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The photophysical properties of a ruthenium-substituted phthalocyanine

P. Charlesworth[a], T.G. Truscott[a], R.C. Brooks[b], B.C. Wilson[c]

[a]*Chemistry Department, Keele University, Keele, Staffordshire, ST5 5BG, UK*
[b]*Johnson Matthey Biomedical Research, West Chester, PA, USA*
[c]*Ontario Cancer Institute and Ontario Laser and Lightwave Research Centre, Toronto, Ont., Canada*

Abstract

Laser flash photolysis, pulse radiolysis and time-resolved near-IR luminescence were used to study the photoproperties of a novel water-soluble metallophthalocyanine with no peripheral substitution. The phthalocyanine induces a significant reduction in tumour size or complete tumour eradication by photodynamic therapy (PDT). Yet, although this phthalocyanine sensitizes the production of singlet oxygen with a quantum yield of 0.3 in methanol, the quantum yield of singlet oxygen production in air-saturated D_2O is less than 0.01 (the detection limit of our equipment). This is explained in terms of the combined effect of the triplet state lifetime of the phthalocyanine (1.2 μs in nitrogen-saturated methanol and 0.65 μs in nitrogen-saturated water) and the near tenfold reduction in the oxygen concentration from methanol to water. Therefore we propose that, in aqueous solution, a non-singlet-oxygen-mediated mechanism, probably electron transfer, prevails and leads to the observed PDT effect.

Keywords: Laser flash photolysis; Singlet oxygen; Phthalocyanine; Photodynamic therapy; Pulse radiolysis

1. Introduction

Phthalocyanines (Pc) are currently of interest as second generation sensitizers for the photodynamic therapy (PDT) of cancer and, because of their strong red absorption band, are well suited to take advantage of the maximal tissue penetration and low light scattering properties of organelles in the 600 to 1000 nm "phototherapeutic window" [1].

Unfortunately, many phthalocyanines and metallophthalocyanines are insoluble in water and biologically compatible organic solvents. Previously, soluble forms of phthalocyanines have been prepared by the addition of sulphonic acid or carboxylic acid moieties to the periphery of the macrocycle [2]. These synthetic routes often lead to mixtures of isomers that are not easily resolved [3]. An approach which avoids this problem is to prepare a metallophthalocyanine in which the periphery is unsubstituted, and the nature of the axial ligands determines the desired property [4]. In this work, we report the photophysical properties of one such phthalocyanine (JM2929) which utilizes a ruthenium central metal atom with two triphenylphosphine monosulphonate ligands (Fig. 1).

JM2929 has been shown to be as effective as Photofrin (Quadra Logic Technologies Inc, BC) and more effective than aluminium phthalocyanine tetrasulphonate in vitro in the PDT killing of RIF-1 cells[1]. This compound has been tested in vitro against several cell lines with similar results and has been shown to kill by an oxygen-mediated process [6]. Additionally, in mice with subcutaneous RIF-1 tumours, full thickness regression was obtained with JM2929 [6][1].

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[1]For example, in radiation-induced fibrosarcoma (RIF) cells in vitro, 90% colonogenic cell kill was achieved with 18 h incubation in JM2929 at a concentration of 5 μg ml^{-1} and approximately 10^4 J m^{-2} of broad-band red light irradiation. This is comparable with the photocytotoxicity of Photofrin in these cells and considerably more toxic than chloroaluminium sulphonated phthalocyanine (tri–tetra sulphonation) under the same conditions. Similarly, for RIF grown subcutaneously in mice (approximately 8 mm in diameter), full thickness necrosis was obtained with RuPc at 20 mg kg^{-1} body weight injected intravenously and irradiated 24 h later with 150 J cm^{-2} of 670 nm light, a result comparable with the Photofrin PDT response at 10 mg kg^{-1} in this model [5].
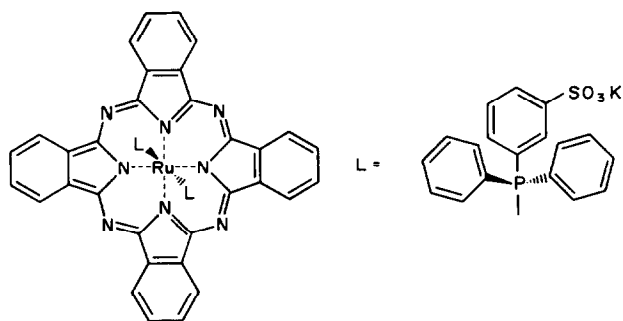
Fig. 1. Structure of dipotassium[bis(triphenylphosphinemonosulphonate)Ru(II)phthalocyanine] (JM2929).

At face value the activity of JM2929 would appear to be consistent with a singlet-oxygen-mediated mechanism. The diamagnetic d_6 ruthenium should extend the triplet lifetime of the system and enhance singlet oxygen production as reported for other metallophthalocyanines [7,8]. We have found that some of the photophysical characteristics of JM2929, namely the singlet and triplet lifetimes in aqueous solution, are in conflict with this explanation of the activity of JM2929. We conclude that a type I reaction is the primary mechanism for the photomediated cell killing seen with this compound.

2. Experimental details

Dipotassium[bis(triphenylphosphinemonosulphonate)Ru(II)phthalocyanine] (JM2929) was used for photophysical studies as received from Johnson Matthey Inc.; the full synthesis and characterization are reported elsewhere [9].

Transient absorption measurements were made in nitrogen-saturated solution by a laser flash photolysis method similar to that described by McVie et al. [10]. The monitoring source (Kratos 250 W xenon arc lamp) was focused through the front 2 mm of a sealable cuvette (10 mm path length) onto the front slits of the monochromator (Applied Photophysics, $f=3.4$, fitted with a Hamamatsu R928 red-sensitive photomultiplier). The photomultiplier output was fed via an automatic back-off unit to a Tektronix 2342A digital oscilloscope (5 ns per point, 1024 points per screen) and terminated with a 50 Ω load resistor. Pulsed laser excitation was obtained from the frequency doubled (532 nm) or frequency tripled (355 nm) outputs of a Q-switched Nd:YAG laser (Spectron lasers; 16 ns pulse width). Transients were signal averaged on the oscilloscope and transferred to a personal computer for analysis.

Singlet oxygen was detected directly by time-resolved near-IR luminescence at 1270 nm using the method of Rodgers and Snowden [11]. The 1270 nm luminescence was detected, following 532 nm pulsed laser excitation, with a room-temperature germanium pho-

todiode amplified with a Judson pre-amplifier and fed to a Tektronix 2342A digital oscilloscope. Transients were signal averaged and transferred to a personal computer for analysis. The indirect method of Kraljic and El Mohsni [12] was also used for detecting singlet oxygen in aqueous solution. In this method, the bleaching of 5×10^{-5} M p-nitroso-N,N'-dimethylaniline (RNO), in the presence of 8×10^{-3} M imidazole in air-saturated, pH 7.4 phosphate buffer, was monitored at 440 nm as a function of the phthalocyanine concentration on irradiation with 650 nm laser light.

In order to measure the photobleaching of JM2929, 2 ml aqueous samples with an initial concentration of 2.76×10^{-5} M were irradiated with 650 nm light from an argon-ion-pumped dye laser with an incident power of 100 mW (irradiance, approximately 50 mW cm^{-2}). At various times during irradiation, the absorption spectrum of the sample was measured (400–750 nm) and the absorbance at 650 nm was plotted against the irradiation time. The effect of oxygen on the photobleaching was assessed by purging the sample with dry nitrogen for 10 min prior to irradiation, with the absorption spectrum being measured before and immediately after purging.

The radical anion and radical cation transient difference spectra were determined by pulse radiolysis at the Paterson Laboratories, Christie Hospital, Manchester; the experimental arrangement has been described previously [13]. The radical anion transient difference spectrum was determined in pH 7.4 phosphate-buffered formate solution purged with dry nitrogen, and the radical cation was determined in pH 7.4 phosphate-buffered sodium azide solution. The radical cation was also generated chemically using bromine in methanol as an oxidant. To 4 ml JM2929 (1.47×10^{-5} M) was added successive 2 μl aliquots of 1.5×10^{-2} M Br$_2$ in methanol (0.5 equivalents) to give 0.5, 1 and 2 equivalents; the effect of the generation of the radical cation was followed by visible absorption spectroscopy. To confirm that the changes observed were related to the oxidation of the ring, 5 μl of 1.2×10^{-2} M CCl$_4$ (1 equivalent), which is capable of oxidizing only free phosphines to their corresponding oxides, in methanol was added to 4 ml of JM2929 (1.47×10^{-5} M) as a control.

Absorption and fluorescence measurements were made using Perkin–Elmer Lambda 2, Cary 219 and Perkin–Elmer LS-50 spectrophotometers. Fluorescence measurements were made using an absorbance of about 0.1 at the excitation wavelength (650 nm).

3. Results and discussion

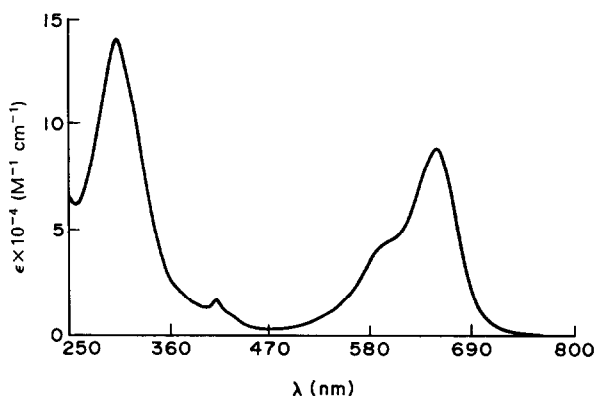The ground state absorption spectrum of JM2929 (Fig. 2) in water has a broad Q band with λ_{max} at 650

Fig. 2. Ground state absorption spectrum of JM2929 in water at a concentration of 100 μM.
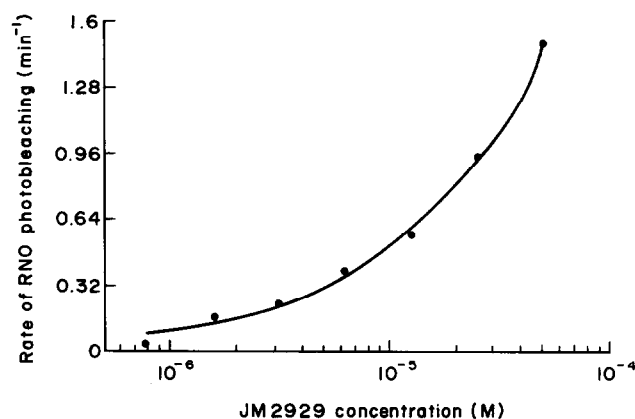

Fig. 3. Photobleaching of p-nitroso-N,N'-dimethylaniline (RNO) as a function of the phthalocyanine concentration in air-saturated phosphate buffer.
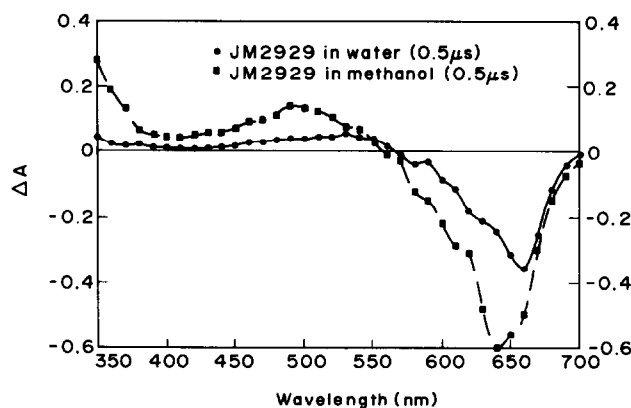

Fig. 4. Triplet difference absorption spectra for JM2929 in water and methanol (laser pulse energy ≈ 10 mJ; initial concentration of sample ≈ 10 μM).

nm ($\epsilon = 8.82 \times 10^4$ M^{-1} cm^{-1}) and shows no change in shape or peak shift with increasing concentration up to 1.0×10^{-4} M. This clearly suggests that the triphenylphosphine ligands prevent aggregation, but reduce the "fine structure" that is commonly associated with the electronic spectra of metallophthalocyanines.

Within the detection limit of our spectrophotometer, fluorescence emission was not observed in either methanol or water. Indeed, preliminary picosecond transient absorption measurements indicated that the singlet state lifetime is 60–100 ps in methanol, probably as a result of the heavy atom effect due to the ruthenium central metal atom.

Quantum yields of singlet oxygen production measured against [tetrakis(4-sulphonatophenyl)porphine] (TPPS) in D$_2$O [14] and haematoporphyrin dihydrochloride (HP·2HCl) in methanol [15] were $\Phi_\Delta < 0.01 \pm 10\%$ and $\Phi_\Delta(\text{CH}_3\text{OD}) = 0.3 \pm 10\%$ respectively. However, although no singlet oxygen luminescence was detected in D$_2$O even under oxygen-saturated conditions, no long-lived products were observed under the described conditions and no overall change in the ground state absorbance was seen. It is interesting to note, however, that the transient absorption maximum is spectrally comparable with the end product obtained in the photobleaching experiment.

JM2929 clearly sensitizes the photobleaching of RNO (Fig. 3) at high phthalocyanine concentrations, suggesting that either a very small amount of singlet oxygen is generated (below the detection limit of our system) or, as reported by Sharpatyi and Kraljic [16], RNO photobleaching is the result of electron transfer or other products.

In view of the difference between the singlet oxygen yields in CH$_3$OD and D$_2$O, the triplet state transient absorption properties were measured (Fig. 4) in methanol and water. Although a strong triplet state transient absorption was observed in both solvents, the unimolecular decay rate constants lifetimes in the absence of oxygen were $k = 8.8 \times 10^5$ s^{-1} (1.2 μs) and 1.5×10^6

s^{-1} (0.65 μs) for methanol and water respectively, and are typical of many ruthenium complexes such as the Ru(II) polypyridyls. Fig. 5 shows the transient decays measured for JM2929 in methanol and water, in the presence and absence of oxygen. Clearly, there is no significant difference in the aqueous decay curves between the air- and nitrogen-saturated solutions a rate constant of $k_q(\text{O}_2) \approx 1 \times 10^9$ M^{-1} s^{-1} was obtained for the quenching of the JM2929 excited state by oxygen in air-saturated solution.

According to the Stern–Volmer equation, the bimolecular rate constant (k_q) for the dynamic quenching of the excited state by oxygen is given by Eq. (1), where k_1 is the observed rate constant in the absence of oxygen, k_2 is the observed pseudo-first-order rate constant in the presence of oxygen and [O$_2$] is the oxygen concentration

$$k_q = \frac{(k_2 - k_1)}{[\text{O}_2]} \tag{1}$$

From Eq. (1), it is clear that the unimolecular decay rate constant and the oxygen concentration in the air-
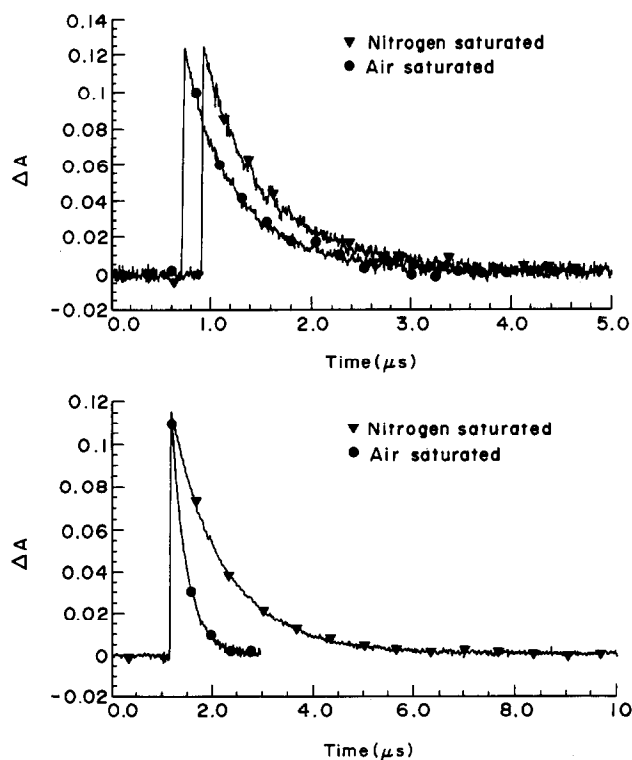
Fig. 5. Transient decays for JM2929 in water (top) and methanol (bottom) showing the effect of oxygen on the rate of decay of the excited state.
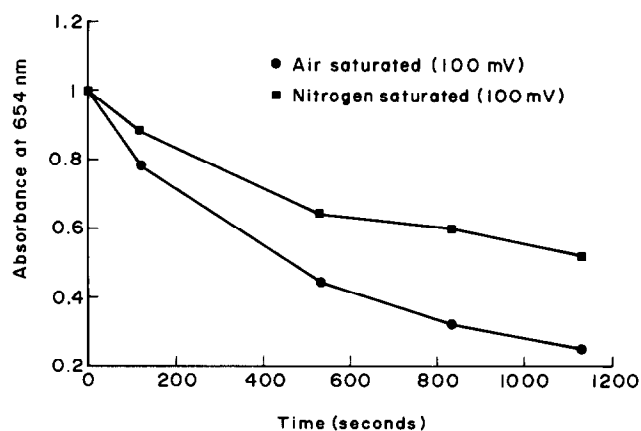

Fig. 6. Photobleaching of air- and nitrogen-saturated aqueous solutions of JM2929, induced by laser irradiation, at 650 nm, as a function of time. Each curve was normalized to the pre-irradiation absorption.

saturated solvent play a pivotal role in determining the efficiency of singlet oxygen production in the solvent. In D_2O, for example, the combined effect of a low oxygen concentration in air-saturated solution (2.65×10^{-4} M) and a short triplet lifetime results in a very low efficiency of singlet oxygen production. In methanol, however, the nearly tenfold increase in oxygen concentration (2.12×10^{-3} M) combined with a longer triplet lifetime results in an increased efficiency of singlet oxygen production relative to that in D_2O. This suggests one explanation for the observed reduction in the singlet oxygen quantum yield in D_2O relative to methanol.

Ground state photobleaching studies show that JM2929 (Fig. 6) is bleached in both nitrogen- and air-saturated aqueous solutions, although no significant spectral shape changes are observed, except for a slight increase in the absorption at about 470 nm. The rate of photobleaching in the presence of air was initially faster than that in nitrogen; thus the photobleaching effect can be accelerated by oxygen but is not dependent on its presence. There was no significant recovery of the absorbance post-irradiation, indicating that the photobleaching is irreversible. In every case, simply purging the solution with nitrogen resulted in a 20%–30% pre-irradiation drop in the absorbance. A more detailed study of this phenomenon suggests that the axial phosphate ligands on the phthalocyanine may induce mild

surfactant properties in the molecule. It was found that, when an aqueous solution of JM2929 was bubbled with a gas, significant foaming occurred and the bubbles appeared to draw the phthalocyanine out of solution, depositing it on the top and sides of the vessel. It was also noted that, after a period of time, particles of "phthalocyanine" could be observed in the remaining solution. This was not observed in methanol.

Both the photobleaching of JM2929 in the absence of oxygen and the JM2929-mediated photobleaching of RNO require electron transfer. These findings suggest that the observed oxygen-dependent photodynamic activity is not a singlet-oxygen-mediated process, due to the "short" triplet state lifetime and near-zero singlet oxygen quantum yield in aqueous solution, but rather a type I process possibly involving superoxide anion. Recent studies have shown photodynamic activity for copper benzochlorin with a triplet state transient lifetime of less than 20 ns [17], reaffirming that the electron transfer pathway may be of importance in photodynamic activity. Alternatively, the non-singlet-oxygen-dependent oxidation of 3-methylindole by manganese phthalocyanine has been reported as a model for tryptophan-2,3-dioxygenase [18]. This process involves the formation of $Mn^{3+}(Pc)-O_2^{\bullet}$ as the reactive species. However, literature data and direct experimental evidence rule against this mechanism in favour of a type I reaction. In type I reactions, interaction between the substrate and the electronically excited (usually triplet state) sensitizer leads to electron or hydrogen atom transfer giving rise to a radical pair which may undergo subsequent reaction with oxygen yielding an oxidized substrate.

The oxidation of low-spin Ru(II) to form an analogous species, extrapolated from the reduction potentials of similar complexes [19], is expected to be difficult. It has been shown by Dolphin et al. [20] using electrochemistry, UV–visible and electron spin resonance

(ESR) spectroscopy that diamagnetic Ru(II) phthalocyanine-bis(pyridine) complexes oxidize first at the phthalocyanine ring to give rise to a radical cation. JM2929 shows the same spectrophotometric and ESR changes as reported by Dolphin et al. when treated with chemical oxidants such as bromine (Fig. 7) or when photolysed using a 650 W visible photoflood lamp. It is interesting to note that the final oxidized species has an extremely broad band in the 450 to 550 nm range, suggesting that the formation of the radical cation is responsible for the increase in absorbance seen around 470 nm in the photobleaching experiments.

In view of the possibility of electron transfer reactions, the radical anion and cation were measured by pulse radiolysis (Fig. 8) as this provides an unambiguous route to the individual species. It was found that the radical anion had a half-life of about 2–3 ms, whilst that of the radical cation was greater than 20 ms. Both species show peak maxima centred around 550 nm; however, the radical cation tends towards depletion below 400 nm, whilst the anion remains as an absorption band.
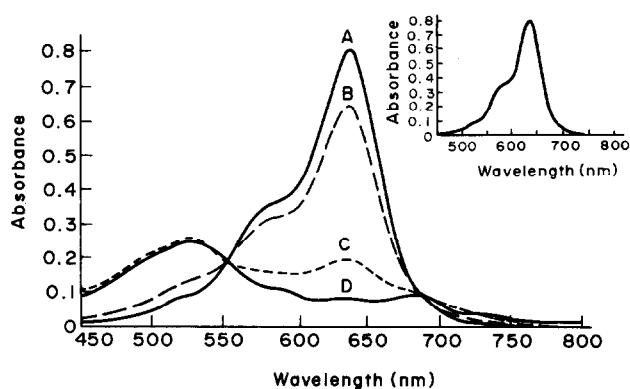

Fig. 7. Visible spectral changes during titration of JM2929 to the radical cation with bromine (15 mM in methanol): A, JM2929 before titration; B, JM2929+0.5 equivalent of bromine; C, JM2929+1 equivalent of bromine; D, JM2929+2 equivalents of bromine; inset: JM2929+1 equivalent of CCl₄ (12 mM in methanol).
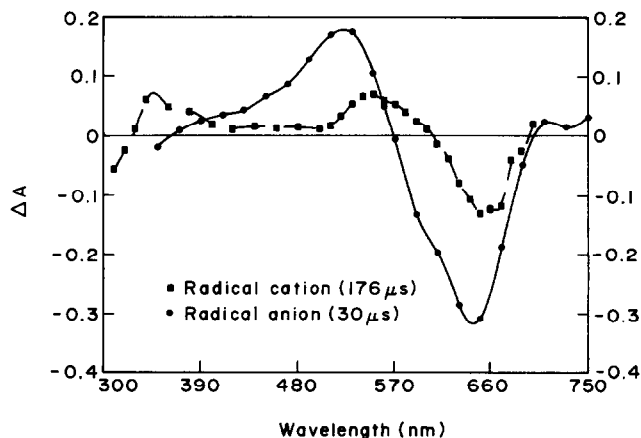

Fig. 8. Radical difference absorption spectra for radical anion and radical cation in aqueous solution.

The reason for the differences between the species is as yet unknown, and further work is required to study specifically the electron transfer reactions of this system.

It is clear that, in aqueous systems, the photophysical characteristics of JM2929 are significantly different from those of previously studied biologically active metallophthalocyanine sensitizers. At face value, the short triplet lifetime and inability to detect, by direct luminescence methods, singlet oxygen production by JM2929 in D₂O or other aqueous systems is inconsistent with reports that metallophthalocyanines containing diamagnetic metals are photobiologically active [7,9]. However, JM2929 is active both in vivo and in vitro, and its activity is oxygen dependent at concentrations and light doses similar to other established photosensitizers such as haematoporphyrin derivative [5].

4. Conclusions

Clearly, an electron transfer (type I) process is important in these observations. For JM2929 to proceed by a type II mechanism, it must be sequestered in the biological environment in such a manner that its triplet state lifetime is extended sufficiently to allow the photosensitized generation of singlet molecular oxygen. Indeed, Rodgers [21] has commented recently that a type I process may be favoured in biological systems, although for sensitizers such as Photofrin, the type II mechanism cannot be excluded [22].

This molecule, which is only one within a large class [6,9], may provide a tool to elucidate further the role of type I reactions in PDT, due to the lack of interference by singlet oxygen processes. In understanding the mechanism(s) of JM2929, the role of oxygen needs to be studied further to determine if the lethal damage is caused by superoxide anion generated by the phthalocyanine, or if oxygen functions as a "fixative" for other radical damage.

References

[1] W.M. Star, J.P.A. Marijnissen and M.J.C. van Gemert, Light dosimetry: status and prospects, J. Photochem. Photobiol. B: Biol., 1 (1987) 149–167.

[2] J.R. Darwent, P. Douglas, A. Harriman, G. Porter and M.C. Richoux, Metal phthalocyanines and porphyrins as photosensitizers for reduction of water to hydrogen, *Coord. Chem. Rev.*, *44* (1982) 83–126.

[3] H. Ali, R. Langlois, J.R. Wagner, N. Brasseur, B. Paquette and J.E. van Lier, Biological activities of phthalocyanines—X. Synthesis and analysis of sulfonated phthalocyanines, *Photochem. Photobiol.*, *47* (1988) 713–717.

[4] G. Jori, Far-red-absorbing photosensitizers: their use in the photodynamic therapy of tumours, *J. Photochem. Photobiol. A: Chem.*, *62* (1992) 371–379.

[5] G. Singh et al., manuscript in preparation.

[6] R.C. Brooks, G.E. Bossard, M.C. Darkes, G.W. Henson, M.J. Abrams, S.P. Fricker, G. Singh and B.C. Wilson, Novel water soluble phthalocyanines as photosensitizers, *Proc. Am. Assoc. Cancer Res.*, *34* (1993) Abstract—2134 358.

[7] R. Langlois, H. Ali, N. Brasseur, J.R. Wagner and J.E. Van Lier, Biological activities of phthalocyanines IV. Type II sensitised oxidation of L-tryptophan and cholesterol by sulphonated metallophthalocyanines, *Photochem. Photobiol.*, *44* (1986) 117–123.

[8] I. Rosenthal, C.M. Krishna, P. Reisz and E. BenHur, Role of molecular oxygen in photodynamic effect of phthalocyanines, *Radiat. Res.*, *107* (1986) 136–142.

[9] G.E. Bossard et al., *Inorg. Chem.*, in preparation.

[10] J. McVie, R.S. Sinclair and T.G. Truscott, Triplet states of copper and metal-free phthalocyanines, *J. Chem. Soc., Faraday Trans. II*, *78* (1978) 1870–1879.

[11] M.A.J. Rodgers and P.T. Snowden, Lifetime of $O_2(^1\Delta_g)$ in liquid water as determined by time-resolved infrared luminescence measurements, *J. Am. Chem. Soc.*, *104* (1982) 5541–5543.

[12] I. Kraljic and S. El Mohsni, A new method for the detection of singlet oxygen in aqueous solutions, *Photochem. Photobiol.*, *28* (1978) 577–581.

[13] J.P. Keene, Pulse radiolysis equipment, *J. Sci. Instrum.*, *41* (1964) 493–496.

[14] J.B. Verlhac, A. Gaudemer and I. Kraljic, Water-soluble porphyrins and metalloporphyrins as photosensitizers in aerated aqueous solutions. 1. Detection and determination of quantum yield of formation of singlet oxygen, *Nouv. J .Chim.*, *8* (1984) 401–406.

[15] R.W. Redmond, K. Heihoff, S.E. Braslavsky and T.G. Truscott, Thermal-lensing and phosphorescence studies of the quantum yield and lifetime of singlet molecular oxygen ($^1\Delta_g$) sensitized by haematoporphyrin and related porphyrins in deuterated and non-deuterated ethanols, *Photochem. Photobiol.*, *45* (1987) 209–213.

[16] V.A. Sharpatyi and I. Kraljic, Detection of singlet oxygen in radiolysis of aerated aqueous solutions, *Photochem. Photobiol.*, *28* (1978) 587–590.

[17] S.H. Selman, J.A. Hampton, A.R. Morgan, R.W. Keck, A.D. Balkany and D. Skalkos, Copper benzochlorin, a novel photosensitizer for photodynamic therapy: effects on a transplantable urothelial tumour, *Photochem. Photobiol.*, *57* (1993) 681–686.

[18] K. Uchida, M. Sona, S. Naito, T. Onishi and K. Tamaru, Manganese phthalocyanine as a model of tryptophan-2,3-dioxygenase, *Chem. Lett.*, *4* (1978) 471–474.

[19] A.B.P. Lever and J.P. Wilshire, Redox potential of metal phthalocyanines in nonaqueous media, *Can. J. Chem.*, *54* (1976) 2514–2516.

[20] D. Dolphin, B.R. James, A.J. Murray and J.R. Thornback, Synthetic and oxidation studies of ruthenium(II) phthalocyanine complexes, *Can. J. Chem.*, *58* (1980) 1125–1132.

[21] M.A.J. Rodgers, Reflections on type I photodynamic damage, *J. Photochem. Photobiol. B: Biol.*, *18* (1993) 296–298.

[22] I. Rosenthal and E. Ben-Hur, Phthalocyanines in photobiology, in C.C. Lcznoff and A.B.P. Lever (eds.), *Phthalocyanines; Properties and Applications*, VCH Publishing, London, 1989, pp. 393–425.