molecules/cell, we can estimate that the lower limit of the threshold to produce phototoxicity is near $10^9$ singlet oxygen molecules/cell, summed over the entire irradiation time. This type of estimate is important to allow a quantitative comparison of the relative biological effectiveness for killing with PSs. Assuming the cells to be an average of 15-$\mu$m diameter, the volume of a cell is $1.8 \times 10^{-12}$ liter and the singlet oxygen dose is then near $10^{21} O_2(\Delta g)$ molecules/liter (i.e., the equivalent of over $10^{-3}$ mol/liter if summed over the total irradiation time). These values can be compared with the estimates of Nichols and Foster (40), who measured the threshold singlet oxygen dose for Photofrin in tumor spheroids to be $0.32 \times 10^{-3}$ mol/liter, or Georgakoudi et al. (41), who corrected this estimate for the photobleaching effect and estimated the value as 12 mM. Given the variation in the localization site between
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Photofrin and these purpurins and that different cell lines were used, the order of magnitude agreement observed here is encouraging. This comparison of singlet oxygen yield required for cell death is a useful index of phototoxicity and one that will likely vary depending on the localization of the sensitizer in vitro. Interestingly, the confocal fluorescence studies show the PS localized in the lysosomes and that the fraction of the cell that is composed of lysosomes is perhaps <1% of the cell. This suggests that the singlet oxygen produced in the lysosomes is near 0.1 mol/liter, summed over the entire irradiation time. Further study of this threshold dose concept may improve the understanding of photodynamic efficacy and the correlation to absorbed photodynamic dose.

The interpretation of singlet oxygen production as the mediator of cell toxicity has implicitly assumed that oxygen is in abundant supply and that the photochemical depletion is not significant. We have validated that the concentration of oxygen does not fall significantly during this irradiation in two ways: first, we have compared our results with several published studies; and secondly, we calculated the consumption rate for the experimental conditions used in the study. Moor et al. (42) used both calculations and direct measurements to conclude that in vitro oxygen depletion is not significant, even at optical fluence rates of 500 mW/cm², which are a factor of 16 times higher than used in this study. Aveline and Redmond (21) also used direct measurements of oxygen consumption in vitro with μM levels of deuteroporphyrin and different brominated derivatives and did not observe appreciable oxygen consumption until the oxygen in solution was reduced to 0.5% (down from our value of 22%).

Calculation of the oxygen consumption rate can be done assuming that in normal cell culture the oxygen concentration is near that of air-saturated water, which corresponds to 0.26 mm, and the maximal cellular concentration of PS was 0.147 fmol/cell and 0.058 fmol/cell for SnET2 and SnOEBC, respectively. Using 35-mm Petri dishes at 70% confluence gives us approximately 0.6 million cells/dish, which leads to an overall concentration near 0.12 μM when normalized for the 0.5-ml volume of saline covering the cells. The photon absorption probability rate can be calculated from the product of the photon absorption cross section of the PS, σ, times the light fluence rate, ϕ, when converted to the appropriate quantized units. Given a molar absorbance of 2 × 10⁴ M⁻¹ cm⁻¹, this corresponds to a cross section of σ = 3.3 × 10⁻¹⁷ cm²/molecule. The light fluence rate was 0.03 W/cm², which corresponds to 1 × 10¹⁷ photons cm⁻² s⁻¹. Then the photon absorption probability rate/molecule is the product σϕ = 3 photons/molecule/second, such that most molecules will absorb three photons every second. However, the concentration of PS is 0.12 μM, whereas the oxygen concentration is near 0.26 mm. Thus, even assuming a singlet oxygen quantum yield of 1, the consumption of oxygen over the 100 s of the experiment leads to a total of 0.036 μM consumed. Thus, a conservative estimate of the consumption rate is an order of magnitude lower than the rate needed to cause depletion of oxygen in vitro.

In summary, this study shows that even for PSs with very similar chemical structures and photophysical properties in homogenous media, the photosensitization potential does not have a simple relationship with any single variable including cellular content. PS subcellular distribution and photophysics within cells are critical determinants of photosensitization efficiency. When such comprehensive measurements are made in the relevant environment, reasonable agreement can be obtained between phototoxicity and calculations of singlet oxygen production (21), and then it may be possible to quantitatively predict the absorbed photodynamic dose to cells and tissue.

ACKNOWLEDGMENTS

The authors would like to thank Hans-Christian Luedemann for his expert assistance in the transient diffuse-reflectance measurements, and to Drs. Brian C. Wilson, Lothar Lilge, and Michael S. Patterson for thoughtful discussions.

REFERENCES


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