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Communication

Tritolylporphyrin dimer as a new potent hydrophobic sensitizer for photodynamic therapy of melanoma★✪

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We report the synthesis, photochemical and photophysical properties and preliminary studies on biological effect of a new tritolylporphyrin dimer (T–D). Absorption and emission properties of T–D suggest its possible use in photodynamic therapy. T–D is capable of singlet oxygen production with 0.8 quantum yield. It also has a high photostability. The photodynamic properties of the dimer were examined following the growth of SKMEL 188 (human melanoma) cells irradiated with red light (cut off < 630 nm). The surviving fraction of the cells decreased about 3-fold (*vs.* non-irradiated cells) for an 81 J/cm^2 dose. Our results suggest that tritolylporphyrine dimer T–D may be an interesting hydrophobic sensitizer for photodynamic therapy.

For more than 80 years it has been known that haematoporphyrins cause photosensitivity in man. The efficacy of tumour photodynamic therapy (PDT) with haematoporphyrin derivatives (HpDs) as a photosensitizer is now well established (Graczyk, 1999).

HpD – related commercial products, partially purified HpDs known as Photofrin I and

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Abbreviations: APTP, 5-(4-aminophenyl)-10,15,20-tritolylporphyrin.; CPTP, 5-(4-carboxyphenyl)-10,15,20-tritolylporphyrin; DHE, dihaematoporphyrin ether; Me_2SO, dimethylsulfoxide; F–10, RPMI, cell culture media (Gibco BRL); HpDs, haematoporphyrin derivatives; NaCl/P_i, phosphate-buffered saline; PAC, photoacoustic calorimetry; PDT, photodynamic therapy; S–91, Cloudman melanoma; SKMEL 188,human melanoma cells; T–D, tritolylporphyrin dimer (10,15,20-tritolylporphyrin-5-(4-amidophenyl)-[5-(4-phenyl)10,15,20-tritolylporphyrin]); TPP, 5,10,15,20-tetraphenylporphyrin.

Photofrin II are approved for medical use in some countries and have been used to treat thousands of patients all over the world. Some HpDs were also used to treat experimental melanoma, mostly its non-pigmented form (Franken *et al.*, 1985; Gomer *et al.*, 1985; Phillips *et al.*, 1987; Kecik *et al.*, 1993), only in a few cases was the HpDs-based PDT studied using tumours and cells containing different levels of melanin (Nelson *et al.*, 1988; Favilla *et al.*, 1995). Both pigmented and non-pigmented melanoma cells were affected by light dependent toxicity, but the effect was greater for non-pigmented cells.

There are some limitations for using Photofrin II as a photosensitising agent. It is not well defined chemically, it is a mixture of HpDs, and this makes it difficult to control its photosensitising properties, distribution in tissues and stability. It was established some years ago that the most active components in HpDs mixtures are dihaematoporphyrin ethers and/or esters (Dougerthy *et al.*, 1984; Graczyk, 1999) known as DHE. This fact focussed the attention of researchers on the synthesis of some new kinds of porphyrin dimers not possessing the disadvantages of their predecessors, which would be chemically homogeneous and more stable. In this work we describe the synthesis of a new (Fig. 1) tritolylporphyrin dimer (T–D). Some of its photophysical and photochemical properties essential from a PDT point of view as well as its phototoxic activity against melanoma cells in culture are described.

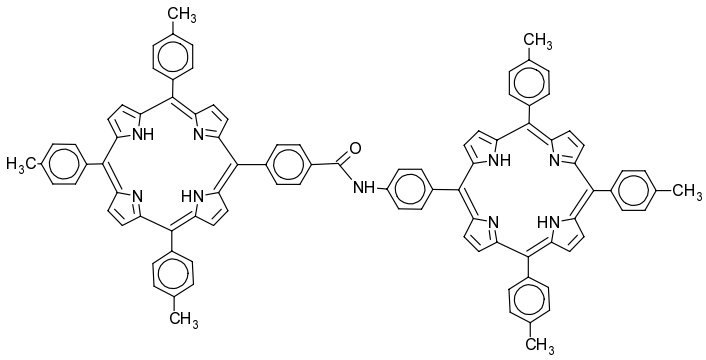

Figure 1. 10,15,20-tritolylporphyrin-5-(4-amidophenyl)-[5-(4-phenyl)-10,15,20-tritolylporphyrin](T–D).

MATERIALS AND METHODS

Synthesis of porphyrin dimer (T–D). The reagents for the reaction were obtained by the conventional method of Adler *et al.* (1967) by the condensation of mixed aromatic aldehydes with pyrrole in boiling propionic acid. From the reaction mixtures 5-(4-aminophenyl)-10,15,20-tritolylporphyrin (APTP) and 5-(4-carboxyphenyl)-10,15,20-tritolylporphirin (CPTP) were separated. The porphyrin dimer was prepared by a procedure similar to that described in the literature (Faustino *et al.*, 1997). CPTP was converted to acidic chloride by dissolving it (32 mg) in a mixture of dried toluene (4 cm^3) and dried pyridine (1 cm^3) with an excess of thionyl chloride (0.15 cm^3, 0.21 mmol). The solution was stirred and refluxed under argon for 2 h. The solvents were evaporated and the resulting residue was dissolved in chloroform (5 cm^3) in the flask, which was then placed in an ice-bath and a solution of APTP (31 mg) dissolved in chloroform (8 cm^3) was added dropwise for 15 min. After about 30 h reaction at room temperature no substrates were observed on TLC plates (0.2 mm silica gel, chloroform). After that period the reaction mixture was evaporated to dryness. The resulting product was dissolved in chloroform (15 cm^3) and washed with 2% hydrochloric acid, water, 5% sodium hydrogen carbonate and again water. The organic phase was dried with anhydrous magnesium sulphate and evaporated to dryness. Sixty mg of crude product was obtained and was purified on a silica gel column (70–210 mesh, 2 × 40 cm); a mixture of chloroform and methanol (20 : 1 v/v) was used as eluent. The first eluted fraction was the dimer. The purity of the dimer was checked by TLC (silica gel 0.2 mm plate, chloroform, R_F = 0.85). The structure of the substance was confirmed by using ESI Mass Spectroscopy, ^{1}H-NMR and IR. The yield of the dimer was 50 mg (80%).

Absorption and fluorescence spectra. Absorption spectra were recorded with a two-beam Shimadzu UV-VIS 2101 PC spectrophotometer and a Hewlett-Packard 8453 diode array spectrophotometer. Fluorescence spectra were determined using a computer controlled Spex spectrofluorimeter Fluorolog-3, model FL3-22 (Xenon lamp 450 W). Phosphorescence spectra were measured using a SPEX 1934D3 phosphorimeter (Xenon flash lamp). Fluorescence quantum yield Φ_F (under nitrogen) was measured relative to tetraphenylporphyrin (TPP), which has the quantum yield of fluorescence in toluene $\Phi_F = 0.1 \pm 0.01$. Phosphorescence emission spectra in the range 600–900 nm (excitation in Soret band 421 nm) were recorded in frozen toluene glass (77 K).

Rate constant of triplet state quenching. To determine the transient absorption spectra and the rate constants of triplet state decay in the presence or absence of oxygen an Applied Photophysics Laser Flash Photolysis spectrophotometer (model LKS 60, Xenon arc lamp, 150W/CR OFR OSRAM) was used. The absorption spectrum of the triplet state of T–D in the range 300–800 nm in toluene and the kinetic curves of decay of the triplet state of T–D after excitation (Nd:Yag laser, 532 nm) were registered.

The triplet state quenching rate constant was determined according to equation 1:

$$k_q = \left(\frac{1}{\tau_T(O_2)} - \frac{1}{\tau_T(N_2)} \right) \cdot \frac{1}{[O_2]} \qquad (1)$$

where: $\tau_T(O_2)$, $\tau_T(N_2)$ indicate the lifetimes of the T–D triplet state (determined from the decay curves) in the presence of oxygen and in nitrogen, respectively; $[O_2]$ is the concentration of oxygen in the system studied (1.81×10^{-3} M) (Pineiro *et al.*, 1998).

Typical results are shown in Fig. 3 and summarised in Table 1.

Quantum yield of singlet oxygen formation. Preliminary experiments of quantum yield of singlet oxygen formation by T–D were carried out using PAC (Photoacoustic Calorimetry) (Pineiro *et al.*,1998). The quantum yield of singlet oxygen formation was found to be $\Phi_{1_{\Delta_q}} = 0.8$, which is a typical value for this class of compounds.

Photobleaching experiments. Solutions of T–D in dimethylsulfoxide (Me_2SO) (10^{-4} M) were irradiated with an LH313K halogen lamp in air for different irradiation times: from 1 min to 3 h. No change in optical spectrum was observed. This fact indicates that the deactivation of singlet oxygen is of a purely physical nature.

Cytotoxicity assay. To check the cytotoxicity of T–D, human melanoma cells (SKMEL 188) and mouse melanoma cells (S91) were incubated for 24 h in phosphate-buffered saline ($NaCl/P_i$) containing various concentrations of T–D. The porphyrin dimer was dissolved in Me_2SO, then diluted in $NaCl/P_i$ to reach different concentrations of T–D (10^{-8}– 10^{-5} M). Thereafter the cells were maintained in F10 (SKMEL) and RPMI (S91) media with 10% and 5% serum, respectively, for 48 h. Cytotoxicity in the dark was monitored by counting the number of cells in the treated and untreated cultures.

Photodynamic effect measurements. To evaluate the influence of T–D-based PDT on cell growth, the cells in the presence of the photosensitiser (10^{-7} M) were irradiated using an LH313K lamp with a filter cutting off wavelengths below 630 nm. Different doses of energy between 13.5–82 J/cm^2 (irradiation time: 5–30 min) were used. After irradiation, cells were incubated in the culture medium for 36 h. The surviving fraction was monitored by counting the number of PDT treated and untreated cells. Data points are the means of two or more experiments. Bars represent standard error (±S.E.M.).

RESULTS AND DISCUSSION

It is now recognised that an adequate photosensitising agent should posses specific chemical and biological properties. It should be easy to synthesise, relatively stable, exhibit minimal dark toxicity, have a substantial absorbance band above 600 nm and an efficient yield of singlet oxygen generation. It should also be a chemically pure substance. Of course, decisive are always the results of biological experiments, showing the ability of the compound to generate light induced

cell toxicity. We present and discuss the results from this point of view.

Synthesis of porphyrin dimer (T–D)

The synthesis of T–D proceeds with sufficient efficiency (80%). The isolation and purification of the compound is relatively simple and cheap. The product obtained is homogeneous, chemically well defined, and is not a mixture of many porphyrins, which is an advantage in relation to Photofrin II.

Photophysics and photochemistry of porphyrin dimer

The relevant data obtained from absorption and fluorescence spectra as well as from laser flash photolysis of T–D are summarised in Table 1, Figs. 2 and 3. In the concentration range used in the spectroscopic and biological studies the Lambert-Beer law (measured in toluene) was obeyed and the tendency to aggregate was not found. This fact does not exclude the possibility of aggregation inside the cells, which is often found for this kind of compounds. For T–D the strongest absorption band is, like for other porphyrins, near 400 nm (423 nm, $\varepsilon_{423} = 4.73 \times 10^5\ M^{-1}cm^{-1}$), and there are additional bands with progressively decreasing absorbance at 517, 552, 592, and 649 nm. The last absorption band at 649 nm is the most important regarding PDT, because only the red light has sufficient tissue penetration ability (Wilson *et al.*, 1985). The absorption coefficient of T–D at this wavelength is not very high, $\varepsilon_{649} = 7.55 \times 10^3\ M^{-1}cm^{-1}$, but it is sufficient to absorb the light effectively. Other porphyrins have simi-

Table 1. Some photophysical and photochemical data of T–D (toluene, room temperature, air).

Wavelength used for PDT molar absorption coefficient	λ = 649 nm; $\varepsilon_{649} = 7.55 \times 10^3\ M^{-1}cm^{-1}$
First excited singlet state energy	$E_S = 183.2 \pm 0.3\ kJ \cdot mol^{-1}$
Maximum of fluorescence quantum yield of fluorescence	$\lambda^F_{max} = 652$ nm; $\Phi_F = 0.11 \pm 0.01$
Rate constant of triplet state quenching	$k_q = 1.74 \cdot 10^9 \pm 1.25 \cdot 10^5\ M^{-1}s^{-1}$
Triplet state life-times:	
in the presence of oxygen (1.81×10^{-3}M) in deaerated toluene	$\tau(O_2) = 312 \pm 9.8$ ns $\tau(N_2) \geq 19 \times 10^3$ ns
Singlet oxygen generation yield	$\Phi_{1_{\Delta_q}} = 0.8$

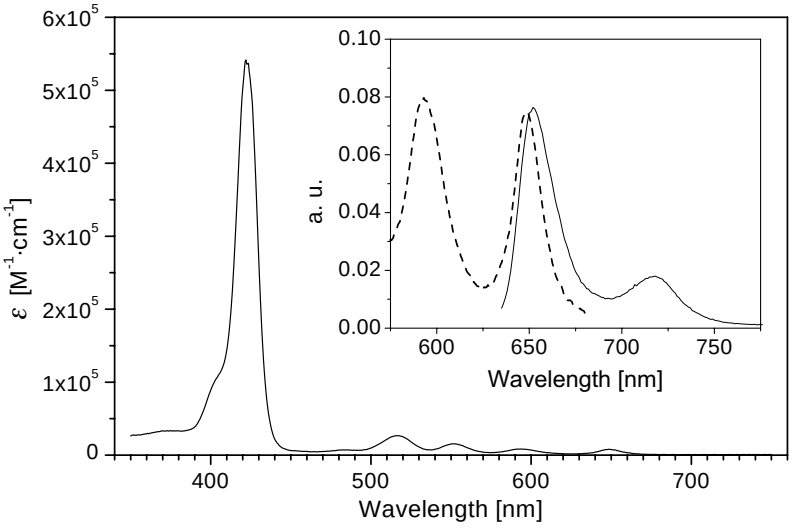

Figure 2. UV-VIS absorption spectrum of T–D in toluene at 5×10^{-6} M.

The wavelengths above 630 nm were used in PDT experiments. Inset: Long-wavelength part of the absorption spectrum of T–D (broken line) and the fluorescence spectrum of T–D in toluene (solid line). The observed Stokes' shift is small, about 3 nm.

lar values of absorption coefficients at these wavelengths. The spectroscopic singlet-state energy (E_S, Table 1) was obtained using standard procedure, from the intersection of normalised absorption and fluorescence spectra (Fig. 2 – inset). The fluorescence quantum yield of T–D in deaerated toluene is $\Phi_F = 0.11 \pm 0.01$, which is a reasonable value for a sensitizer to be used in PDT. The shape of the transient absorption spectrum and the rate constant of its decay strongly suggest the triplet state origin. All triplet state decay curves (Fig. 3) are independent of the monitoring wavelength and reveal the same type of monoexponential kinetics. The life-times of the triplet state in the presence and absence of oxygen, as well as the rate constant of its quenching by oxygen ($\Phi_{1_{\Delta_g}}$), were calculated according to the procedure described in Methods (mean values are shown in Table 1). The presented photochemical and photophysical data of T–D are similar to the data for other porphyrins (Pineiro *et al.*, 1998; Faustino *et al.*, 1997; Graczyk,1999).

Bioassay

The photosensitising efficiency of T–D is shown in Fig. 4. The surviving fraction of the human melanoma cells (SKMEL 188) decreased almost 3-fold (*versus* untreated cells) with an energy dose 81 J/cm^2. The results for mouse melanoma cells

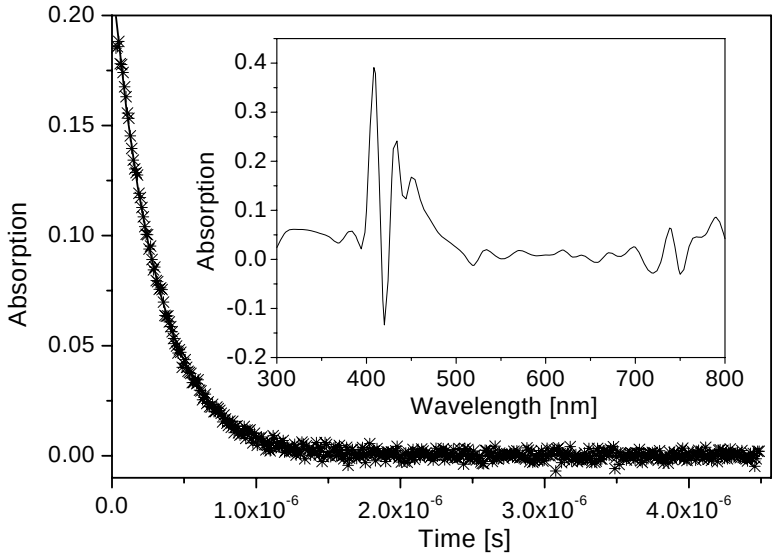

Figure 3. Typical decay curve of the triplet state of T–D in toluene.

Inset**:** Typical transient absorption spectrum of T–D in toluene after 100 ns since the exciting pulse (excitation wave λ = 532 nm, A_{532} = 0.1). In addition to the bands connected with absorption by the triplet state of T–D, some bands with negative values of absorbance connected with depopulation in the ground state were observed. All experiments were carried out at room temperature (approx. 22°C).

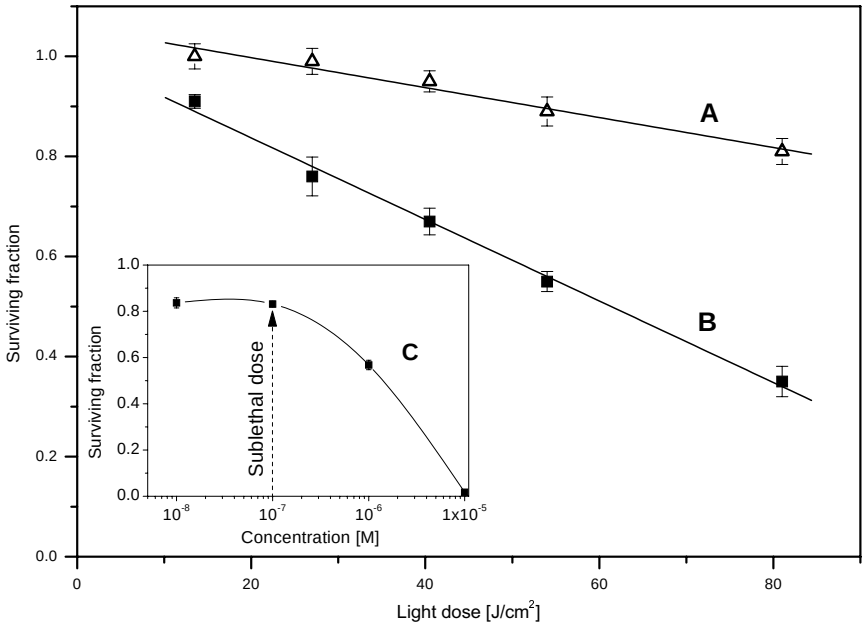

Figure 4. Effect of photodynamic treatment [B] with T–D as the sensitizer on survival of SKMEL 188 cells.

Cells were treated with T–D (10^{-7}M) illuminated with the indicated dose (J/cm^2) and further incubated in growth medium in the dark for 48 h. Light control **[A]** the cells were illuminated without T–D. In the inset the cytotoxicity of T–D in the dark is presented **[C]**.

(S91) were almost the same (data not shown). Light alone caused small changes in the surviving fraction of the cells (Fig. 4, curve A). The toxicity of the dimer in the dark is presented in Fig. 4 (curve C). The concentration of T–D of 10^{-7}M was chosen as sublethal and used for the PDT experiment. Comparing our bioassay results to the others, which used HpDs or Photofrin as photosensitising agents (Penning *et al.*, 1994; Chang *et al.*, 1999) one can see that we obtained a comparable photokilling effect using a significantly lower dye concentration (30-fold) and irradiation dose (2-fold).

The newly synthesised photosensitizer T–D has quite a lot of advantages compared to HpDs being in use to date. Chemical homogeneity, low tendency to aggregate, good solubility in hydrophobic phases with a relatively long-living triplet state to generate sufficient singlet oxygen quantities ($\Phi_{1_{\Delta_q}}$ = 0.8) make T–D an effective compound acting *via* the classical kind II photokilling mechanism.

Our compound also has some disadvantages, common for all HpDs: a relatively small extinction coefficient around 630 nm lowers the photoexcitation, thus requiring the administration of large amounts of energy in order to obtain a satisfactory phototherapeutic response. However, the above-mentioned photochemical features of T–D and its behaviour in the PDT experiment *in vitro* permit us to finally conclude that this porphyrin dimer is a promising candidate for a new PDT-agent and is worth further investigations, especially *in vivo*.

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