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Wavelength-dependent Effect of Tetra(m-hydroxyphenyl)chlorin for Photodynamic Therapy in an 'Early' Squamous Cell Carcinoma Model

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Abstract. The purpose of the present study was to correlate the wavelength of the irradiation source with the phototoxic activity of tetra(m-hydroxyphenyl)chlorin (mTHPC) in healthy and neoplastic mucosae. The hamster tumour model for early squamous cell carcinoma was used in these experiments. In vitro and in vivo studies have shown that mTHPC absorbs significantly at 652 nm (1, 2). This wavelength is used currently in clinical mTHPC photodynamic therapy (PDT) trials. In order to study the wavelength dependence of the phototoxic effect on normal and tumour tissues, irradiation tests were performed 4 days after injection of 0.5 mg kg^{-1} mTHPC. An argon-ion pumped dye laser was used as the light source. The light dose of 12 J cm^{-2} was delivered at a light dose rate of 150 mW cm^{-2}. The wavelength was varied between 642.5 and 665 nm at 2.5-nm increments. The PDT damage was evaluated in serial Haematoxylin and Eosin stained sections using a tissue-damage scale. Light between 647.5 and 652.5 nm induced the highest damage to both the healthy and tumour mucosae. At wavelengths equal to or below 645 nm, and equal to or above 655 nm, tissue damage decreased. Wavelengths below 642 nm and above 660 nm did not induce any visible tissue damage. These results suggest that the in vivo optimal wavelength range for PDT with mTHPC is between 647 and 652 nm. This information is essential for selecting an appropriate light source.

INTRODUCTION

Photodynamic therapy (PDT) is emerging as a minimally invasive alternative for the treatment of early squamous cell carcinomas (SCCs) in hollow organs (3–6). In head and neck cancers, the advantage of PDT compared to conventional modalities, such as surgery, radiation or chemotherapy, lies in the prevention of excessive tissue loss and of significant functional disorders (7).

Successful PDT involves the optimization of a large number of parameters. Among them are the type of photosensitizer (PS) including the mode and vehicle used for its administration, the drug dose, the wavelength of the light, the light dose, the light dose rate and the drug-light interval. In addition to these parameters, the wavelength of the light used in PDT is obviously crucial for optimizing the therapeutic effect. As it is difficult to evaluate all of these variables in a clinical context, the use of an animal model may provide preclinical data relevant to clinical trials of PDT. For this reason, 'early' SCCs, chemically induced using 7,12-dimethylbenz(a)anthracene (DMBA) in the cheek pouch of the Syrian hamster (8), have been chosen for this study. In both histology and immunohistochemistry, this tumour model mimics human carcinogenesis in the upper aero-digestive tract and oesophagus (9–11).

Wavelength dependence has been studied in vivo and in vitro with different second-generation PSs such as phthalocyanines

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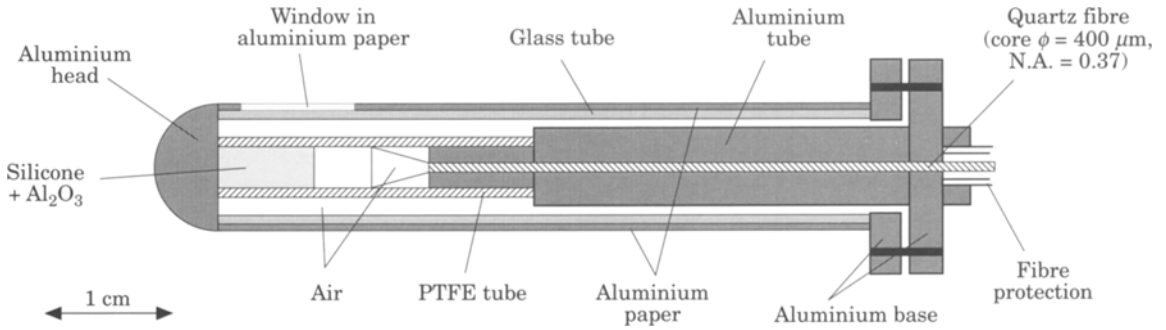

Fig. 1. Light distributor used for irradiation of the hamster cheek pouch mucosa.

(12, 13), BPD-MA (14) and 5-ALA (15). With many PSs, wavelength shifts are observed between the absorption maxima measured in solvents and the most effective PDT wavelength in vivo (12, 13). Such shifts can be due to different factors such as the localization of the PS in different tissues and tissue compartments as well as tissue absorption properties, the production of singlet oxygen, or the physico-chemical environment (16).

To measure the PDT 'action spectrum' around 652 nm, irradiation tests between 642.5 and 665 nm were performed. Results were compared with the fluorescence excitation spectrum of mTHPC measured in vivo, and its absorption spectrum measured in vitro (1, 2). To the best of the authors' knowledge, this information is not available for mTHPC.

MATERIALS AND METHODS

Animal model

Carcinogenesis in the hamster cheek pouch was chemically induced (BRL, Fuellinsdorf, Switzerland) according to methods that have been described previously (17). Briefly, the early SCC (carcinoma in situ and microinvasive carcinoma) was produced by topical application of 0.5% oily DMBA (Sigma Chemicals Co. St Louis, USA) solution in the left cheek pouch mucosa thrice weekly over 10 weeks. The contralateral cheek pouch, which was not painted with DMBA, served as control. The animals were housed at room temperature with a 12-h light/dark cycle. Food and drinking water were given ad libitum. All experiments were performed under intraperitoneal anaesthesia (ketalar 150 mg kg^{-1} and xylesine 15 mg kg^{-1}) and in accordance with protocols approved by the experimental animal ethics committee.

Photodynamic therapy

Tetra(m-hydroxyphenyl)chlorin (mTHPC) was supplied in powder form by Scotia Pharmaceuticals Ltd (Guildford, UK). Prior to utilization, it was dissolved in a solution of 30% v/v polyethylene glycol 400, 20% v/v ethanol and 50% v/v H_2O. Photodynamic therapy on tumour-bearing and contralateral healthy cheek pouch mucosae was performed 4 days after an intracardiac injection of 0.5 mg kg^{-1} body weight mTHPC (18). Irradiation conditions were adapted to those applied in clinical trials (19). The light dose of 12 J cm^{-2} was delivered at a rate of 150 mW cm^{-2}. This light intensity is not sufficient to induce a thermal effect in tissues (20). Photodynamic therapy was carried out with wavelengths between 642.5 and 665 nm at 2.5-nm increments.

Light source and light delivery

An argon-ion pumped dye laser system [Spectra-Physics Model 2045 (20 W continuous wave in the visible) and Spectra-Physics Model 375 B dye laser], operated with 4-dicyanomethylene-2-methyl-6-(p-dimethylaminostyryl)-4H-pyran (DCM) dye (LC 6500 from Lambda Physics) was used as the light source. The DCM dye allows the laser to be tuned between 600 and 695 nm. The wavelength is tuned with a birefringent filter (Spectra-Physics, Model 573-94) located in the dye laser cavity, and the wavelength is verified with a monochromator [Jobin-Yvon, Model H 10, Precision: 0.2 nm full width half maximum (FWHM)]. The light was applied using a 1 cm diameter cylindrical distributor equipped with a lateral circular window (Fig. 1). The light delivered by an optical quartz fibre to the light diffuser is expanded through a small air space, and passes into a cylindrical rod that contains a transparent

silicone polymer loaded with Al_2O_3 particles in order to scatter the light. The aluminium end mirror and the concentration of particles along the main axis of this cylinder are chosen to give a homogenous light distribution at the surface of the outer cylinder. Careful positioning of the light diffuser in the hamster cheek pouch in direct contact with the buccal mucosa offers a high reproducibility in the light dosimetry.

Analysis of PDT-induced tissue damage

The animals were killed, using an overdose of the same anaesthetics as mentioned above, 96 h after irradiation, which corresponds to the time of maximal mucosal damage. The procedure used for taking biopsies has been described previously (21). The entire tumoral and healthy cheek pouches were resected, and lesions were divided into two equal specimens. One was fixed in 5% buffered formalin (pH 7.0), paraffin-embedded, sectioned in 5-μm thick slices and stained with Haematoxylin and Eosin (H&E) for standard histological examination. For each animal, the whole necrotic area was examined in serial sections stained with H&E to evaluate the depth of the mucosal destruction. The other part of the excised lesion was frozen immediately in liquid nitrogen by contact with an isopentane slush, and cut at cryogenic temperatures in 5-μm thick slices. These were used in some cases for additional histochemical myosin-ATP-ase and NADH-diaphorase staining techniques, which are especially useful for assessing the degree of damage to striated muscles (22, 23). Following the enzymatic reaction, counterstaining with Haemalun allowed the delineation between necrotic and healthy layers to be demonstrated.

In order to evaluate the histological depth of PDT damage on different mucosal layers, such as epithelium, lamina propria or striated muscle, a four-graded tissue-damage scale was used. On this scale, Grade 1 corresponds to the destruction of the epithelium. In Grade 2, the necrosis extends to the lamina propria. Grade 3 corresponds to a necrosis that reaches the striated muscle, and in Grade 4, all mucosal layers are destroyed. Tissue damage up to Grade 2 was estimated as an insufficient effect. Grades 2 and 3 were judged as an 'optimal' PDT response, and Grade 4 necrosis was considered to be an overdose response. The grades of necrosis and evaluation of the tissue damage are summarized in Table 1. This non-linear, morphological scale has been chosen because massive (although not always observed) interstitial oedema of the mucosal wall hinders reproducible micrometric measurements of the tissue damage. This means that when a marked oedema is present, the absolute measurement of the depth of necrosis could be increased artificially as compared to the same necrosis in a non-oedematous cheek pouch.

Table 1. Tissue damage scale

Grade of tissue damage	Histology
0	No tissue destruction
1	Destruction of the epithelium
2	Destruction of the epithelium and the lamina propria
3	Destruction of the epithelium, the lamina propria and the striated muscle
4	Destruction of all layers resulting in transmural necrosis

Grades 0 and 1 are estimated as insufficient, 2 and 3 as optimal and 4 as overdose responses.

Statistical analysis

The results of irradiation performed on the healthy and tumour mucosae were evaluated as depths of tissue damage graded from 0 to 4 using the scale described above. The statistical significance of the differences in tissue responses achieved at various wavelengths was determined using a non-parametric Mann-Whitney U-test ($\alpha \leq 0.05$).

RESULTS

Macroscopically, the first changes in the irradiated mucosae were observed 24 h after PDT as a diffuse oedema of the whole cheek pouch. The first visible tissue reaction matching the irradiation window appeared 48 h later, and achieved a maximum at 96 h after PDT. Figure 2 illustrates the results of the irradiation in terms of tissue damage to the healthy and neoplastic mucosae at

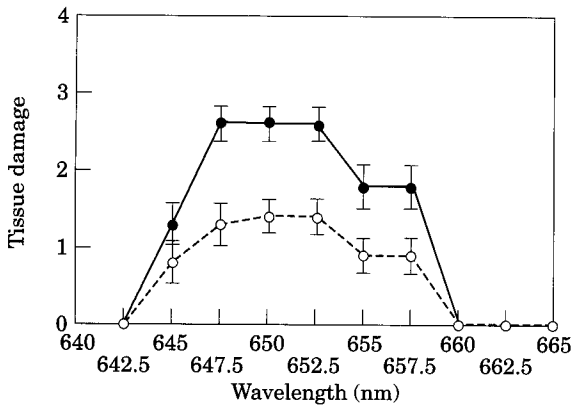

Fig. 2. Damage induced by photodynamic therapy (PDT) on healthy and neoplastic hamster cheek pouch mucosae 4 days after injection of 0.5 mg kg^{-1} mTHPC. The fluence of 12 J cm^{-2} was delivered at a fluence rate of 150 mW cm^{-2}. The wavelength was varied from 642.5 to 665 nm in 2.5-nm increments. Light delivered between 647.5 and 652.5 nm induced maximal damage to both tumour-bearing (●) and healthy (○) mucosae. At these wavelengths, significantly higher PDT damage was noted for early squamous cell carcinoma as compared to the healthy mucosa ($\alpha \leq 0.05$). At 645, 655 and 657 nm, less pronounced tissue damage was observed for both mucosae. Light applied at wavelengths below 642.5 and over 660 nm did not induce any visible tissue damage. Data points represent mean tissue damage for five animals, as determined by the rating scale described in the text. Error bars are ±1 standard deviation. The significance of the differences in tissue responses achieved at various wavelengths was determined using a non-parametric Mann-Whitney U-test ($\alpha \leq 0.05$).

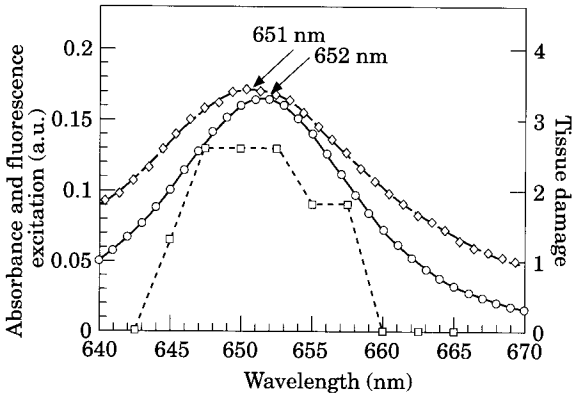

Fig. 3. The in vitro absorption spectrum of 5 μM mTHPC in phosphate-buffered saline (PBS) solution supplemented with 10% bovine serum (○) as well as the in vivo fluorescence excitation spectrum (◇) and the photodynamic therapy tissue damage 'action spectrum' (□) are compared. The fluorescence excitation peaks are at nearly the same wavelengths as the absorption peaks (absorption in vitro 652±1 nm and fluorescence excitation in vivo 651±0.5 nm). Fluorescence excitation and absorption curves have patterns in the red region (647–652 nm) that are similar to those of the tissue-damage curve.

wavelengths ranging from 642.5 to 665 nm. Light delivered between 647.5 and 652.5 nm induced maximal damage to both the healthy and tumour-bearing mucosae. At these wavelengths, significantly higher damage from PDT was noted for early SCC as compared to the healthy mucosa ($\alpha \leq 0.05$). With the same light dose delivered at 645, 655 and 657 nm, less pronounced tissue damage was observed for both mucosae ($\alpha \leq 0.05$). Light at wavelengths below 642.5 and over 660 nm did not induce any visible tissue damage.

DISCUSSION

This in vivo study has shown that in the red part of the spectrum, wavelengths between 647 and 652 nm induce the greatest amount of tissue damage in both the healthy and tumour-bearing mucosae. The range of wavelengths (647–652 nm) over which PDT of early SCC is most effective in this particular tumour model suggests that for early cancers in hollow organs, fairly broad-band light sources can be used while retaining a maximum phototoxic effect.

The mTHPC-PDT tissue damage curve can be compared to either the in vitro absorption spectrum or the in vivo excitation signal as shown in Fig. 3. The in vitro absorption curve of 5 μM mTHPC in phosphate-buffered saline (PBS) supplemented with 10% bovine serum shows its absorption maximum in the red to be 652 nm (1, 2, 24). The in vivo fluorescence excitation spectrum of mTHPC measured with a fibre-based optical multichannel analyser (25) on the healthy and tumour-bearing cheek pouch mucosae has a fluorescence excitation peak at nearly the same wavelength (M. Forrer, unpubl. data). The slight difference between these spectra can be attributed to two factors: first, the difference in precision of the two methods used; and second, the influence of the optical tissue absorption and scattering on the fluorescence excitation spectra (26). The fluorescence absorption and excitation spectra are similar in form to the tissue-damage curve. The region of pronounced tissue damage is situated between 647.5 and 652.5 nm. These wavelengths correspond to equal fluorescence excitation and absorption intensities on either side of the fluorescence excitation and absorption maxima. In contrast to observations reported for some of the other compounds used in PDT (1), there seems to be no wavelength shift between the absorption, excitation and tissue-damage response curves in the red part of the spectrum.

In the present experiments, the highest PDT efficacy was achieved at wavelengths of

650 ± 2.5 nm using an argon-ion pumped dye laser with a 0.2 nm FWHM. Recent developments in diode laser technology offer high-power devices with several nanometre bandwidths (27). The results obtained here suggest that such diode lasers can be used as appropriate light sources without a significant loss of PDT efficacy.

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