Inactivation of Tumours and Viruses *via* Efficient Photoisomerisation

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A novel mechanism, based upon photoisomerisation, is proposed to account for the efficient and selective photodynamic therapeutic activity of Merocyanine 540, an anionic cyanine dye.

Photosensitising dyes have found many applications in biomedicine,[1—3] including (i) inactivation of polio virus in sewage and drinking water, (ii) preparation of noninfectious virus antigens, (iii) sterilisation of blood, blood products and bone marrow, (iv) killing of leukaemic cells and certain tumours, (v) treatment of herpetic skin and eye lesions, and (vi) selected areas of antiviral activity. In no case has the mechanism for photoactivity been clarified, although the possible involvement of singlet oxygen[4] has been stressed on innumerable occasions. Many different dyes have been tested, of which Merocyanine 540, a lipophilic dye,[5] shows great promise as a photosensitiser for selective killing of occult tumour cells in autologous re-emission bone marrow grafts,[6,7] elimination of leukaemic cells[8,9] and lymphoma,[10] and sterilisation of blood.[11] Here, we demonstrate that, in contrast to popular ideas,[5,12] the highly efficient photodynamic therapeutic activity shown by this dye is not associated with intermediate formation of singlet oxygen. Instead, the photoeffect appears to involve localised disruption of neoplastic cells due to efficient photoisomerisation of lipid-bound Merocyanine 540.

Merocyanine 540, an anionic cyanine dye, shows high selectivity for electrically-excitable, leukaemic and immature hemopoietic cells, where it resides preferentially within the lipid.[5,12,13] It shows less affinity for proteins, carbohydrates, or nucleic acids although it does bind to serum and normal cells. The dye aggregates in water and in nonpolar solvents. Consequently, Merocyanine 540 will be widely distributed throughout the biological substrate and, in order to understand its probable role in photodynamic therapy, it is necessary to determine photophysical properties for the dye in many different environments.

In methanol, or other organic solvents of moderate polarity, Merocyanine 540 exists as a monomer, for which the excited singlet state, although short-lived[14,15] (τ_s 0.23 ns), is reasonably fluorescent (ϕ_f 0.13). Intersystem-crossing to the long-lived excited triplet state (τ_t 220 μs) occurs with low quantum efficiency (ϕ_t 0.04). The triplet state reacts with oxygen with a bimolecular rate constant of 3×10^9 dm^3 mol^{-1} s^{-1} and excitation (λ 532 nm) of Merocyanine 540 in O_2-saturated methanol produces singlet oxygen (ϕ_Δ 0.03). However, the major photoprocess involves geometric isomerisation of the molecule.[15] This latter process, which is a type of internal conversion from the first excited singlet to the ground state (ϕ_{ic} 0.83), forms a long-lived isomer with a quantum yield of 0.41. Spectroscopic studies imply that isomerisation involves *trans*-to-*cis* rearrangement of the central double bond, the rate constant being 3.6×10^9 s^{-1}. The resultant *cis*-isomer reverts to the original *trans*-isomer in the dark with a rate constant of 105 s^{-1} and an activation energy of 63 kJ mol^{-1}.[15]

In less polar solvents (*e.g.* mixtures of methanol and cyclohexane) comparable photophysical properties are observed, although the dye aggregates when the solution dielectric constant falls below *ca.* 6. These aggregates retain quite similar photochemical behaviour to that characterised for the corresponding monomer. Thus, the excited triplet state is formed in low quantum yield (ϕ_t 0.03) and the principal photoprocess involves rapid internal conversion from the first excited singlet state through equilibration of the central double bond. In O_2-saturated solution, illumination of the aggregate generates singlet oxygen with a quantum yield of 0.02.

Zwitterionic forms can be proposed for Merocyanine 540 in water or very polar organic solvents.[16] The presence of such forms perturbs the absorption spectrum and reduces the quantum yields for fluorescence (ϕ_f 0.042) and formation of the triplet excited state (ϕ_t 0.02). The triplet state (τ_t = 240 μs) is quenched by oxygen ($k = 1.8 \times 10^9$ dm^3 mol^{-1} s^{-1}) but formation of singlet oxygen was not observed in O_2-saturated water ($\phi_\Delta < 0.01$). Again, rapid equilibration of the central double bond occurs upon illumination (ϕ_{ic} 0.94) and, in N_2-saturated water, secondary rearrangements occur on the ms timescale which result in destruction of the dye.

Illumination of Merocyanine 540 bound to human serum albumin (HSA), fresh peripheral blood mononuclear cells, or acute promyelocytic leukaemia cell line HL-60 cells in O_2-saturated D_2O gave negligible yields of singlet oxygen ($\phi_\Delta < 0.01$). This is due to inefficient triplet state formation. For dye bound to HSA, $\phi_f = 0.27$, $\phi_t = 0.02$, and the triplet found in deoxygenated solution (τ_t 300 μs) is quenched upon aeration of the solution (τ_t 8 μs). Merocyanine 540 bound to HSA forms the photoisomer with a quantum yield of 0.28. The protein-bound isomer decays very slowly by two competing first-order processes having rate constants of 37 and 3.1 s^{-1}.

Incorporating Merocyanine 540 into neutral micelles increases both fluorescence (ϕ_f 0.31) and triplet state (ϕ_t 0.05) quantum yields although rapid internal conversion (ϕ_{ic} 0.64) still predominates. In micelles, rates of formation (k 2.4×10^8 s^{-1}) and decay (k 73 s^{-1}) of the photoisomer are decreased owing to the higher local viscosity and the quantum yield for formation of the isomer (ϕ 0.17) is reduced relative to fluid solution. In microemulsions[14] and liposomes,[15] there are further increases in ϕ_f and concomitant decreases in yield of photoisomer; ϕ_t remaining below 0.05. Again, the principal route for deactivation of the excited singlet state involves equilibration of the central double bond, the transition state partitioning to ground state *trans*- or *cis*-isomers. It is most probable that the lipid-bound Merocyanine 540 will demonstrate photochemical behaviour similar to that observed in these microheterogeneous media.

These studies show that illumination of Merocyanine 540 does not produce appreciable yields of singlet oxygen in solution, in aggregates, bound to a protein or intact cell, or incorporated into a micelle or liposome. In all environments, the major photoprocess involves geometric rearrangement from the first excited singlet state of the dye. Because of this rapid internal conversion, triplet state formation remains inefficient and never accounts for more than 5% of the photon balance. As such, the highly efficient photodynamic therapeutic activity[5—13] of Merocyanine 540 cannot arise from a triplet state reaction (such as formation of singlet oxygen) but must be a consequence of a singlet excited state process.

Equilibration of the central double bond is the principal photoreaction observed with Merocyanine 540 and it is likely

that this process is related to the cell killing ability. In this respect, two factors should be considered. Firstly, efficient internal conversion has the effect of dissipating quite large amounts of heat (*ca.* 200 kJ mol^{-1}) in the immediate surroundings. This local heating could disrupt the membrane, even causing a phase change in certain cases. Secondly, the large-scale structural change accompanying both forward and reverse isomerisations could further disrupt the membrane structure. This physical disturbance could damage the cell, allowing passage of ions through the membrane. In this way, only Merocyanine 540 which is incorporated into a lipid membrane would exert any photodynamic therapeutic activity; both surface-bound and free dye would be quite ineffective. This hypothesis, taken together with the preferred binding of Merocyanine 540 to cancer cells,[5—13] explains the exceptionally high selectivity with which the dye kills cancerous cells whilst remaining relatively inert towards healthy cells.[8,9] Acceptance of this novel reaction mechanism should permit development of new and improved dyes for such types of biomedical application.

The C.F.K.R. is supported jointly by the Biotechnology Resources Programme of the N.I.H. and by the University of Texas at Austin. This work was supported in part by grants from the Office of Naval Research, B.R.F. cell biology fund, and the Leukemia Society of North Central Texas.

Received, 7th April 1989; Com. 9/01440C

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