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Parameters Affecting Photodynamic Activity of Foscan® or Meta-tetra(hydroxyphenyl)chlorin (mTHPC) In Vitro and In Vivo

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Abstract. In vitro photodynamic activity of Foscan® or meta-tetra(hydroxyphenyl)chlorin (mTHPC) was approximately five times lower in the presence of protein in comparison with serum-free medium. Photocytotoxicity does not depend on the incubation time of the dye with cells. The in vitro results are discussed from the point of view of photodynamically active aggregated species. The photodynamic activity of mTHPC was elevated in vivo by macroscopic measurement of the necrosis depth after tumoral resection using an in vivo staining procedure with Evans blue dye. Only tumours from treated animals presented measurable necrosis areas, mostly localised in the surface around the irradiated site with a mean depth of 3.0 ± 0.3 mm. The photodynamic activity was found to be significantly higher when using low irradiance (32 mW/cm^2) than when using a higher one (160 mW/cm^2). These results were not related to intratumoral mTHPC photodestruction analysed by in vivo fluorescence spectral analysis.

Keywords: Human colon adenocarcinoma cells; Meta-tetra(hydroxyphenyl)chlorin; Nude mice; Photodynamic therapy

INTRODUCTION

Photodynamic therapy (PDT) is based on the selective uptake of a photosensitiser by tumour tissue [1] followed by irradiation of this tissue with visible light. Photochemically induced tumour destruction occurs mainly through singlet oxygen production [2,3].

A few years ago, second generation photosensitisers were synthesised, among which was meta-tetra(hydroxyphenyl)chlorin(mTHPC) [4] with enhanced absorption of light in the red region of the visible spectra ($\lambda_{max} = 652$ nm), which favours deeper light penetration in tissue. mTHPC was found to be more tumour specific than conventional porphyrins [5,6] and to produce 6–10 mm depth of necrosis [7,8] in tumour-bearing mice. In addition, skin photosensitisation, usually encountered as a side effect, was reported to be shorter (2.5 weeks) [9].

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In vivo, the mode of action of first and second generation photosensitisers is considered to be very complex. It is based on direct (cell killing) and indirect (vasculature damage) photodynamic effects [10]. The latter occurs through damaging endothelium cells leading to hypoxia [11], or through the decrease in partial pressure of oxygen (PO_2) that has been reported to appear after PDT [12]. In addition to hypoxia, other factors may contribute to the biological efficacy of photodynamic therapy, such as photosensitiser concentration in the tumour tissues, light fluence and light fluence rate, as well as the photobleaching occurring during irradiation.

The recent attempt to classify the data on the fluence-rate effects in PDT showed a reverse fluence-rate effect; tumour damage decreased as the fluence rate increased from 50 to 100 mW/cm^2 [13].

The present study focuses on the influence of different parameters on the in vitro and in vivo efficacy of mTHPC. We had a particular interest in the influence of proteins on mTHPC-phototoxicity, given the high affinity of porphyrins and chlorins for proteins [14–16], as well as

in the influence of the incubation time on the dye photoinduced cytotoxicity, which reflects the kinetics of cellular uptake of mTHPC. In order to evaluate the in vivo efficacy of mTHPC, different fluence rates for a given dose of light were applied to tumour-bearing mice with subsequent evaluation of necrosis.

MATERIAL AND METHODS

Photosensitiser

mTHPC was kindly provided by Scotia Pharmaceuticals (Guildford, UK) and was dissolved according to the laboratory recommendation in polyethylene glycol (PEG) 400 (30%), ethanol (20%) and water (50%). For in vitro experiments, mTHPC was diluted in Dulbecco's MEM culture medium (Gibco, Cergy, Pontoise, France) without proteins or supplemented with 2% fetal calf serum (FCS) (Dutscher, Brumath, France).

Cell Line

HT-29 colon adenocarcinoma cell line was maintained in Dulbecco's MEM supplemented with 10% heat-inactived FCS, 1% penicillin/streptomycin (Gibco BRL) at 37°C in a 5% CO_2 atmosphere to reach exponential growth, then trypsinised to obtain cell suspensions. Aliquots of 2×10^4 viable cells per well were plated into 96-well dishes in 200 µl of culture medium. Cells were allowed to attach to the dishes for 72 h at 37°C before being tested.

Tumour Models

Three-week athymic female mice (Swiss, nu/nu, IFFA CREDO, France) were inoculated subcutaneously with 8×10^6 HT29 cells. Three to four weeks later, as the tumour reached a diameter of 8–10 mm, mTHPC (0.3 mg/kg body weight) was injected intraperitoneally, and mice were irradiated. Control groups of animals did not receive either mTHPC, or light.

Photodynamic Activity

In Vitro Assays

Cells were incubated with the photosensitiser (0.3–10 µg/ml) in Dulbecco's MEM supplemented with 2% FCS for 3 or 24 h (unless otherwise indicated) at 37°C and washed twice with ice-cold phosphate-buffered saline (PBS). Fresh medium was then added and cells were irradiated either at 5 J/cm^2 or at 10 J/cm^2.

Light (650 nm) was emitted from a kiton red dye laser (Spectra-Physics 375 B, Les Ulis, France) pumped by an argon laser (Spectra-Physics 2030). The wavelength used for irradiation was controlled with a monochromator (Jobin Yvon, France). The laser beam was transmitted through a 600 µm silica–silicon optic fibre (SEDI, Evry, France). The output power was fixed to 1 W and controlled before each irradiation using a power meter integrated to the laser control desk. The homogenous light spot was adjusted to 14 cm in diameter and exposure time varied in order to obtain different fluences ranging from 5 to 10 J/cm^2.

The cell survival was assessed by 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, Saint Quentin Fallavier, France) colorimetric assay [17]. The MTT assay is widely used in cell proliferation and cytotoxicity assays. Most cellular bioreduction of MTT is associated with mitochondrial enzymes. The colorimetric assay provides data equivalent to clonogenic assay [18–20].

Briefly, 50 µl of 125 mM MTT was added into each well and the dishes were incubated for 3 h at 37°C to allow MTT metabolisation. After incubation, the formazan crystals were dissolved by adding 50 µl of 25% sodium dodecylsulphate (SDS) solution into each well. The absorbance of the resulting solution (540 nm), proportional to the cell concentration, was measured using a Multiskan MCC 340 plate reader (Flow Laboratories, Les Ulis, France).

The results were expressed as absorbance values relative to untreated controls. The photosensitiser concentrations yielding 50% of cytotoxicity (IC_{50}) were calculated using the median effect principle [21].

Light alone or mTHPC alone (0.1–10 µg/ml) had no significant effect on cytotoxicity. The mean absorbance values (540 nm) for control (mTHPC only or light alone) were, 0.78 ± 0.04 and 0.71 ± 0.02, respectively.

Only after a long incubation period (24 h) was mTHPC found to be slightly cytotoxic, the IC_{50} was 13 µg/ml.

The results are presented as mean values of a minimum of eight wells in triplicate assays and were statistically compared using

Student's t-test with a significance limit at $p<0.05$.

In Vivo Assays

Three days after mTHPC injection [22], each animal was anaesthetised by intramuscular injection of 12.5 mg/ml ketamine (2 × 0.25 ml) (Parke Davis, Courbevoie, France), and irradiated at 650 nm as for cell dishes. The output power was fixed at 100 or 500 mW, to obtain 32 mW/cm^2 and 160 mW/cm^2, respectively. A single irradiation was performed with a spot reaching 20 mm in diameter at the skin surface above the tumour. The light dose (10 J/cm^2) was applied by adjusting the duration of irradiation. Tumour necrosis was evaluated using Evans blue dye method, this dye reflects also the mechanisms of tumour destruction [23]. Immediately after PDT, mice were injected (i.p.) with 0.5 ml of Evans blue dye (0.6%) then killed 24 h later with an overdose of halothan (Paris, France); at this time (24 h) an optimal PDT response was observed [24,25]. The tumour was then ablated, fixed in Bouin solution for 30 min, and cut longitudinally. Stained and unstained areas were measured with calipers. Tumour damage was identified as unstained necrotic areas, since Evans blue dye cannot diffuse in tissues with vasculature damage.

Intratumoral mTHPC Photobleaching

In vivo quantification of mTHPC fluorescence was performed using a CP 200 spectrograph (Jobin-Yvon) connected with an optical multichannel analyser. Three optical fibre probes were used for excitation (550 µm of core diameter), fluorescence collection and backscattered power measuring (200 µm of core diameters). The distance between the axes of optical fibres was 375 µm. The excitation light at 413 nm was emitted by 300 W xenon lamp through a bandpass filter.

In order to compare the spectra, the measurements were normalised according to the autofluorescence before mTHPC administration. The fluorescence in experimental groups of mice was measured before irradiation and 5 min after, to estimate the flourescence of mTHPC lost during irradiation. Animals were maintained in the dark during all the experiments.

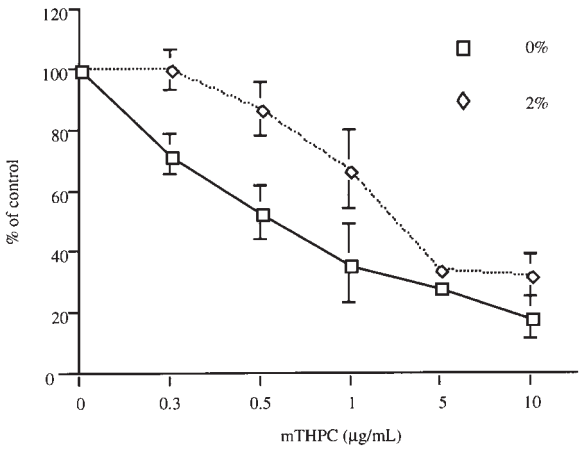

Fig. 1. Influence of serum on mTHPC photocytotoxicity. HT29 cells were incubated for 3 h with mTHPC diluted in medium containing 0 (□) or 2% (◇) of FCS, and photoirradiated at 5 J/cm^2 with 650 nm. The MTT assay was performed 48 h after exposure. Results are mean values (±SEM) of triplicated experiments.

RESULTS

In Vitro Assays

Influence of Proteins on Photodynamic Activity

Photocytotoxicity diminished in the presence of protein content (FCS) in culture medium. A significant difference was evidenced between the IC_{50} obtained in serum free and 2% FCS-containing medium: 0.8 µg/ml versus 4.0 µg/ml (Fig. 1). It should be noted that as the dye concentration during incubation increases, the difference between both treatments decreases, being negligible at high mTHPC concentrations (5 and 10 µg/ml).

Influence of Time Delay on Photodynamic Efficiency

In order to study if photodynamic activity of mTHPC is delayed, the photocytotoxicity of cells exposed to concentration gradient of photosensitiser was evaluated immediately after irradiation (10 J/cm^2), 24 and 48 h later (Fig. 2) yielding IC_{50} as follows: 4.6 µg/ml, 0.8 µg/ml and 0.8 µg/ml, respectively.

Influence of Incubation Time on mTHPC Phototoxicity

Before exposure to light (10 J/cm^2), cells were incubated with mTHPC for either 3 h or 24 h. Increase in incubation time did not result in an increase in cytotoxicity, at least for the initial interval of concentrations (Fig. 3). The

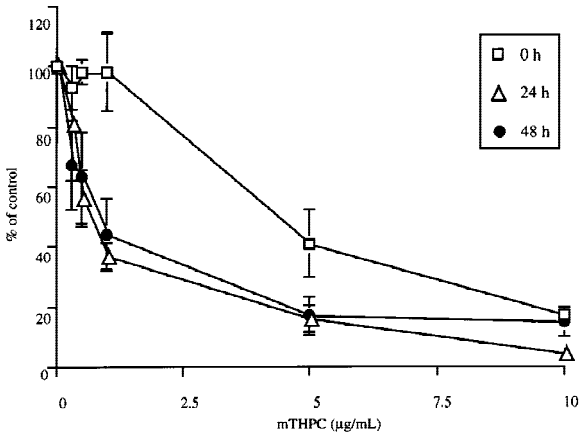

Fig. 2. Time-delayed mTHPC photocytotoxicity on HT29 cells. MTT assay was performed immediately (□), 24 (△) or 48 (●) h after exposure of HT29 cells to mTHPC (3 h) and light (10 J/cm^2) at 650 nm. Results are mean values (±SEM) of triplicated experiments.

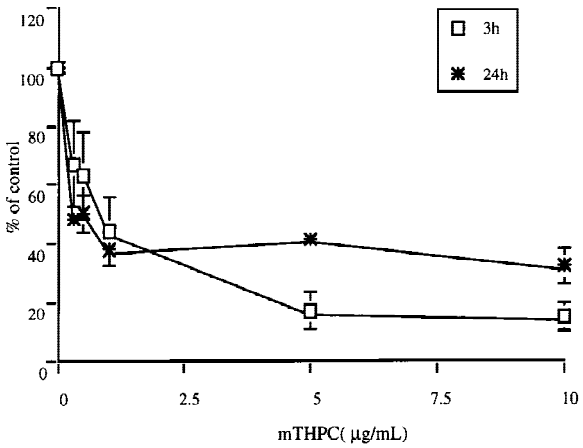

Fig. 3. Influence of incubation time on mTHPC photocytotoxicity. HT29 cells were incubated for 3 h (□) or 24 h (✱) and were irradiated at 650 nm for a dose light of 10 J/cm^2. The MTT assay was performed 48 h after exposure. Results are mean values (±SEM) of triplicated experiments.

statistical analysis showed that the difference in IC_{50} was not significant for both incubation times. Our results are not consistent with those reported recently [26] with a phototoxicity increasing proportionally to incubation time.

In Vivo Assays

Influence of Light Fluence Rate on Tumour Tissue Necrosis

Tumour-bearing mice were divided into two groups irradiated at 10 J/cm^2 at two different fluence rates: 32 mW/cm^2 or 160 mW/cm^2. The morphological observation of the first group of mice (n=9) treated at 32 mW/cm^2 showed a necrosis depth of 3.0 ± 0.3 mm, the second group (n=5) treated at 160 mW/cm^2 showed no significant difference as compared with the control group (n=3) without light and photosensitiser (Fig. 4). The tumours treated at 32 mW/cm^2 presented an area of haemorrhage at the surface with underlying deep necrosis (3.0 ± 0.3 mm). On the contrary, tumours treated at 160 mW/cm^2 presented diffusive stained and unstained areas with neither haemorrhage area, nor significant necrosis.

Fluorescence was measured before and 5 min after PDT treatment in both groups of mice. The decrease in intratumoral mTHPC fluorescence, as a result of irradiation, was the same in both groups (Fig. 5). The fluoresence after irradiation was compared with the initial value for each group and was 49 ± 3% and 40 ± 7% for the mice irradiated at 32 mW/cm^2 and 160 mW/cm^2, respectively.

DISCUSSION

The Foscan® or mTHPC, is currently under clinical investigation [27]. Its strong absorption at 652 nm allows the treatment of larger tissue volumes. Indeed, tumour necrosis as deep as 10 mm has been achieved with mTHPC PDT [7,8]. Besides spectral aspects, the photosensitiser presents favourable photosensitising properties compared to Photofrin II [28] (high yield of cell photoinactivation, high rate of photobleaching).

The present study focuses on the influence of different parameters on photodynamic activity with mTHPC in vitro and in vivo cytotoxicity. The photodynamic activity of mTHPC was found to be influenced by proteins contained in the culture medium. mTHPC is known to possess a high protein and lipid affinity, resulting in monomerisation, this fact is documented by the changes in fluorescent spectra upon addition of FCS [26]. In addition, our spectroscopic results demonstrate that the ratio of the molar extinction coefficients of mTHPC solution (data not shown) at 415 nm with and without 2% FCS was 2. In comparison with aqueous solutions, the fluorescence maxima was significantly enlarged in the presence of serum (the ratio of fluorescence intensity with and without 2% FCS was 8). These facts indicate that the presence of serum leads to mTHPC monomerisation. In all probability, the difference in photocytotoxicity reflects the different modes in mTHPC cellular uptake. In

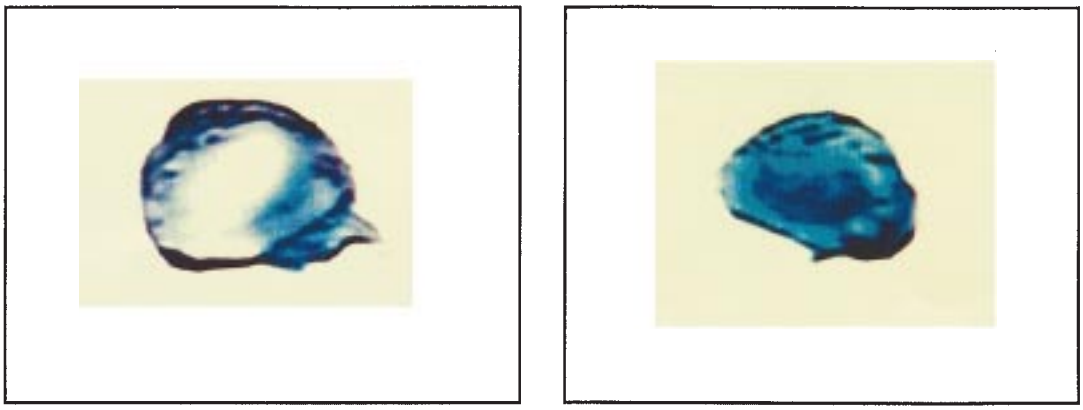

Fig. 4. Tumour necrosis after photodynamic treatment (mTHPC was injected in mice at 0.3 mg/kg body weight, 72 h later photoirradiaton was performed). Depth of necrosis was evaluated with Evans blue 24 h after PDT treatment at 32 mW/cm^2 (a) and 160 mW/cm^2 (b).

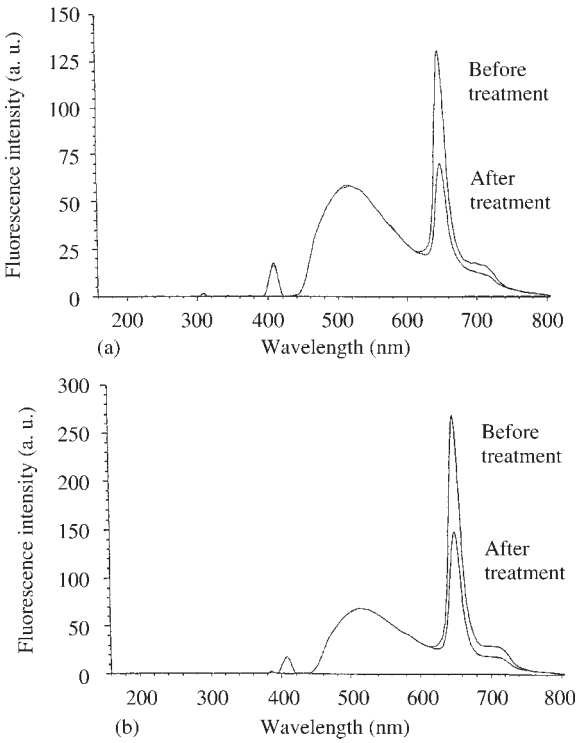

Fig. 5. In vivo fluorescence spectrum of mTHPC before PDT treatment and 5 min after photoirradiation at (a) 32 and (b) 160 mW/cm^2.

the absence of serum, the dye is taken up by direct pinocytosis. This mechanism is well known for the aggregated forms of free porphyrins [29]. Also, as has been shown earlier, for several tetrapyrrolic dyes (Photofrin II, phthalocyanins), the cellular uptake of photosensitiser is considerably enhanced in serum-free conditions, because the cellular clearance is slower [30] and the rate of incorporation is faster [31].

The results of the present work show, that in the presence of serum, the uptake depends on mTHPC concentration: at low concentrations of mTHPC (up to 1 μg/ml), the dye binds to serum proteins and the aggregated fraction is negligible. Conversely, at higher concentrations (5 and 10 μg/ml) the fraction of free, aggregated photosensitiser increases, finally resulting in the same photocytotoxicity as for serum-free mTHPC. This hypothesis is supported by the fact that the percentage of mTHPC bound to each fraction of serum proteins does not depend on the initial concentration of the dye (in the range 0.1–10 μg/ml) [15].

mTHPC photodynamic activity was not influenced by the cell incubation time before irradiation (Fig. 3). We postulated that cellular uptake was the same at 3 h and at 24 h. The optimum incubation time is usually chosen according to the fluorescence measurements in living cells. Ma et al. [32] have shown that the maximum accumulation of mTHPC in cells occurs 24 h after incubation. In our previous work, using flow cytometry, we have also found, that the increase in fluorescence in mTHPC-loaded HT29 cells reaches a plateau 24 h after incubation and lasts up to 48 h [33]. It is noteworthy that the increase in fluorescence does not necessarily reflect the real concentration of the dye, but mostly

changes in the photosensitiser monomeric species. The aggregated species of photosensitisers are known to have a lower fluorescence quantum yield than the corresponding monomers [32]. Probably, 24 h of dye incubation results in better monomerisation, and consequently, higher values of intracellular mTHPC fluorescence [33]. If we are right in our tentative explanation, that photodynamic activity occurs mostly through accumulation of aggregated species of mTHPC, the difference between our fluorescence pattern data and photocytotoxicity can be explained easily.

The present study showed that the photodynamic activity of mTHPC was not immediate, but occurred 24 h after irradiation. Probably, the time-delayed photocytotoxicity reflects the fact that PDT can also induce cell death in the hours following PDT for instance, by apoptosis [34]. On the other hand, the time-delayed photocytotoxicity could be explained by the fact that the lesions induced by photochemical reactions are not immediately lethal.

In vivo use of Evans blue dye is a direct and easy method of evaluating the mechanism of tumour destruction and measuring necrosis depth after PDT treatment. The low fluence-rate mTHPC PDT (32 mW/cm^2) induced tumour necrosis of 3.0 ± 0.3 mm depth, whereas no necrosis was observed at high fluence-rate (160 mW/cm^2). This effect may be attributed to different rates of photobleaching at chosen light fluence rates.

To verify this hypothesis, the in vivo decay in fluorescence was measured before irradiation and 5 min after and was found to be the same in both cases. Thus, the photodestruction of intratumoral mTHPC during PDT treatment does not depend on the fluence rates used (32 or 160 mW/cm^2) (Fig. 5), and the difference between necrosis induced by 32 or 160 mW/cm^2 cannot be attributed to mTHPC photobleaching. Also, the photodynamic effect cannot be explained as a consequence of the slowing of blood flow which occurs after tumour irradiation. Indeed, a very high decrease in tumour perfusion was observed for high fluence rate, as well as for low fluence rates [13]. The most plausible explanation is the different rate of oxygen consumption depending on the applied fluence rate. Rapid oxygen consumption during high fluence-rate PDT leads to oxygen depletion, which protects cells from PDT damage [35]. Low fluence-rate PDT lowers the rate of oxygen consumption, thus extending the oxygenated region in the tumour [36].

In conclusion, using low fluence-rate PDT Foscan® may improve therapeutical index, especially for a thin superficial cancer such as œsophagus epidermoid carcinoma.

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