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SINGLET OXYGEN GENERATION OF PORPHYRINS, CHLORINS, AND PHTHALOCYANINES

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Abstract—The production of singlet oxygen was measured indirectly for three classes of photosensitizers: porphyrins (Photofrin II, TPPS₄), chlorins (MACE, DACE), and a phthalocyanine (CASPc). Buffered solutions of sensitizers and singlet oxygen acceptors were irradiated with a CW dye laser and the oxygen depletion was monitored electrochemically with a Clark-type microelectrode. A comparison of oxygen-depletion rate constants and quantum efficiencies yields the order of efficiency of the sensitizers: TPPS₄ > MACE > PII > DACE > CASPc. For singlet oxygen acceptors the order was: furfuryl alcohol > imidazole > tryptophan. CHO cell suspensions were also used as acceptors. Here the order of efficiency (per absorbed photon) was PII > MACE ≈ CASPc. Expressed in terms of oxygen depletion per cell the order was CASPc ≈ PII > MACE. When performing cell clonogenicity studies the order of efficiencies, expressed as percentage cell kill per unit weight of sensitizer, was CASPc > PII > MACE ≈ DACE. The discrepancy between the efficiencies of sensitizers to generate singlet oxygen and their cytotoxicity was explained in terms of photodegradation (for the chlorins), intracellular localization (for PII), and contributions from a Type I mechanism (for CASPc).

INTRODUCTION

Photodynamic therapy (PDT)‡ of malignant tumors involves in intricate combination of biological, photophysical and photochemical processes (Gomer, 1987; Dougherty, 1987). These include: (i) uptake of drug (D); (ii) selective retention of D in the tumor and (iii) irradiation of the drug-containing tumor with light. While the biological mechanisms playing a role in the first two processes are largely unknown, the third process is believed (Weishaupt *et al.*, 1976) to lead to the formation of singlet molecular oxygen O₂(¹Δ_g), hereafter referred to as ¹O₂, a short-lived, highly reactive species and a known tumoricidal agent (Straight and Spikes, 1985). This may be represented by the following simplified scheme:

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‡Abbreviations: CASPc, chloro-aluminum sulfonated phthalocyanine; CHO, Chinese hamster ovary cells; DACE, di-L-aspartyl chlorin e₆; DCM, 4-dicyanomethylene-2-methyl-6(p-dimethyl amino-styryl)-4H-pyran; DHE, dihematoporphyrin ether/ester; EDTA, (ethylenedinitrilo)tetraacetic acid disodium salt; FCS, fetal calf serum; Fur, furfuryl alcohol; Im, imidazole; MACE, mono-L-aspartyl chlorin e₆; MEM, minimal essential medium; PBS, phosphate buffered saline; PDT, photodynamic therapy; PII, Photofrin II; TPPS₄, tetraphenylporphine tetrasulfonate.

where the superscripts 1, 3,* denote a molecule in, respectively, a singlet, a triplet, and an electronically excited state. In this study, D is a porphyrin-type photosensitizer and Ac denotes an acceptor; i.e., a molecule that is oxidized by singlet oxygen and is stable with respect to ambient, ground state oxygen O₂(³Σ_g⁻), hereafter referred to as O₂. Currently, the primary photosensitizer used for clinical applications is Photofrin II (PII). In this work we have used imidazole, tryptophan and furfuryl alcohol as acceptors in order to compare the relative efficiencies of various photosensitizers to generate singlet oxygen. All compounds are evaluated with respect to PII. In addition, we describe the successful utilization of *in vitro* cell suspensions as singlet oxygen acceptors. This approach was pursued so that a pre-clinical, cost-effective test model for new drugs could be developed, thus limiting time-consuming *in vitro* studies of potential photodynamic drugs. The efficiency, on the molecular level, of different drugs can be tested in this manner and drug/light dose relationships (Fingar *et al.*, 1987) can be derived. Deviations from the linear relationship expected between measured singlet oxygen generation in buffered solutions and observed cytotoxicity may point to important differences in the uptake mechanism and localization of drugs in cells. In the present study we have investigated a selected porphyrin (TPPS₄), two novel chlorin derivatives (MACE and DACE), and one phthalocyanine compound (CASPc). They have all been shown to possess favorable properties for photosensitization. Their photodynamic performance *in vitro* will be compared to and contrasted with that of PII.

In order to determine the amount of ¹O₂ produced, buffered solutions of D + Ac were irradiated

in the presence of O_2 . The resulting decrease in O_2 concentration was monitored electrochemically by a Clark-type microelectrode (Baumgartl and Lubbers, 1983). Since Ac interacts specifically with 1O_2 , the disappearance of O_2 was correlated directly to the amount of 1O_2 produced.

Electrochemical detection has several advantages over alternative methods (e.g. spectroscopic monitoring of the disappearance of Ac) (Gottfried *et al.*, 1988; Keene *et al.*, 1986; Lambert *et al.*, 1986). The controlled-potential technique used in this work is universal and independent of the spectroscopic properties of Ac and it can also be used in an *in vivo* environment where high background scattering would impair absorbance/fluorescence measurements. In addition, when the constituents of cells serve as acceptors, spectroscopic techniques become extremely difficult and the extent of reaction (4) must be judged, after irradiation, by qualitative evaluation of cellular non-viability (e.g., clonogenicity assays).

MATERIALS AND METHODS

Photofrin II (Photomedica Inc., Raritan, N.J.) served as the reference material. The stock solution, 2.5 mg/mL, was stored at -70°C and solutions of PII were freshly prepared when needed and kept in the dark. It is well known (Grossweiner *et al.*, 1982) that the lipophilic PII is strongly aggregated in aqueous solutions. Therefore, solutions of PII in PBS (except those used in cells) were premixed with Triton X-100, a high molecular weight surfactant. In all calculations involving the molarity of PII we assumed, perhaps somewhat arbitrarily (Keir *et al.*, 1987), that PII (with Triton or in cells) acted as a monomeric species with a molecular weight of 1250 dalton, corresponding to that of its major active component, DHE (Kessel, 1986), and having molar extinction coefficients $\epsilon_{630} = 4.4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ and $\epsilon_{405} = 1.5 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

Tetraphenylporphyrine tetrasulfonate (Porphyrin Products, Logan, UT) is a stable, synthetic porphyrin, molecular weight 1239 dalton and $\epsilon_{630} = 4.3 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, exhibiting pronounced selective retention in tumors (Winkelman, 1962); its photosensitization capability is well studied (Gottfried *et al.*, 1988; Sacchini *et al.*, 1987; Evensen and Moan, 1987). It was used in this work because it shares several characteristics with the newly developed experimental photosensitizers, CASPe, MACE and DACE; all being monomeric, anionic and hydrophilic. As a result, they are expected to possess similar cellular uptake mechanisms.

Chloro-aluminum sulfonated phthalocyanine (Ciba-Geigy, Switzerland) is a well-characterized sensitizer used in PDT (Spikes, 1986). It was used as supplied by the manufacturer, without further purification. Previous studies indicate that this material has an average of three sulphonic acid groups per molecule (Tralau *et al.*, 1987). It has a molecular weight of 835 and possesses a strong absorption band at 675 nm ($\epsilon_{675} = 7.3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ measured by us in PBS). It is photostable and, in contrast with PII, exhibits no skin photosensitivity (Roberts *et al.*, 1989a).

Mono-L-aspartyl chlorin ϵ_6 (molecular weight 711) and DACE (molecular weight 826) are experimental chlorin derivatives which absorb at 654 nm, $\epsilon = 2.1 \times 10^4$ and $\epsilon = 1.2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, respectively. They produce no skin photosensitization and their *in vitro* (Roberts *et al.*,

1988) and *in vivo* (Nelson *et al.*, 1987) photosensitizing characteristics have been previously described.

Furfuryl alcohol (Eastman Kodak Co., Rochester, NY), Im (Sigma Chemical Co., St. Louis, MO) and Tryptophan (Sigma) were used as received. Triton X-100 (Eastman Kodak) is highly viscous and was first diluted with PBS to a 10% (vol/vol) solution before adding it to PII to make 1% (vol/vol) solutions. Chinese Hamster Ovary cells (CHO-K1, *Cricetulus griseus*, ATCC CCL 61) were grown in MEM (Gibco, Grand Island, NY) supplemented with 0.3 mg L-glutamine/mL, 100 U penicillin G/mL, 100 μg streptomycin/mL, and 10% FCS (Gibco). Prior to each experiment, CHO cells were incubated for 24 h with 15 mg/mL doses of the various sensitizers. Following incubation, cells were removed from the flask, using 0.1% trypsin with 0.025% EDTA, gently centrifuged, and resuspended in PBS at an initial concentration of 1×10^6 cells/mL.

Irradiations were performed with a Cooper Lasersonics (Santa Clara, CA) model 770 DL argon pumped dye laser, using DCM dye which permits tuning over the therapeutic wavelengths, 630 nm, 654 nm, and 675 nm. For irradiation at 405 nm we used a Coherent (Palo Alto, CA) model 90-K krypton ion laser.

Disposable plastic cuvettes, $1 \times 1 \times 4$ cm, were filled with 2 mL solutions of D+Ac in PBS and placed in a water bath kept at $22 \pm 1^\circ\text{C}$. A 400- μm diameter fused-silica optical fiber was coupled to the laser and terminated under water at a distance of 1 cm from the cuvette and 0.7 cm above the bottom. This resulted in an irradiated area of 1 cm^2 in the mid-lower section of the sample cell. Power densities were 60 mW/cm² in the red spectral region and 10 mW/cm² at 405 nm, as measured with a Coherent model 210 power meter. The irradiation time for each sample was 3 min.

The concentration of dissolved oxygen was measured electrochemically (Baumgartl and Lubbers, 1983) using a Clark-type microelectrode (Micro-Sense, Ramat Gan, Israel). The principle of operation of the electrode is based on diffusion of oxygen molecules through the sample and across the silicon membrane of the microelectrode. Oxygen is then reduced at the cathode creating an electric current in the outer circuit. Because of the small dimensions of the electrode tip (typically 10 μm diam) the intrinsic oxygen depletion caused by the measurement was minimal and no stirring of the solution was required. The current, in the pico-ampere range, was measured by a specially designed, adjustable amplifier (Diamond Electro-Tech Inc., Ann Arbor, MI), which also maintained the cathode at a potential of -0.75 V dc. The analog output of the microelectrode was transferred onto a strip-chart recorder. The electrodes exhibited a linear response over the entire $[O_2]$ range, from zero up to $1.3 \times 10^{-3} \text{ M}$ (obtained by bubbling O_2 through the solution). Electrodes were calibrated in oxygen-free PBS either by bubbling N_2 through the solution for 30 min prior to immersion or by adding sodium dithionite, $\text{Na}_2\text{S}_2\text{O}_4$, to the solution. Between each run the electrode was immersed several times in distilled water to remove any sensitizer adsorbed onto the silicon membrane, and then in aerated PBS. The reading in the latter case was taken to correspond to the standard value, $[O_2] = 2.7 \times 10^{-4} \text{ M}$, in aerated water at 22°C (Dean, 1985); all measurements of oxygen depletion were relative to this value. At each point in time a measurement of $[O_2]$ in an open cuvette involves a dynamic equilibrium between O_2 depletion through reaction 4 and replenishment of O_2 by diffusion from non-irradiated portions of the sample and from the atmosphere. All measurements were therefore performed in a reproducible manner by positioning the electrode tip with a micro-manipulator in exactly the same spot in the middle of the cuvette. In addition, since electrochemical measurements are sensitive to temperature variations (signals

Figure 1. Typical electrode response during 3-min, 50-mW irradiation at 630 nm. (A) PII; (B) PII + imidazole; (C) PII/Triton X-100 + imidazole.

change by 5%/K), the sample cuvettes were placed in a large-volume water bath in order to dissipate heat that may have been generated by energy absorbed from the incident laser beam. Using this approach, stable electrode readings were obtained during the entire 3-min irradiation period for all acceptor-free photosensitizer control solutions.

RESULTS

Figure 1 shows a typical electrode response. Blank measurements, performed on solutions of PBS alone, D in PBS, and sensitizer-free CHO cell suspensions, resulted in no change in electrode current during irradiation (Fig. 1A). For solutions of D and Ac it can be seen (Fig. 1B) that when the laser was switched on there was an immediate decrease in $[O_2]$. For some D+Ac systems, the depletion rate, $d[O_2]/dt$, was constant over the entire irradiation period. For other systems, other power densities, or other concentrations of D or Ac, the depletion followed a bi-phasic pattern: a fast initial depletion followed by a slower one. This may have been due, in part, to the rapid disappearance of O_2 at the electrode tip, thus changing oxygen mass transport and reaction rate conditions. For PII solutions, addition of the surfactant Triton X-100 dramatically increased the rate of oxygen consumption (Fig. 1C). This response is due to the fact that, upon addition of a surfactant, aqueous solutions of lipophilic PII change from highly aggregated to monomeric or low oligomeric form (Lambert *et al.*,

1986). Reduced PII aggregation facilitates generation of 1O_2 , thus causing the observed enhancement in oxygen consumption (Gottfried *et al.*, 1988). For this reason, all measurements involving PII were performed in 1% (vol/vol) Triton X-100 solutions.

A discussion of the dynamic equilibrium of $[O_2]$ in the region close to the tip of the microelectrode is beyond the scope of the present study. Therefore, we will analyze the results phenomenologically using the cumulative depletion rate, $\Delta[O_2]/\Delta t$, determined at the end point of the 180-s measurement period. Although the initial depletion rate, derived from the initial slope of Fig. 1C, $d[O_2]/dt$, may be the more accurate criterion for comparing photosensitizers, the cumulative depletion rate provides a more precise, time-averaged indicator of biological/clinical effectiveness. In Figs. 2 A–F oxygen depletion is depicted for each photosensitizer as a function of sensitizer and acceptor concentration. These data were obtained, as described above, by laser irradiation of cuvettes which contained a known concentration of sensitizer, [D], and acceptor, [Ac]. The relative oxygen consumption efficiency per unit weight of drug can be determined by inspection of the x-axis values. For example, a comparison of MACE (Fig. 2C) with PII (Fig. 2B) indicates that comparable O_2 depletion was achieved in both cases, despite a 16-fold higher concentration (by weight) of PII. A summary of relative O_2 depletion efficiency, averaged for all D concentrations, is presented in Fig. 4. Comparisons are given for each photosensitizer, relative to PII, on a per-unit-weight basis.

The relative efficiency of different singlet oxygen acceptors can be evaluated in the same manner. For example, comparison of the acceptors imidazole and furfuryl alcohol reveals that for imidazole/MACE samples (Fig. 3), between two and four times higher drug concentrations were necessary to effect the same O_2 depletion observed with furfuryl alcohol/MACE mixtures (Fig. 2C). When tryptophan was used as an acceptor, even larger concentrations of D were required to elicit comparable responses to imidazole. These results suggest that acceptor efficiency can be ranked in the following order: furfuryl alcohol > imidazole > tryptophan. The practical implication of this ranking is that smaller concentrations of D and Ac can be used when furfuryl alcohol is the acceptor. For this reason, experiments were primarily conducted with furfuryl alcohol acceptor.

A summary of measured photosensitizer characteristics is presented in Table 1. These results compare absorbance, moles of photons (Einstein) absorbed, and estimated triplet drug concentration ($[^3D^*]$) as a function of photosensitizer concentration in PBS. All values are reported for specified wavelengths; total incident energies (E_{incid}) are based upon three-minute sample irradiations. Estimations of $[^3D^*]$ were made using Eq. 5:

Figure 2. Oxygen depletion as a function of photosensitizer and furfuryl alcohol concentrations; irradiation 3 min: (A) 10 mW/cm², 405 nm. (B) 60 mW/cm², 630 nm. (C) 60 mW/cm², 654 nm. (D) 60 mW/cm², 675 nm. (E) 60 mW/cm², 638 nm. (F) 60 mW/cm², 675 nm. [FUR] for all cases is shown in frame A.

$$[^3D^*] = \phi_{isc}(K/V)\tau \quad (5)$$

where ϕ_{isc} is the intersystem crossing quantum efficiency, defined by the ratio of the rate constant for intersystem crossing (k_{isc}) and the sum of k_{isc} and competing rate constants for radiative (e.g. fluorescence) and non-radiative processes, K is the rate of photon absorption (photons/second), V is the irradiation volume, and τ is the effective, non-radiative lifetime of $^3D^*$. A typical value of τ for porphyrin-type photosensitizers in air-saturated aqueous solutions is 2 μ s (Pottier and Truscott, 1986) and ϕ_{isc} ranges typically from 0.4 to 0.9. We

assumed similar values for the other photosensitizers. Based on 1-ml irradiation volumes and incident power densities used, $[^3D^*]$ values were estimated to be in the pM (10^{-12} M) region (see Table 1).

The observed rate constants (k_{obs}) for the depletion of O₂ were calculated by evaluating $d[O_2]/dt$ (from $\Delta O_2/\Delta t$) and solving for k_{obs} in Eq. 6:

$$-d[O_2]/dt = -d[Ac]/dt = k_{obs}[^3D^*][Ac] \quad (6)$$

Equation 6 can be derived by treating $^3D^*$ and 1O_2 as rapidly reacting intermediates and applying the

Table 1. Measured characteristics of photosensitizers

Drug	Units	Values			Remarks
PII/Triton	mg/l	5	10	20	for mol wt = 1250 (a) at 630 nm $E_{\text{incid}} = 10.8 \text{ J}$
[PII]/Triton	μM	4	8	16	
[$^3\text{PII}^*$]/Triton	pM	25	49	94	
A	Absorbance	0.0175	0.035	0.070	
E_{abs}	Einstein $\times 10^{-6}$	2.2	4.4	8.5	
PII/Triton	mg/l	0.4	0.8	1.2	for mol wt = 1250 (a) at 405 nm $E_{\text{incid}} = 1.8 \text{ J}$
[PII]/Triton	μM	0.3	0.6	0.9	
[$^3\text{PII}^*$]/Triton	pM	6.6	13	19	
A	Absorbance	0.046	0.092	0.138	
E_{abs}	Einstein $\times 10^{-6}$	0.6	1.3	1.7	
DACE	mg/l	1.25	2.5	5	mol wt = 826 (a) at 654 nm $E_{\text{incid}} = 10.8 \text{ J}$
[DACE]	μM	1.5	3	6	
[$^3\text{DACE}^*$]	pM	26	51	99	
A	Absorbance	0.0176	0.0352	0.0703	
E_{abs}	Einstein $\times 10^{-6}$	2.3	4.6	8.9	
CASPC	mg/l	1.25	2.5	5	mol wt = 835 (a) at 675 nm $E_{\text{incid}} = 10.8 \text{ J}$
[CASPC]	μM	1.5	3	6	
[$^3\text{CASPC}^*$]	pM	150	270	430	
A	Absorbance	0.1094	0.2188	0.4377	
E_{abs}	Einstein $\times 10^{-6}$	13.7	24	39	
MACE	mg/l	0.31	0.62	1.25	mol wt = 711 (a) at 654 nm $E_{\text{incid}} = 10.8 \text{ J}$
[MACE]	μM	0.44	0.88	1.76	
[$^3\text{MACE}^*$]	pM	14	28	54	
A	Absorbance	0.0092	0.0184	0.0368	
E_{abs}	Einstein $\times 10^{-6}$	1.3	2.5	4.9	
TPPS	mg/l	1	2	4	mol wt = 1239 (a) at 638 nm $E_{\text{incid}} = 10.8 \text{ J}$
[TPPS ₄]	μM	0.83	1.66	3.32	
[$^3\text{TPPS}_4^*$]	pM	5.2	10.4	21	
A	Absorbance	0.0036	0.0071	0.0142	
E_{abs}	Einstein $\times 10^{-6}$	0.47	0.94	1.88	

(a) Based on triplet lifetime $\tau = 2 \mu\text{s}$ and $\phi_{\text{isc}} \approx 1$ (see text).

Figure 3. Oxygen depletion as a function of MACE and imidazole concentrations. Irradiation 3 min, 60 mW/cm², 654 nm.

steady state approximation to their disappearance. Actually, k_{obs} is a composite of several rate constant terms which describe the entire photochemical process. It includes the relationship between [$^3\text{D}^*$] and

Figure 4. Per-unit-weight photosensitizer efficiencies, relative to PII, based on O₂ depletion caused by furfuryl alcohol quenching of singlet oxygen.

[$^1\text{O}_2$], given by the ratio of k_d and all competing [$^3\text{D}^*$] decay processes. Singlet oxygen generation quantum efficiency is derived from Eq. 7 (Mura-secco *et al.*, 1985; Gottfried *et al.*, 1988)

$$\begin{aligned} \phi_{\Delta} &= n^1\text{O}_2/n_{\text{abs},\Delta} \\ &= (n\text{O}_2/n_{\text{abs},\Delta})\{k_d + (k_c + k_p)[\text{Ac}]\}/k_c[\text{Ac}] \end{aligned} \quad (7)$$

where $n^1\text{O}_2/n_{\text{abs},\lambda}$ is the number of singlet oxygen molecules produced and $n^1\text{O}_2/n_{\text{abs},\lambda} = n\text{Ac}/n_{\text{abs},\lambda}$ is the number of ground state oxygen molecules depleted per absorbed photon. It should be noted that ϕ_Δ is larger than the quantum efficiency for oxygen depletion since chemical quenching is only one of the various pathways for deactivation of $^1\text{O}_2$. The rate constant for chemical quenching, k_c , described by Eq. 4, is $4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ for imidazole (Monroe, 1985) and $1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ for furfuryl alcohol (Murasseco *et al.*, 1985). The remaining terms in Eq. 7 are the rate constants for physical quenching (k_p) and self deactivation (k_d) of singlet oxygen. Typical aqueous solution values for k_p and k_d are $0.4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (Reddi *et al.*, 1984) and $2.5 \times 10^5 \text{ s}^{-1}$, respectively. Since in the present experiments $[\text{Ac}] \approx 10^{-4} \text{ M}$, the quantity $(k_c + k_p)[\text{Ac}] \ll k_d$, and Eq. 7 reduces to:

$$\phi_\Delta = (n\text{O}_2 n_{\text{abs},\lambda}) k_d / k_c [\text{Ac}] \quad (8)$$

Values for k_{obs} and ϕ_Δ are listed in Table 2; they were calculated using Eqs. 6 and 8 while assuming, as mentioned above, that $[\text{O}_2]$ for our air-saturated aqueous solutions was $2.7 \times 10^{-4} \text{ M}$. As expected, for a given photosensitizer, ϕ_Δ values are essentially independent of excitation wavelength and type of Ac.

Cell suspension measurements were conducted based on this observation. Due to the low absorbance of porphyrins at 630 nm, irradiation at this wavelength resulted in signal-to-noise ratios which were near the detection limit for O_2 electrochemical measurements. Therefore, when monitoring the oxygen depletion for low concentrations of PII or cell suspensions, irradiation experiments at 630 nm were supplemented with experiments using 405 nm.

Standard cell clonogenicity studies were performed in order to evaluate the cytotoxicity of PII, CASPc, MACE and DACE. The efficiency of each photosensitizer, relative to PII, is compared on a per-unit-weight basis for 25, 50 and 75% survival rates. The results, illustrated in Fig. 5, indicate that for a given irradiation intensity, four times the dose of PII was required to kill CHO cells at the same (25%) level of survivability as a dose of CASPc. In contrast, at the same survivability level (25%),

Table 2. Oxygen depletion rate constants (k_{obs}) and singlet oxygen quantum efficiencies (ϕ_Δ)

Sensitizer	λ (nm)	Acceptor	$k_{\text{obs}} \times 10^{-7}$ ($\text{M}^{-1} \text{ s}^{-1}$)	ϕ_Δ
PII	405	furfuryl	4.9 ± 1.2	0.26
PII	630	furfuryl	5.0 ± 1.4	0.25
PII	630	imidazole	2.3 ± 0.53	0.28
MACE	654	furfuryl	9.5 ± 3.0	0.48
MACE	654	imidazole	2.3 ± 0.47	0.47
DACE	654	furfuryl	4.0 ± 0.60	0.20
CASPc	675	furfuryl	0.35 ± 0.09	0.085
TPPS ₄	638	furfuryl	13 ± 3.3	0.63

Figure 5. Per-unit-weight photosensitizer efficiencies, relative to PII, based on *in vitro* dose response determined by clonogenicity assay.

MACE and DACE were, respectively, only 14 and 10% as effective as PII.

Chinese hamster ovary cells were further evaluated with respect to their ability to serve as acceptors for photochemically generated oxygen intermediates. Cells were incubated for 24 h with equal concentrations (15 mg/ml) of three photosensitizers; PII, MACE, and CASPc. Cellular uptake was determined by spectrophotometric analysis of the supernatant and was found to be roughly equivalent for each drug (Table 3). At least three measurement solutions containing varying cell suspension concentrations were prepared for each drug. Cellular oxygen consumption was measured with a microelectrode for each suspension during laser irradiation. The rate of oxygen depletion for various drug types is listed in Table 3. These values range from $(8.0 \pm 0.85) \times 10^7$ to $(2.3 \pm 0.21) \times 10^8$ molecules $\text{O}_2 \text{ cell}^{-1} \text{ s}^{-1}$ for MACE and CASPc, respectively. It is interesting to note that a cellular uptake of 1.6 pg D corresponds to about 10^9 molecules of D localized in/on one CHO cell (for $D \approx 1000$ dalton). Thus, the oxygen consumption rate implies that, on the average, for 10 molecules D one molecule O_2 is consumed per second in the cellular oxygenation process. These oxygen depletion rates are smaller than those observed for furfuryl alcohol and imidazole mediated reactions. It should be emphasized that the *in vitro* cellular oxygen consumption reaction is not restricted to a single quenching mechanism. The high chemical reactivity of furfuryl alcohol toward $^1\text{O}_2$ ensures that the measured O_2 depletion in solution is the result of a Type II (singlet oxygen depletion) mechanism (Murasseco *et al.*, 1985). In the case of cellu-

Table 3. Photosensitizer uptake and oxygen depletion rates for CHO cells

Sensitizer	Uptake (g/cell)	O_2 consumed (molecules $\text{cell}^{-1} \text{ s}^{-1}$)
PII	1.2×10^{-12}	$(2.1 \pm 1.3) \times 10^8$
MACE	1.6×10^{-12}	$(8.0 \pm 0.85) \times 10^7$
CASPc	1.4×10^{-12}	$(2.3 \pm 0.21) \times 10^8$

Figure 6. Per-absorbed-photon photosensitizer efficiencies based on oxygen depletion by CHO cell suspensions.

lar oxygenation, however, the measured O_2 depletion may be the result of either Type I or Type II mechanisms owing to the lack of homogeneity of cellular structure and chemical composition.

DISCUSSION

On a per-absorbed-photon basis, TPPS₄ appears to generate more singlet oxygen in buffered solution than the other photosensitizers. The order of efficiency is: TPPS₄ > MACE > PII > DACE ≫ CASPc, as determined by comparing the ϕ_Δ values listed in Table 2.

The per-photon efficiency of O_2 cellular depletion (Fig. 6) follows a different trend than in buffered solutions: PII > MACE = CASPc. The fact that PII is more efficient than CASPc is not surprising since, in buffered solutions, ϕ_Δ for PII is roughly three times larger than for CASPc. In contrast, MACE exhibits an anomalously poor cellular oxygen consumption. This incongruence is illustrated by the fact that the ϕ_Δ value for MACE is roughly twice that of PII, while per-photon cellular oxygen consumption is an order of magnitude less. On a per-weight basis the MACE cellular oxygen consumption rate (Table 3) is less than half that of PII and CASPc. This may be understood when one considers its susceptibility to photodegradation and its metabolic instability. Previous experiments (Roberts *et al.*, 1989a) show MACE to be particularly sensitive to photodegradation when complexed with proteins or in cells.

Mono-L-aspartyl chlorin ϵ_6 is localized in enzyme-containing lysosomes of cells by endocytosis (Roberts *et al.*, 1989b). Studies on cellular retention rate suggest that MACE, which contains a naturally occurring enzymatic substrate (aspartic acid), is rapidly degraded, probably as a result of attack by hydrolases (Roberts and Berns, 1989). The combination of high susceptibility to photodegradation and enzymatic degradation will result in a photochemically inefficient sensitizer. These characteristics explain, in part, the low phototoxicity of MACE

(Fig. 6) despite the high quantum yield (Table 2). In PBS solutions, however, at the low laser fluences used in the present work, MACE is relatively photostable. This can be deduced from Fig. 2C which shows that, at constant MACE concentrations, increases in [Ac] result in proportional % O_2 depletions. If MACE participated in the quenching reaction (i.e. self-photooxidation) a sub-linear relationship would have been observed.

Interestingly, in the case of CASPc-mediated reactions, the ability of cells to consume 1O_2 is markedly increased over that of buffered furfuryl alcohol solutions. This is illustrated by the contrast between the low k_{obs} and ϕ_Δ values (Table 2), and the relatively large rate of oxygen consumption in CASPc-incubated cells. Conceivably, CASPc used in this study is not entirely monomeric in aqueous solutions and this may, in part, account for the comparatively low ϕ_Δ values. Corroborative evidence for this is given by the fact that the Ciba-Geigy supplied CASPc has been described as possessing an average of 3 sulphonic acid groups per molecule (Tralau *et al.*, 1987) and, as such, exhibits slightly poorer water solubility than the tetra-sulfonated compound. Furthermore, $\epsilon_{675} = 7.3 \times 10^4$, measured by us in PBS, is smaller than that reported for pure, highly water-soluble tetra-sulfonated CASPc, $\epsilon_{675} = 1.5 \times 10^5$ (Darwent *et al.*, 1982).

Alternatively, it should be noted that cellular oxygen consumption rates are reflective of any oxygen intermediate (singlet oxygen as well as superoxide, O_2^-). Thus, 1O_2 generation alone may not explain the photodynamic efficacy of CASPc. Perhaps, for some phthalocyanines, e.g. CASPc (Ben-Hur, 1987; Ferraudi *et al.*, 1988), there is a Type I (superoxide generation) component to cytotoxicity, although this may not be the general case (Valduga *et al.*, 1988). Further evidence for this hypothesis is provided by the low value of k_{obs} for CASPc in buffered solutions (Table 2). Following irradiation, $^3D^*$ can collide with O_2 to produce 1O_2 (Eq. 3), or it can participate in a Type I reaction (Foote, 1984). By comparing k_{obs} values, the propensities of photosensitizers to participate in Type I and Type II reactions can be evaluated. From the results listed in Table 2, it appears that reactions involving CASPc may occur, to some extent, via Type I reaction intermediates which are not detectable with furfuryl alcohol as an acceptor. In contrast, TPPS₄-initiated oxidations occur predominantly through singlet oxygen intermediates and, therefore, furfuryl alcohol-mediated values of k_{obs} and ϕ_Δ are high. Additional support for this reasoning can be derived by examining the raw data in Fig. 2. For all photosensitizers, increases in [D] are accompanied by a concomitant increase in oxygen consumption (at a given [Ac]). In the case of CASPc, however, a sub-linear relationship is observed (Fig. 2F), indicating, perhaps, that CASPc itself quenches $^3D^*$ and O_2^- is produced.

Cell clonogenicity data (Fig. 5) show that, per-unit-weight, CASPc is four times more cytotoxic than PII. While this dose-response relationship may be of importance to *in vivo* studies, more detailed conclusions can be drawn regarding cellular uptake mechanisms. For equal weights of CASPc and PII the number of absorbed photons is vastly different. Inspection of Table 1 reveals that for 5 mg/ml, the ratio $E_{\text{abs}}(\text{CASPc})/E_{\text{abs}}(\text{PII}) = 39/2.2 \approx 18$. If we assume that the overall quantum yield of the combined (Type I+Type II) reaction mechanisms of CASPc is equal to that of PII one would expect also an 18-fold higher cytotoxicity. The fact that CASPc is 'only' four times more cytotoxic alludes to the importance of subcellular localization: PII localizes in the mitochondria (Berns *et al.*, 1982), crucial organelles for cellular viability, whereas CASPc localizes in the 'less critical' lysosomes (Roberts and Berns, 1989).

In conclusion, these studies demonstrate that the microelectrode measurement of oxygen consumption can be used to rapidly and cost-effectively screen photosensitizers even in very small amounts. Although measurements of relative efficiencies are comparatively simple, they can be designed to provide a considerable amount of useful information. However, analyses of buffered aqueous systems do not characterize the *in vitro* oxidizing capabilities of photosensitizers and this technique alone should not be used to predict the therapeutic value of a given drug. In fact, the photochemical characteristics of PDT sensitizers in solution, if not combined with *in vitro* and/or *in vivo* studies, can be misleading. As we have demonstrated, recording and comparing oxygen consumption rates, using *in vitro* systems as well as solutions, provides a clearer picture of the mechanism of action of each drug. These results show the important roles that sensitizer localization and stability play in comparing sensitizer *in vitro* efficiencies.

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