Photosensitizing efficiencies of poryphyrins, chlorins, and phthalocyanines.

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ABSTRACT

A Clark-type microelectrode is used to measure oxygen consumption rates in laser-irradiated solutions of photosensitizer and photosensitizer-containing cells. The presence of a singlet oxygen-specific acceptor molecule, furfuryl alcohol, permits indirect determination of relative singlet oxygen generation efficiencies from oxygen consumption data. Solution and cell measurements are performed which compare photosensitizing efficiency of Photofrin-II (PII), tetraphenylporphine tetrasulfonate ($TPPS_4$), mono-L-aspartyl chlorin e_6 (MACE), and chloroaluminum sulfonated phthalocyanine (CASPc). Relative singlet oxygen generating efficiency, per-unit-weight and per-absorbed-photon, were determined to be: MACE > CASPc > $TPPS_4$ > PII and $TPPS_4$ > MACE > PII > CASPc, respectively. When these results are compared to oxygen consumption in photosensitizer-containing cells, differences in the order and magnitude of photosensitizing efficiencies are observed. The relative oxygen consumption rate in cells was: PII ≈ CASPc > MACE >> $TPPS_4$. Additional information concerning cell killing efficiency is derived from clongenicity assays. These data indicate that consideration of singlet oxygen generating ability in solution must be considered in conjuntion with cellular assays in order to provide an *in vitro* estimate of photosensitizer efficacy.

1. INTRODUCTION

During photodynamic therapy, irradiation of photosensitizer-containing cells results in the formation of short-lived cytotoxic oxygen intermediates. These compounds, predominantly singlet molecular oxygen ($O_2(^1\Delta_g)$ or $^1O_2$), are highly reactive tumoricidal agents.[1,2] When $^1O_2$ encounters a suitable acceptor molecule (Ac), an oxidation reaction may occur:

$$^1O_2 + Ac \rightarrow AcO_2 \qquad k_c, \text{ chemical quenching} \tag{1}$$

As AcO_2 is formed, ground state oxygen ($O_2(^3\Sigma_g^-)$ or $^3O_2$) is depleted. The rate of $^3O_2$ (and Ac) disappearance can be expressed by:

$$-d[^3O_2]/dt = -d[Ac]/dt = k_{obs}\,[^3D^*][Ac] \tag{2}$$

where k_{obs} is the observed (measured) rate constant for O_2 depletion and $[^3D^*]$ is the concentration of excited-state triplet photosensitizer. Equation 2 can be derived by applying the steady-state approximation to the disappearance of the rapidly-reacting intermediates $^3D^*$ and $^1O_2$.

In this work, a Clark-type microelectrode[3] was used to measure the disappearance of oxygen from laser-irradiated solutions of photosensitizer (D) and Ac. Four photosensitizers were evaluated: 1) Photofrin-II, PII; 2) tetraphenylporphine tetrasulfonate, $TPPS_4$; 3) mono-L-aspartyl chlorin e_6, MACE; and 4) chloroaluminum sulfonated phthalocyanine, CASPc. Two types of Ac were investigated: furfuryl alcohol (Fur), a singlet oxygen-specific acceptor[2], and cell suspensions. Since Fur interacts specifically with $^1O_2$, the disappearance of $^3O_2$ can be correlated directly to singlet oxygen production. When cell components serve as acceptors, however, there is a possiblity that more than one oxidation mechanism can be invoked.

Experiments were conducted by varying D and Ac concentrations, irradiating each mixture for 3 minutes, and recording $^3O_2$ disappearance ($\Delta\%\ O_2$) as a function of [Ac], [D], and Einsteins (moles of photons) absorbed. Relative singlet oxygen quantum efficiencies (ϕ_Δ) were estimated from the following relationship:[4]

$$\phi_\Delta = n^1O_2 / n_{abs,\lambda} \propto K(n^3O_2 / n_{abs,\lambda}) \quad (3)$$

where $n^1O_2 / n_{abs,\lambda}$ and $n^3O_2 / n_{abs,\lambda}$ are, respectively, the number of singlet and triplet oxygen molecules produced and consumed per absorbed photon, and K is a constant which accounts for the influence of [Ac] and competing kinetic pathways on $^1O_2$ deactivation.

In the case of cell suspension measurements, oxygen consumption rate (molecules $^3O_2$ consumed per cell per second) and photosensitizer uptake (pg/cell) were determined for PII, CASPc, and MACE in chinese hamster ovary (CHO) cells. In addition, an indication of cell killing efficiency on a per-unit-weight basis was obtained by performing standard cell non-viability (clonogenicity) assays for each photosensitizer.[5] These cellular results were compared to singlet oxygen generation efficiency predictions derived from furfuryl alcohol solution quenching data. This multi-parametric approach was pursued so that a simple, rapid, pre-clinical screening system for new drugs could be developed, thus, perhaps, minimizing the need for extensive *in-vitro* studies.

2. MATERIALS AND METHODS

2.1. Chemicals

Stock solutions (approximately 2.5 mg/ml) of each photosensitizer were prepared in phosphate-buffered saline (PBS) and stored in the dark at -70 °C. All solution (non-cellular) measurements using PII (Photomedica, Raritan, N. J.) were conducted in 1% (v/v) Triton X-100 in order to monomerize the highly lipophilic drug. Under these conditions, PII was assumed to be composed of its major active component, di-hematoporphyrin ester/ether.[6] Both cellular and solution measurements of other photosensitizers were conducted by making appropriate dilutions in PBS.

$TPPS_4$ (Porphyrin Products, Logan, UT) and CASPc (Ciba-Geigy, Switzerland) were used, as supplied, without further purification. $TPPS_4$ is a synthetic porphyrin and is reported to be stable, hydrophilic, and monomeric in aqueous solution. Its photosensitizing capability has been previously described.[7] CASPc is a well characterized compound,[8] which, as supplied by Ciba-Geigy, has an average of 3 sulphonic acid groups per molecule.[9]

MACE, a chlorin derivative, was generously provided by Dr. Kevin Smith of the University of California, Davis. The *in vivo*[10] and *in vitro*[5] photosensitizing characteristics of MACE have been previously described.

2.2. Preparation

Oxygen consumption measurements of photosensitizer and furfuryl alcohol (Eastman Kodak Co., Rochester, NY), were conducted by irradiating solutions which contained varying concentrations of D and Ac .

Experiments utilizing Chinese Hamster Ovary (CHO) cells involved 24-hour incubation of cells with 15 mg/ml doses of each photosensitizer. Following incubation, cells were centrifuged, rinsed, and resuspended in PBS at a concentration of approximately 1×10^6 cells/ml. Oxygen consumption measurements were performed on at least 3 dilutions of the initial cell suspension.

Standard cell clonogenicity studies were conducted on photosensitizer/cell suspensions which received equivalent light doses, but were prepared by incubating with varying concentrations of photosensitizer. Cell survivability was evaluated using previously described techniques.[5]

2.3. Measurements

All oxygen measurements were conducted electrochemically using 10-20 μm diameter Clark-type microelectrodes (Micro-Sense, Ramat Gan, Israel). By maintaining the cathode at a constant potential of -0.75 V dc, oxygen, which diffuses across the electrode's silicon membrane, is reduced and a picoampere current is generated. Potential control and current detection were accomplished using an adjustable amplifier (Diamond Electro-Tech Inc., Ann Arbor, MI). A strip-chart recorder was used to record the analog output of each electrode.

Electrodes were calibrated at $[O_2] \approx 0$ either by bubbling N_2 through PBS measurement solutions for 30 minutes or by saturating PBS solutions with sodium dithionite, $Na_2S_2O_4$. Pure oxygen was bubbled through calibration solutions.in order to obtain $[O_2] = 1.3 \times 10^{-3}$ M. A linear electrode response was observed over the entire working range of $[O_2]$. Between runs,

electrodes were rinsed in air-saturated distilled water and PBS ($[O_2] \approx 2.7 \times 10^{-4}$ M at 22 °C).[11]

All samples (solutions and cell suspensions) were placed in diposable plastic cuvettes and immersed in a large-volume water bath (22 ± 1 °C) in order to dissipate heat that may have been generated due to absorbed light. Irradiations were performed at 630, 654, and 675 nm using a Cooper Lasersonics (Santa Clara, CA) model 770 DL argon-pumped dye laser (with DCM dye) and at 405 nm with a Coherent (Palo Alto, CA) model 90-K krypton laser. A 400-μm diameter quartz optical fiber terminated with a microlens was used to deliver light to each sample. The fiber was positioned so that a $1cm^3$ section of the sample cell was irradiated. The electrode was reproducibly positioned, with a micromanipulator, in the center of the irradiated volume. Each sample was exposed to laser light for three minutes at power densities of 60 mW/cm^2 (630, 654, and 675 nm) and 10 mW/cm^2 (405 nm). No measureable oxygen consumption was observed during control measurements of D and Ac (Fur and cells) alone.

3. RESULTS AND DISCUSSION

Oxygen consumption measurements were conducted for each D at three different photosensitizer concentrations ranging between 0.3 and 20 mg/l. At each [D], at least 3 different [Fur] (10^{-4} - 10^{-3} M) were used. Results from a portion of these experiments are illustrated in Figures 1 and 2. These data indicate that oxygen consumption, as measured by % oxygen reduction from pre-irradiation levels, is linearly proportional to both [Fur] and [D] (at constant [D] and [Fur], respectively). Since $[^3D^*] \propto [D]$, this observed bi-molecular dependence provides experimental confirmation of the oxygen depletion rate relationship described by equation 2. Relative singlet oxygen generating efficiency, per-photosensitizer-unit-weight, can therefore be derived by comparing the slopes of Figure 2.

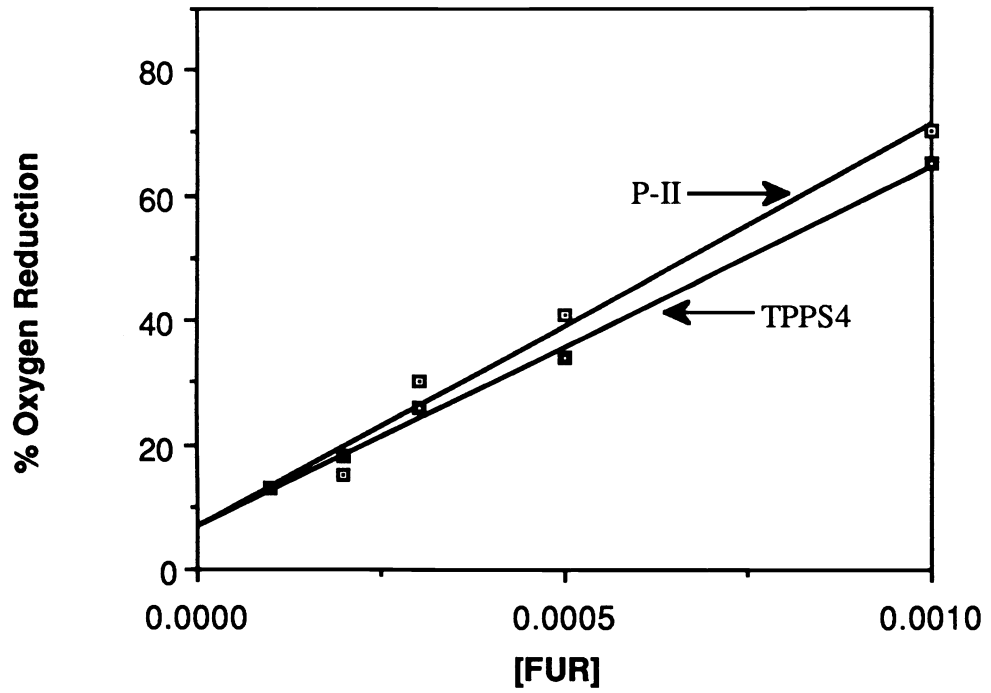

Figure 1. % O_2 reduction vs. [Fur] (moles/L) for PII (630 nm, 60 mW/cm^2); $TPPS_4$ (638 nm, 60 mW/cm^2).

Relative singlet oxygen generating efficiency per absorbed photon can be determined, in a similar manner, from the relationship between oxygen depletion and absorbed light. Figure 2 is, therefore, redisplayed as % oxygen reduction vs. moles absorbed photons (einsteins) in Figure 3. Einsteins absorbed were calculated, for each [D] and λ, from experimentally-determined Beer's law relationships. In fact, Figure 3 graphically illustrates the relationship described by equation 3 where, at constant [Ac] and irradiation time, $n^1O_2 = (n_{abs,\lambda})\Phi_\Delta \propto n^3O_2$; and slopes from n^3O_2 vs. $n_{abs,\lambda}$ yield relative ϕ_Δ values.

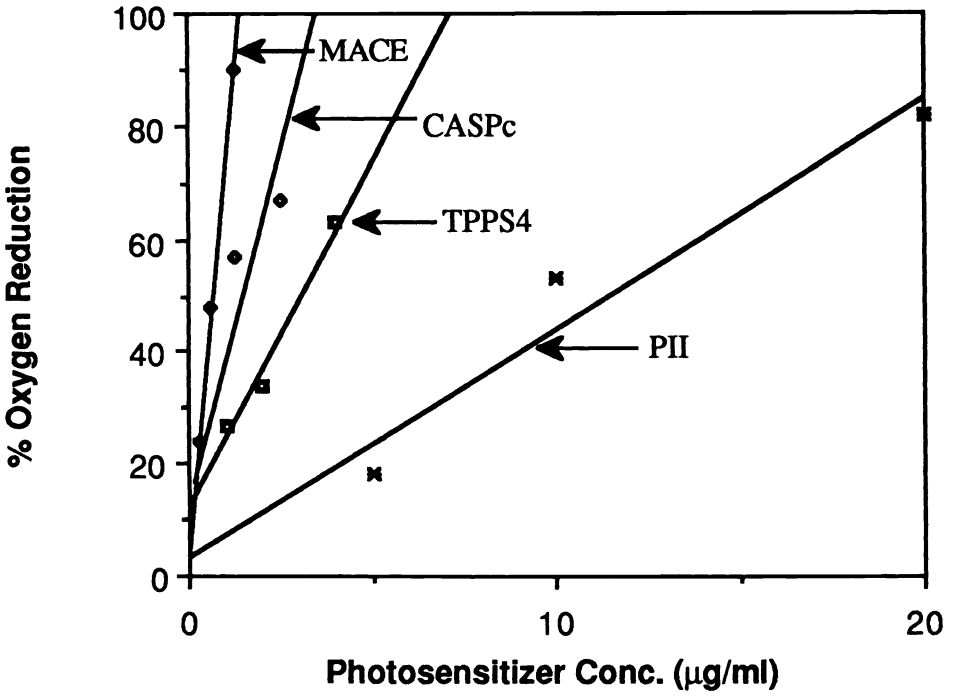

Figure 2. % Oxygen reduction vs photosensitizer conc. for PII (630 nm); $TPPS_4$ (638 nm), CASPc (675 nm), and MACE (654 nm); 60 mW/cm^2 irradiations.

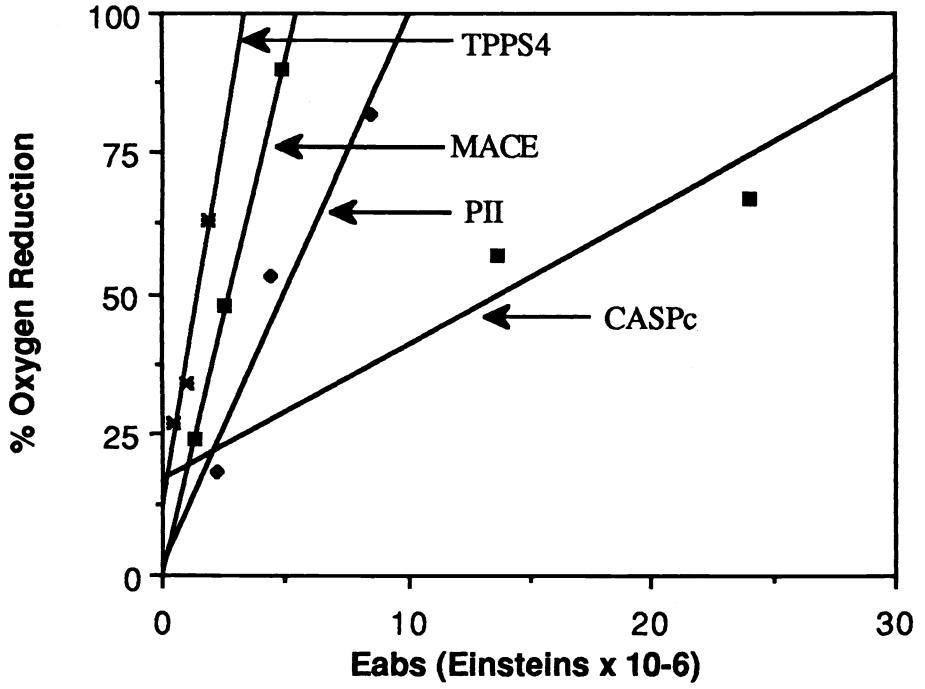

Figure 3. % Oxygen reduction vs. moles absorbed photons (einsteins).

A comparison of relative photosensitizer singlet oxygen generating efficiencies per unit weight and per absorbed photon (relative ϕ_Δ) in solution are illustrated in Figure 4. These results, derived from the relationships between the slopes of Figures 2 and 3, suggest that per-unit-weight and per-absorbed-photon efficiency rankings are: MACE > CASPc > $TPPS_4$ > PII and $TPPS_4$ > MACE > PII > CASPc, respectively.

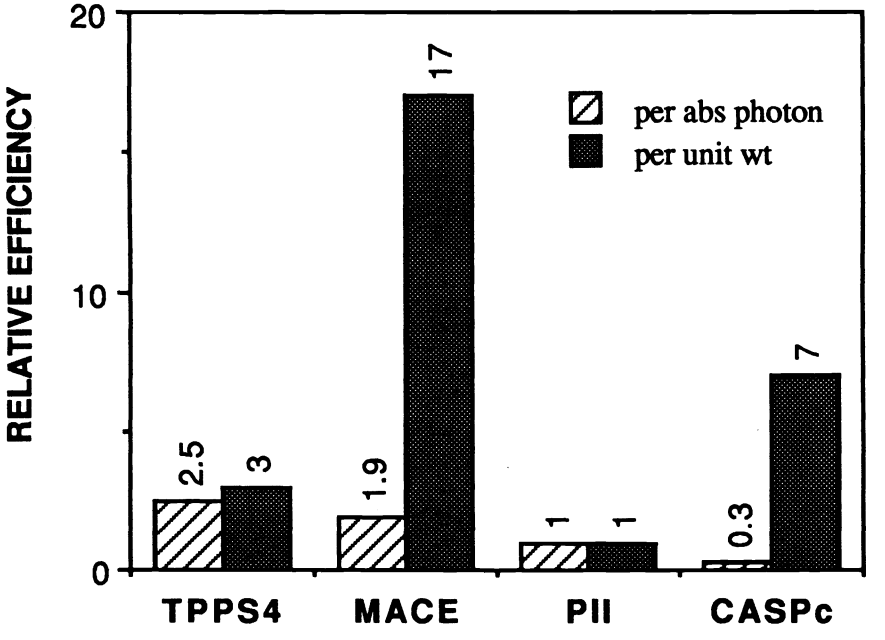

Figure 4. Relative singlet oxygen generation efficiency per unit weight and per absorbed photon.

In order to assess the relevance of these solution measurements to cellular studies, oxygen consumption of irradiated photosensitizers in CHO cells and clonogenicity assays were pursued. Relative oxygen consumption rates and photosensitizer uptake for PII, MACE, CASPc, and $TPPS_4$ are summarized in Table 1. Uptake was determined by measuring the difference in absorbance between the 15 μg/ml photosensitizer solutions before and after the 24 hour incubation period. When cellular oxygen consumption is considered on a per-unit-weight basis (by taking into account cellular uptake), relative efficiencies are dramatically different from those predicted by solution studies: PII ≈ CASPc > MACE >> $TPPS_4$ (1 : 0.9 : 0.3 : ?). We were unable to record significant O_2 depletion rates for $TPPS_4$-incubated cells, perhaps due, in part, to its relatively low cellular uptake. These efficiency differences may point to variations in subcellular localization, and, in the case of MACE, the possibility that aspartic acid-specific hydrolytic enzymes may degrade intracellular material.[12] Additional discrepanicies may arise due to the fact that cellular receptors are not exclusively singlet oxygen quenchers (as is Fur) and oxygen consumption may reflect the generation of other oxygen intermediates (e.g. superoxide).

Table 1. Photosensitizer Uptake and Relative Oxygen Depletion Rates in Cells

Sensitizer	Rel. O_2 depletion rate/cell	Uptake (g/cell)	Rel. O_2 depletion rate/gram
PII	1	1.2×10^{-12}	1
MACE	0.38	1.6×10^{-12}	0.28
CASPc	1.1	1.4×10^{-12}	0.9
$TPPS_4$	—	0.6×10^{-12}	—

Relative cell-killing efficiency was evaluated by performing clonogenicity assays on CHO cells incubated with varying concentrations of each photosensitizer. Light doses and irradiation wavelengths were identical to those used in *in vitro* oxygen consumption measurements. These results, summarized in Figure 5, indicate that relative (per-unit-weight) cell killing efficiency at 50% survivability levels is: CASPc > PII > MACE >> $TPPS_4$. Although these relative efficiencies are significantly different from those predicted by solution measurements, there is a reasonable correlation between clonogenicity and *in vitro* suspension results. This relationship is not unexpected since cellular oxygen consumption is presumed to be responsible for cell death. In fact, differences between relative efficiencies using these techniques were only observed for CASPc and PII. This inconsistancy may have been due to alternate cytotoxicity mechanisms or, perhaps, to non-linear drug uptake; relative cytoxicity was evaluated using a 0.25-15 μg/ml range of incubation solutions while cellular O_2 consumption was performed on 15 μg/ml-incubated cells. Despite these potential problems, *in vitro* O_2 consumption rates are clearly a better predictor of cell survivability than solution measurements and this relationship should be explored in more detail.

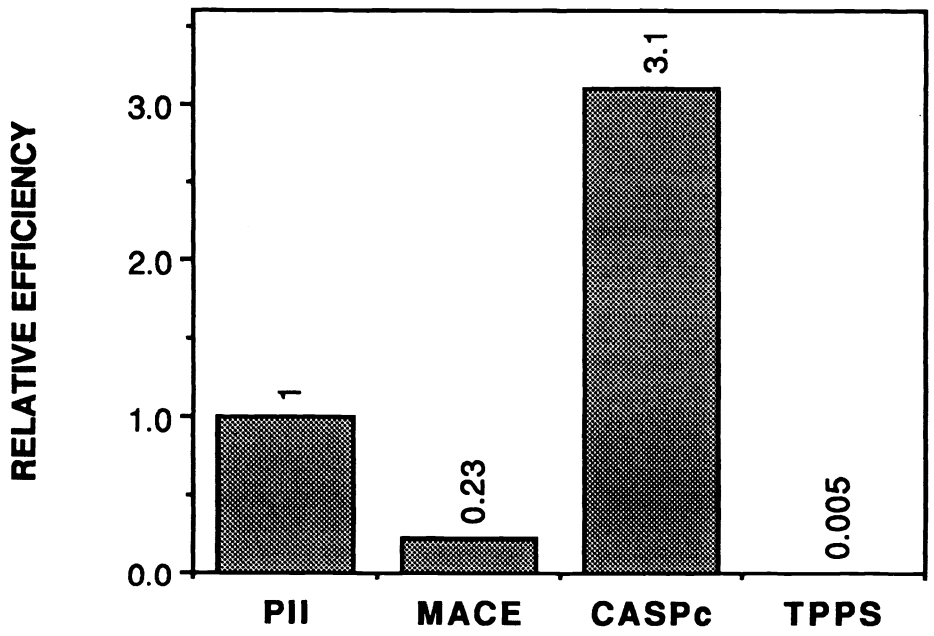

Figure 5. Relative cell-killing efficiency per-unit-weight of photosensitizer; based on clonogenicity data at 50% survival level.

4. CONCLUSIONS

Indirect measurements of photosensitizer singlet oxygen generation can be performed simply and rapidly using microelectrodes and singlet oxygen-specific quenchers. Oxygen consumption rates of photosensitizer-containing cells can be evaluated in a similar manner, although the relative efficiencies of oxygen depletion (per unit weight of photosensitizer) appear to be dependent on the drug's location, i.e. solution or cell. When cell killing efficiency (clonogenicity) is evaluated, solution measurements of singlet oxygen generation efficiency seem to be poor predictors of relative drug efficacy, however oxygen consumption rates in cell suspensions may be suggestive of photosensitizer cytotoxicity.

5. ACKNOWLEDGEMENTS

The authors wish to thank Drs. Kevin Smith and F-Y. Shiau at the University of California-Davis, Department of Chemistry, for their generous gift of mono-L-aspartyl chlorin e_6 (MACE). This work was supported by NIH grants CA-32248 and RR0192. B.J.T. acknowledges support from the Hewitt Foundation for Medical Research.

6. REFERENCES

1. R. C. Straight and J. D. Spikes, "Photosensitized oxidation of biomolecules." In Singlet oxygen: polymers and biomolecules, A. A. Frimer ed.; CRC Press, Boca Raton, pp 92-143 (1985).

2. K. R. Weishaupt, C. J. Gomer, and T. J. Dougherty "Identification of singlet oxygen as the cytotoxic agent in photoinactivation of a murine tumor" Cancer Res. 36, 2326-2329 (1976).

3. H. Baumgartl, and D. W. Lubbers "Microcoaxial needle sensor for polarographic measurement of local O_2 pressure in the cellular range of living tissue. Its construction and properties" In Polarographic oxygen sensors: aquatic and physiological applications, E. Gnaiger and H. Forstner eds.; Springer-Verlag, New York, pp. 37-65 (1983).

4. P. E. Murasecco, E. Oliveros, A. M. Braun and P. Monnier, "Quantum yield measurements of the hematoporphyrin derivative sensitized singlet oxygen production" Photobiochem. Photobiphys. 9, 193-201 (1985).

5. W. G. Roberts, F-Y. Shiau, K. M. Smith, J. S. Nelson, and M. W. Berns "In vitro characterization of mono-L-aspartyl chlorin e6 and diaspartyl chlorin e6 for photodynamic therapy" J. Natl. Cancer Inst. 80, 330-336 (1988).

6. D. Kessel "Proposed structure of the tumor-localizng fraction of HPD" Potochem. Photobiol. 44, 193-196 (1986).

7. V. Gottfried, D. Peled, J. W. Winkelman, and S. Kimel "Photosensitizers in organized media: singlet oxygen production and spectral properties" Photochem. Photobiol. 48, 157-163 (1988).

8. J. D. Spikes "Phthalocyanines as photosensitizers in biological systems and for the photodynamc therapy of tumors" Photochem. Photobiol. 43, 691-199 (1986).

9. C. J. Tralau, A. J. MacRobert, P. D. Coleridge-Smith, H. Barr, and S. G. Bown "Photodynamic therapy with phthalocyanine sensitization: quantitative studies in a transplantable rat fibrosarcoma" Br. J. Cancer 55, 389-395 (1987).

10. J. S. Nelson, W. G. Roberts, and M. W. Berns "In vivo studies on the utilization of mono-L-aspartyl chlorin (NPe6) in photodynamic therapy" Cancer Res. 47, 4681-4685 (1987).

11. J. A. Dean Lange's Handbook of Chemistry, 13th Ed., McGraw-Hill Co., New York (1985).

12. W. G. Roberts and M. W. Berns "In vitro photosensitization-I. Cellular uptake and subcellular localization of mono-Laspartyl chlorin e_6, chloroaluminum sulfonated phthalocyanine, and photofrin II" Lasers in Surgery and Medicine, in press.