Optimizing Photodynamic Therapy: In vivo Pharmacokinetics of Liposomal meta-(Tetrahydroxyphenyl)Chlorin in Feline Squamous Cell Carcinoma

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Abstract

Purpose: The aim of the present study was to optimize and simplify photodynamic therapy using a new liposomal formulation of the photosensitizer meta-(tetrahydroxyphenyl)chlorin [m-THPC (Foscan); liposomal m-THPC (Fospeg)] and to reduce systemic reactions to the photosensitizer.

Experimental Design: To examine the pharmacokinetics of liposomal m-THPC, we determined tissue and plasma variables in feline patients with spontaneous squamous cell carcinoma. In vivo fluorescence intensity measurements of tumor and skin were done with a fiber spectrophotometer after i.v. injection of m-THPC or liposomal m-THPC in 10 cats. Blood samples, drawn at several time points after photosensitizer administration, were analyzed by high-performance liquid chromatography.

Results: None of the liposomal m-THPC-treated cats showed side effects during or after drug injection. Fluorescence intensities, fluorescence ratios (tumor fluorescence divided by skin fluorescence), and bioavailability in the tumor were 2 to 4 times higher with liposomal m-THPC compared with m-THPC. Liposomal m-THPC concentration in the tumor increased constantly to reach a maximum at 4 hours after injection. Plasma concentration and bioavailability were ~3 times higher with liposomal m-THPC compared with m-THPC measured at the time points of highest plasma concentration. The distribution half-life was shorter with liposomal m-THPC, resulting in maximal tumor accumulation up to 5.5 times earlier. Maximal tumor accumulation and maximal fluorescence ratio with liposomal m-THPC occurred at the same time point, indicating maximal selectivity. In both groups, all cats responded to therapy.

Conclusions: Liposomal m-THPC was well tolerated by all cats and seems to have superior pharmacokinetic properties compared with m-THPC. The efficacy of the drug warrants further study.

The first reports on photodynamic therapy (PDT) date back to the beginning of the last century, when researchers observed that a combination of light with hematoporphyrin induces cell death (1). In 1995, the U.S. Food and Drug Administration approved PDT as a novel form of therapy against cancer, and since then, PDT has been used more frequently.

PDT includes two components combined to induce cellular and tissue effects in an oxygen-dependent manner. The first is a “light-sensitive” substance called the photosensitizer. The second is light of a specific wavelength (laser light) to maximally activate the tumor-localized photosensitizer. On activation, a photosensitizer undergoes type I (electron or hydrogen transfer) or type II (local generation of cytotoxic singlet oxygen) photochemical reactions.

Tumor destruction associated with PDT involves three principal mechanisms (2): (a) direct tumor cell kill (3), (b) destruction of tumor-associated vasculature (4–6), and (c) activation of an immune response against tumor cells (7, 8). A short drug-light interval allows the photosensitizer to accumulate predominantly in the vascular compartment. PDT-mediated vascular effects range from transient vascular spasm, vascular stasis, and thrombus formation to total permanent vessel occlusion and can include enhanced vascular leakiness (5). A longer drug-light interval results in maximal concentration of the photosensitizer in the tumor, causing direct tumor cell destruction. This was shown recently for the second-generation photosensitizer meta-(tetrahydroxyphenyl)chlorin [m-THPC (Foscan)] and indicates that the in vivo effects occur via an indirect vascular effect as well as a more direct effect at different drug-light intervals (9, 10).

To optimize PDT, liposomes are presently being tested as carrier and delivery systems with the aim of improving the tumorotropic behavior of photosensitizers.
The present study was thus designed to optimize PDT in cat patients with spontaneous cutaneous squamous cell carcinomas using a new, liposomal formulation of m-THPC [liposomal m-THPC (Fospeg)]. Pet animals with spontaneously developing cancer provide an excellent opportunity to study many aspects of cancer from etiology to treatment. Squamous cell carcinomas are common neoplasms in cats. Similar to human cutaneous squamous cell carcinoma, there is an etiologic correlation between development of the neoplasm and exposure to UV light (11). Several studies have shown the efficacy of PDT in the treatment of feline squamous cell carcinoma (12–15).

The aim of the present study was to investigate the accumulation of liposomal m-THPC in tumor and skin in situ with a spectrophotometer and to define the plasma pharmacokinetics in cats. We hypothesized that, with liposomal m-THPC, a higher tumor-to-skin ratio and an earlier m-THPC concentration peak in plasma and neoplastic tissue could be achieved.

## Patients and Materials and Methods

### Animal patients

Ten pet cats with histologically confirmed cutaneous squamous cell carcinoma were included in the study. Tumors were clinically staged using a modification of the WHO system (14) and included thoracic radiographs, blood analysis (hematology and organ variables), regional lymph node aspiration, and urinalysis. All animal treatments were conducted according to the approval issued by the official veterinary authorities of the Canton of Zurich. All animal owners signed a written informed consent.

### Photosensitizers

The original lipophilic formulation of m-THPC (1.5 mg/mL, 3 mL vials) and the new liposomal formulation of m-THPC (1.5 mg/mL, 3 mL vials) were used (kindly provided by Biolitec AG, Jena, Germany). m-THPC is practically insoluble in all aqueous media. The single component is of >99% purity, with its fluorescence emission peak in the red at 652 nm. The molecular weight of m-THPC is 680.24 Da.

Liposomal m-THPC basically comprises dipalmitoylphosphatidylcholine, dipalmitoylphosphatidylglycerol, and pegylated distearoylphosphatidylethanolamine as liposome-forming compounds. The physical stability of the liposomal formulation was assessed by monitoring the particle size distribution using photon correlation spectroscopy. The mean particle size was ~140 nm. The degree of pegylation was 2.5% to 5% and the degree of m-THPC was 10%. The drug is associated with the lipid membrane of the unilamellar liposomes. The liposomal m-THPC formulation can be diluted with aqueous medium or biological fluids without precipitation.

### Drug application

All animals received 0.15 mg m-THPC/kg body weight out of the same production lot of either m-THPC or liposomal m-THPC. The drug was continuously injected into a cephalic or femoral vein over 10 minutes. Cats that were given m-THPC were premedicated with Clemastin (Tavegyl, Novartis Consumer Health Schweiz AG, Bern, Switzerland; 0.05 mg/kg), a H1 receptor antihistamine. Cats receiving liposomal m-THPC were not premedicated to observe systemic reactions, if any.

For all measurements, time t = 0 corresponded to the end of the injection.

### Spectrophotometric fluorescence measurements

In vivo fluorescence measurements were done noninvasively (a) in normal skin (unpigmented) and (b) in the tumor every hour for the first 10 hours and then at 16, 24, 36 (m-THPC group only), 48, and 72 hours after drug injection. A fiber spectrometer (optical biopsy system, kindly provided by Dr. Martin O’Dwyer, Department of Physics and Astronomy, University of Glasgow, Glasgow, United Kingdom and Biolitec) was used. This instrument is composed of a 405 nm laser diode excitation source, an optical fiber, and a spectrometer. Emission from the tissue was collected, and after suitable filtering, the spectra from 450 to 700 nm were displayed (integration time 500 ms). Intensity values at 652 nm were recorded. Photobleaching at the surface was not relevant due to short acquisition times and a low power at the end of the fiber (30 μW). The tip of the fiber was held perpendicular to the surface of the tissue exerting constant pressure to assure contact. At each location, three individual measurements were obtained and the mean value was determined. The spectrophotometer was calibrated at zero (dark current) before each measurement.

#### Photosensitizer concentration in plasma/urine

To determine the plasma concentration of m-THPC, blood samples were obtained 1, 3, 6, 8, 16, 24, 48, and 72 hours after application of the photosensitizer.

The 1 to 1.5 mL blood samples were collected in sterile CTAD tubes (Becton Dickinson AG, Basel, Switzerland). The tubes were immediately centrifuged for 30 minutes at 2,500 × g and 4°C. The plasma supernatant was stored at −80°C. The plasma m-THPC concentration was measured by high-performance liquid chromatography (Biolitec). A weighed sample of 20 mg was blended with 1.5 mL DMSO/methanol (5:3, v/v) and agitated for 12 hours at 60°C. Afterward, the samples were centrifuged for 5 minutes at 13,000 × g and the clear supernatant (1 mL) was removed for high-performance liquid chromatography analysis [Gold System Module 168, Beckman + fluorescence detector RF-10A XL, Shimadzu (Duissburg, Germany); column, “LiChroCART 250-4” with Purosorher STAR RP-18 end capped, 5 μm, Merck (Darmstadt, Germany); guard column, “LiChroCART 4-4” with Purosorher STAR RP-18e, 5 μm, Merck; temperature, 30°C; mobile phase, acetonitrile/H2O + 0.1% trifluoroacetic acid = 57.5% (v/v)/42.5% (v/v); flow rate, 1 mL/min]. The fluorescence wavelength was set at 410 nm for excitation and 653 nm for emission. The plasma m-THPC concentration was calculated from a calibration curve constructed by plotting the peak height values of m-THPC standard solutions versus their concentrations.

Urine samples were taken at different time points after injection and analyzed by high-performance liquid chromatography for potential m-THPC content.

#### Treatment

PDT of the spontaneous cutaneous carcinomas in client-owned cats was done under anesthesia 48 hours after m-THPC injection and 16 hours after liposomal m-THPC injection. The interval of 48 hours for m-THPC was based on previous experience in cats (12). For liposomal m-THPC, the shorter interval of 16 hours was based on results from laboratory mice, which indicated a more rapid metabolism for the liposomal formulation.

A 652 nm diode laser (Applied Optronics Corp., South Plainfield, NJ) was used as light source. The light was delivered using a quartz optical fiber with a microlens at the tip (Medlight SA, Ecbulens, Switzerland). Noncontact surface illumination of the entire tumor area plus a security margin of 5 mm was implemented. The power at the end of the fiber was measured by a calibrated power meter at 652 nm and the laser was adjusted to obtain a 0.05 W/cm² nonthermal power density on the irradiated surfaces. A dose of 10 J/cm² was delivered and the treatment time (200 seconds) was controlled with the laser’s built-in countdown timer.

Anesthesia was induced by i.v. application of Buprenorphine (Temgesic, Essex Chemie AG, Luzern, Switzerland; 10 μg/kg), Midazolam (Dormicum, Roche Pharma AG, Reinach, Switzerland; 0.2 mg/kg iv), and Propofol (Propofol, Fresenius Kabi AG, Stans, Switzerland). Propofol was given until tracheal intubation was possible. Anesthesia was maintained with Isoflurane (Forene, Abbott AG, Baar, Switzerland; 1-3% in oxygen). Hemoglobin oxygen saturation and heart rate were monitored continuously throughout anesthesia with a pulse oxymeter, and values were recorded before and during illumination. Ringer’s lactate solution was administered i.v. at 4 mL/kg/h.

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5 Dr. Susanna Grae«fe, unpublished data.
Posttreatment, the cats received Buprenorphine (10 μg/kg) every 8 hours for 48 hours. The following 3 weeks they received Piroxicam (Pirocam, Spirig Pharma AG, Egerkingen, Switzerland; 0.3 mg/cat) once daily as pain medication. If necessary, amoxicillin and clavulanic acid (Synulox, Pfizer AG, Zürich, Switzerland; 12.5 mg/kg twice daily) were given.

Toxicity. Acute toxicity was assessed during and after photosensitizer administration. Blood and urine samples were taken 72 hours after injection of the photosensitizer to rule out an effect of the drug on organ variables. Tissue reactions were assessed during, 1 hour after, and 4 days after light treatment. Toxicity was scored according to Common Terminology Criteria for Adverse Events version 3.0.

Statistical analysis. Pharmacokinetic values were obtained by using pharmacokinetic functions for Microsoft Excel. Description of data is given by mean and median ± SD. StatView 5.0.1 software was used for statistical analysis. Data were investigated graphically by box plots. To investigate differences between both drugs with respect to tissue fluorescence and plasma variables, the Mann-Whitney U test was applied. Ps < 0.05 were considered significant.

Results

Animal patients. All patients included in the study were European short hair cats. Four of the patients were female spayed and six were male neutered. There was no statistically significant difference concerning gender, age, or weight between the two groups. All tumors (n = 10) were classified as squamous cell carcinoma by histopathologic examination. All tumors were located on the head, with the majority of tumors occurring on the nasal planum (n = 6). Most of the treated squamous cell carcinomas were low-stage tumors (T1a-T2a; n = 6). None of the cats had evidence of metastatic disease (N0M0).

Spectrophotometric fluorescence measurements. Patients receiving m-THPC showed mean and median maximal tumor fluorescence intensities of 160.88 and 154.89 (relative units), respectively. These maxima were seen between 36 and 48 hours after injection (Table 1). In patients treated with liposomal m-THPC, the mean and median maximal m-THPC fluorescence intensities in the tumor were 275.67 and 279.00 (relative units), respectively. In this group, maxima were reached 5 to 10 hours after injection (Table 1). The fluorescence intensity maxima were not significantly different (P = 0.139), although a distinct difference is obvious (Fig. 1). The intensity increased rapidly over the first 4 hours to reach a maximum and a plateau phase 4 to 6 hours after liposomal m-THPC injection. The time point of intensity maxima differed significantly (P = 0.015) between the two groups. The fluorescence ratio, defined as the mean fluorescence intensity of the tumor divided by the mean fluorescence intensity of normal skin at a given time point, was calculated for each individual animal. For m-THPC, these ratios ranged from 1.79 to 4.81 (mean and median values of 3.02 and 2.45, respectively). For liposomal m-THPC, the ratios ranged from 3.47 to 18.34 (mean and median values of 10.75 and 11.98, respectively). The fluorescence ratio was higher for liposomal m-THPC compared with m-THPC (Fig. 1), although statistical significance was not achieved (P = 0.053). Highest ratios were reached much later after injection of m-THPC than after injection of liposomal m-THPC (Table 1); with liposomal m-THPC, a rapid increase during the first 5 to 10 hours could be observed. For liposomal m-THPC only, the time point of the maximal fluorescence intensities and the time point of the highest tumor-to-skin ratio were identical.

The mean and median bioavailability in the tumor, calculated for the first 16 hours after injection [area under the curve (AUC)], was significantly higher for liposomal m-THPC than for m-THPC (P = 0.030; Fig. 2). The bioavailability in the skin was similar for both photosensitizers (P = 0.305). The AUC16 ratio, calculated from the tumor values divided by the skin values, for m-THPC resulted in mean and median values of

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2.00 and 1.55, whereas the AUC16 ratio for liposomal m-THPC showed mean and median values of 6.94 and 6.50, respectively \((P = 0.053; \text{Table } 1)\).

**Photosensitizer concentration in plasma/urine.** In the m-THPC group, mean and median maximal m-THPC levels of 431.38 and 458.81 ng/mL plasma were reached during the first 8 hours after injection. During this time range, m-THPC levels remained constant or increased slightly. Afterward, the photosensitizer concentration decreased to reach a level near baseline 48 hours after injection. For m-THPC, the calculated distribution half-life (hour 0-16) resulted in mean and median values of 46.74 and 44.62 hours, respectively. The calculated elimination half-life (hour 16-72) showed mean and median values of 20.64 and 20.32 hours, respectively (Table 2). In the liposomal m-THPC group, significantly higher mean and median maximal m-THPC levels were found (1,317.08 and 1,523.30 ng/mL plasma, respectively) compared with the m-THPC group \((P = 0.017)\). The highest m-THPC plasma levels were seen at the first time point measured followed by an immediate and rapid disappearance of the photosensitizer signal (Fig. 3). For liposomal m-THPC, the calculated distribution half-life (hour 0-16) was significantly shorter \((P = 0.017)\), resulting in mean and median values of 9.36 and 7.96 hours. The calculated elimination half-life (hour 16-72) resulted in mean and median values of 22.69 and 19.83 hours, which were comparable with the values obtained with m-THPC \((P = 0.732; \text{Table } 2)\). Plasma drug concentrations returned to near baseline 48 hours after injection.

The mean and median bioavailability, calculated at 16 and 72 hours, was much lower in the m-THPC group compared with the liposomal m-THPC group \((P = 0.053 \text{ and } 0.087, \text{ respectively}; \text{Table } 2)\). m-THPC could not be detected in the urine at any of the time points examined after injection.**

**Treatment response.** All three cats in the m-THPC group had a complete response to therapy. No tumor recurrence could be observed thus far. In the liposomal m-THPC group, four cats had a complete response and three had a partial response. Two of these three cats had tumor recurrences and no further therapy was done, whereas one cat was retreated with PDT and then had a complete response. No tumors have recurred in the cats with a complete response. The longest follow-up time in both therapy groups, however, is only 380 days.

**Toxicity.** During or shortly after m-THPC injection, two of the three cats showed side effects, such as tachypnoe, salivation, and excitation, although they were premedicated. The liposomal m-THPC injection was well tolerated by all cats. None of the liposomal m-THPC–treated animals showed signs of acute toxicity, such as vomiting, diarrhea, salivation, tachypnoe, excitation, or death. There was no obvious difference in skin reaction between the two groups. Only one cat in the liposomal m-THPC group (patient 7), which had extremely high skin fluorescence intensities, developed sunburn-like reactions on the face ~10 days after injection, which healed uneventfully.

Fig. 1. Tumor and skin fluorescence intensities as a function of time after injection of either m-THPC or liposomal m-THPC. Points, median values of all patients; bars, SD.

Fig. 2. Bioavailability of either m-THPC (=1) or liposomal m-THPC (=2) in the tumor during the first 16 hours after injection: box plot investigation.
Discussion

PDT has the potential to be a powerful treatment modality for cancer either applied solitary or in combination with chemotherapy (16), surgery (17), radiotherapy (18), or other strategies, such as hyperthermia (19). However, low selectivity, inconveniently long drug-light intervals, and prolonged generalized photosensitivity are problems encountered with this therapy modality. Modifying the photosensitizing moiety through its physicochemical properties may improve PDT.

In the present study, a new formulation of a commercially available photosensitizer has been studied to address the following hypotheses: the liposomal formulation (a) allows a shorter drug-light interval, (b) results in a higher tumor-to-skin ratio, and (c) shows an earlier plasma peak compared with the conventional formulation.
Maximal tumor fluorescence intensities with liposomal m-THPC were nearly twice as high compared with m-THPC. This is important clinically, as the drug dose needed could probably be lowered. A lower drug dose would result in a shorter generalization photosensitivity of the patients. With liposomal m-THPC, the time of maximal fluorescence intensity in the tumor was shown to be significantly earlier than with m-THPC \((P = 0.015)\). These results suggest that the drug-light interval could be reduced by a factor of 5.5, representing a second relevant advantage for clinics and fulfilling the first hypothesis. For liposomal m-THPC, the time point of maximal tumor accumulation was in accordance with the time of maximal tumor to skin ratio. For m-THPC, this was not the case in the present study. In previously published work using m-THPC, a drug-light interval of 4 to 12 hours resulted in extensive skin and muscle necrosis \((20, 21)\), indicating a low tumor-to-normal tissue ratio at this early time point. In contrary, no obvious changes in normal tissues were observed at any drug-light interval when pegylated m-THPC was used \((22)\). Results of our study clearly show much higher tumor-to-skin ratios in spontaneous feline squamous cell carcinoma with the liposomal drug. In general, the uptake of pegylated photosensitizers is enhanced due to an increased vascular permeability of tumor vessels. In addition, tumor tissue lacks a functional lymphatic system; therefore, extravasated macromolecules cannot return efficiently to the central circulation. By showing a 4 times higher maximal tumor-to-skin ratio for liposomal m-THPC compared with m-THPC, we have proven our second hypothesis. A mean fluorescence ratio of ~12 indicates a distinct selectivity of the new formulation. The lack of statistical significance may be due to the fairly small sample size in each group, although a clear trend was seen \((P = 0.053)\).

The results reported here point at differences of 3 to 4 orders of magnitude for liposomal m-THPC compared with m-THPC in terms of changes in AUC, which means a significantly higher bioavailability for the new liposomal m-THPC formulation \((P = 0.030)\). This suggests again the possibility to decrease the typical administered dosage of m-THPC. In addition, the AUC\textsubscript{16} ratio for liposomal m-THPC is ~4 times higher than for m-THPC, showing a much higher overall accumulation in the tumor than in the skin.

For liposomal m-THPC, the maximal plasma m-THPC levels were ~3.5 times higher than for m-THPC \((P = 0.017)\). With liposomal m-THPC, the maximum plasma concentration was attained at the first time point measured, whereas for m-THPC maximum plasma concentrations were seen ~6 hours after injection. For the lipophilic m-THPC, a delayed concentration maximum has been shown in the plasma of humans, dogs, cats, rabbits, and nude rats \((23)\). Reasons for this phenomenon, such as aggregation of hydrophobic substances (e.g., m-THPC), can be substantially decreased by using liposomal formulations. Therefore, our third hypothesis of an earlier plasma peak after liposomal m-THPC injection was confirmed. The mean half-lives calculated for m-THPC as well as the shape of the graphs are similar to those of a previous study in cats \((24)\). The shorter distribution half-life of liposomal m-THPC could be the reason for the earlier maximal photosensitizer accumulation in the tumor.

We did not see any complication during injection of liposomal m-THPC. During and after the injection of m-THPC, two of three cats showed side effects, such as tachypnoe, salivation, and excitation. Premedication was used because, in previously treated cats, these symptoms were seen at our institution. These side effects led to the discontinuation of m-THPC after 3 cats. Cats in the liposomal m-THPC group were not premedicated; this could have masked even weak side effects. The owners were told to keep the cats away from direct sunlight for another 10 days. The one cat that developed pronounced skin reactions was, in addition to a very low tumor-to-skin AUC\textsubscript{16} housed at high altitude and went outside 1 week after injection.

In this study, we examined tumor, skin, and plasma drug levels to generate an optimized and simplified PDT protocol. In a recent report \((10)\), no significant correlation between tumor drug level and PDT response was found, but correlation between plasma drug level and tumor response was found for m-THPC-PDT. This suggests that illumination in clinical PDT should be done at highest plasma levels, targeting the vasculature more than the tumor cell directly. In another study, m-THPC-PDT had two peaks of activity: an early effect on tumor vasculature synchronous to the plasma peak level followed by a late direct effect at maximum tumor accumulation \((9)\). After having determined the tumor and tissue peaks in the feline species, our next step will be to compare the PDT outcome of the cats treated to date with cats treated optimally (i.e., at the time of highest liposomal m-THPC tumor accumulation versus cats treated at the time of the plasma peak).

All patients of both groups responded to the treatment showing either a complete or a partial tumor remission. Due to the small patient number and the fact that we just now determined the tumor and plasma peaks for liposomal m-THPC in the feline species, a true comparison between the effectiveness of the two formulations is not yet possible. Liposomal m-THPC seems to be at least as effective as m-THPC.

In conclusion, we have shown that the new, liposomal formulation of m-THPC is a safe drug, causing no noticeable acute side effects in any cat in the present study. With liposomal m-THPC, important progress for PDT could be achieved. The combination of a higher selectivity and significantly earlier tumor and plasma peaks will result in a more efficient and eventually more effective PDT protocol.

### Acknowledgments

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