Development of meta-Tetrahydroxyphenylchlorin-Monoclonal Antibody Conjugates for Photoimmunotherapy

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ABSTRACT

A limitation of photodynamic therapy is the lack of tumor selectivity of the photosensitizer. To overcome this problem, a protocol was developed for coupling of meta-tetrahydroxyphenylchlorin (mTHPC), one of the most promising photosensitizers, to tumor-selective monoclonal antibodies (MAbs). mTHPC was radiolabeled with 131I to facilitate the assessment of the in vitro and in vivo behavior. After the modification to 131I-mTHPC-(CH2COOH)3, thus increasing the water solubility and creating a functional moiety suitable for coupling, conjugation was performed using a labile ester. Insoluble aggregates were not formed when mTHPC-MAb conjugates with a molar ratio of up to 4 were prepared. These conjugates showed a minimal impairment of the integrity on SDS-PAGE, full stability in serum in vitro, and an optimal immunoreactivity.

To test the in vivo behavior of the mTHPC-MAb conjugates, the head and neck squamous cell carcinoma-selective chimeric MAb U36 was used in head and neck squamous cell carcinoma-bearing nude mice. Biodistribution data showed that the tumor selectivity of cMB-MAb U36 conjugates mTHPC was increased in comparison with free mTHPC, despite the fact that conjugates with a higher mTHPC-MAb ratio were more rapidly cleared from the blood. Preliminary results on the in vitro efficacy of photodynamic therapy with MAb-conjugated mTHPC showed that mTHPC coupled to the internalizing murine MAb 425 exhibited more phototoxicity than when coupled to the noninternalizing chimeric MAb U36.

INTRODUCTION

PDT is a therapeutic modality for the treatment of superficially localized tumors. In this approach, a photosensitive dye (photosensitizer) is injected i.v., whereafter it accumulates more or less selectively in the tumor. After exposure to laser light in the red or near-infrared region, the sensitizer is excited and is able to produce singlet oxygen, a cytotoxic form of oxygen (1). Direct cell killing (2) and occlusion of tumor blood vessels (3), as well as a strong acute inflammatory reaction (4), can occur. These combined effects result in tumor necrosis. PDT has been applied for noninvasive treatment of many types of cancer, including colon, bladder, lung, and head and neck cancer (5–8).

Until now, Photofrin was one of the most frequently used photosensitizers. Photofrin, which has an absorption maximum at 630 nm, is the commercially produced photosensitizer purified from hematoporphyrin derivative. However, this sensitizer has some drawbacks. The skin toxicity observed with Photofrin-based PDT is rather long lasting, 6–10 weeks. Moreover, Photofrin is a mixture of mono-, di-, and oligomers, and it is not clear which components contribute to the photochemical effects (9).

Alternative photosensitizers have become available recently. One of the most promising second generation photosensitizers is mTHPC. mTHPC is a pure and well-defined compound with better photochemical properties than those of Photofrin. It has a strong absorption band at 652 nm (absorption coefficient, 22400 l·mol⁻¹·cm⁻¹, with 3000 for Photofrin at 630 nm). The longer wavelength light used to excite mTHPC can penetrate deeper into the tissue than 630 nm light, thus allowing treatment of larger tumors. The high photochemical efficiency of mTHPC means that lower light doses (and shorter illumination times) are required for a tumoricidal PDT effect.

Preliminary results of PDT with mTHPC in head and neck cancer patients are encouraging (10). The largest study has been performed by Savary et al. (11) using optimized protocols for PDT with mTHPC for the treatment of early second primary squamous cell carcinoma of the esophagus, bronchi, and mouth. All lesions were carcinoma in situ or microinvasive carcinoma, which had been detected by rigid endoscopy and toluidine blue as a vital stain. Of the 33 lesions treated in this trial, 28 showed no recurrence during the mean follow-up of 14 months. In comparison to surgery and radiotherapy, PDT shows a low morbidity with little fibrosis and scarring.

Despite these promising results, mTHPC-based PDT leaves room for improvement. A limitation is the lack of tumor selectivity, which can result in severe normal tissue damage after PDT of large surface areas. An option to overcome this problem is to couple mTHPC to MAbs directed against tumor-associated antigens. In this way, the photosensitizer will be targeted selectively to the tumor. These mTHPC-MAb conjugates might be especially suitable for the treatment of multiple tumor foci in large areas, as is the case in minimal residual disease after surgical resection of thoracic and peritoneal tumors. The problem of phototoxicity will also be reduced because the accessibility of the skin is limited for MAbs.

The proof of concept for selective delivery of photosensitizers to tumors by MAbs was delivered by Pe`legrin et al. (12) and Folli et al. (13). They showed the effectiveness of a fluorescein-anti-carcinoembryonic antigen MAb conjugate for photoimmunodetection of colon carcinoma in mice and in patients. More recently, they developed an indocyanin-MAb E48 conjugate and evaluated these conjugates in nude mice bearing squamous cell carcinoma xenografts (14). These novel conjugates appeared to be superior for photoimmunodetection in comparison with the fluorescein-MAb conjugates, but unfortunately, neither of these conjugates is suitable for therapy because of their photochemical properties.

In the development of photoimmunonoconjugates for therapy, the synthesis of mTHPC-MAb conjugates has not yet been described. A serious problem in this respect is the poor water solubility of mTHPC. Other factors expected to hamper the development of mTHPC-MAb conjugates suitable for tumor targeting are the potential chemical cross-linkings during conjugation, as well as the impairment of the...
immunoreactivity and pharmacokinetic behavior of the MAb and the photochemical activity of the conjugates. Our institute focuses on the use of MAbs for selective targeting of HNSCC. To this end, MAbs E48 and U36 have been developed (15, 16). Radioimmunoscintigraphy/biodistribution studies in HNSCC patients showed that these MAbs are highly capable of selective tumor targeting (17–19). This observation justifies a study for the use of these MAbs as transport vehicle for selective delivery of mTHPC to HNSCC.

In this report, we describe a protocol for the reproducible synthesis of mTHPC-MAb conjugates and their biodistribution after administration to HNSCC-bearing nude mice. Conjugation and biodistribution studies were performed with dual labeling using 131I-labeled mTHPC and 125I-labeled MAb. Preliminary data on the in vitro efficacy of mTHPC-MAb-mediated PDT will be provided.

### MATERIALS AND METHODS

mTHPC. mTHPC (M, 680.76) was obtained from Scotia Pharmaceuticals (Surrey, UK) as a pure solid. [14C]mTHPC (also provided by Scotia Pharmaceuticals) was synthesized by American Radiolabeled Chemicals, Inc. (St. Louis, MO).

MAbs. Selection and production of MAb U36 and its chimeric (mouse/human) IgG1 derivative (cMAb U36) have been described previously (16, 20). MAb U36 recognizes the v6 domain of the M, 200,000 CD44 splice variant epican (21), which is highly expressed in squamous cell carcinoma of the head and neck, lung, skin, esophagus, and cervix, adenoma carcinomas of breast and lung, as well as in normal stratified epithelium. A clinical radioimmunoscin- 
tigraphy study with 99m Tc-labeled U36 revealed that U36 IgG accumulates selectively and to a high level in squamous cell carcinoma of the head and neck (19), and therefore, the MAb is presently evaluated in radioimmunotherapy studies.

### Table 1

<table>
<thead>
<tr>
<th>OH (s)</th>
<th>pyrrole-CH</th>
<th>TFP-H (m)</th>
<th>benz.-H (m)</th>
<th>-CH2CO-(d)</th>
<th>pyrrole-CH2 (s)</th>
<th>NH (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of protons</td>
<td>4</td>
<td>6</td>
<td>4</td>
<td>16</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>mTHPC</td>
<td>9.81</td>
<td>8.68/8.42/2.85 (d/s/d)</td>
<td>7.65–7.12</td>
<td>4.20</td>
<td>–1.60</td>
<td></td>
</tr>
<tr>
<td>mTHPC-(CH2COOH)3</td>
<td>11.30</td>
<td>8.62/8.37/2.42 (d/s/d)</td>
<td>7.66–7.20</td>
<td>4.83</td>
<td>4.17</td>
<td>–1.64</td>
</tr>
<tr>
<td>mTHPC-(CH2CO-TFP)4</td>
<td>8.60/8.35/2.15 (dd/d/dd)</td>
<td>7.96</td>
<td>7.79–7.39</td>
<td>5.57</td>
<td>4.13</td>
<td>–1.60</td>
</tr>
</tbody>
</table>

*s, singlet; d, doublet; dd, double doublet; m, multiplet. 
*Observed coupling constants (Hz): ① J = 4.9; ② J = 4.9; ③ J = 5.4; ④ J = 5.4; ⑤ J = 10.1; ⑥ J = 5.2; ⑦ J = 4.3; ⑧ J = 12.7; ⑨ J = 5.1.

**Fig. 1.** HPLC profiles [absorbance (415 nm) and radioactivity] during the partial hydrolysis of [131I]mTHPC-(CH2CO-TFP)4. At the start, HPLC analysis showed: A, peak 1, mTHPC-(CH2COOH)CH2CO-TFP; and B, peak 1, [131I]mTHPC-(CH2COOH)CH2CO-TFP and peak 1’, [131I]mTHPC-(CH2COOH); and C, peak 6. During hydrolysis: C, peak 1, mTHPC-(CH2CO-TFP); peak 2, mTHPC-(CH2COOH)CH2CO-TFP; peak 3/4, two isomers of mTHPC-(CH2COOH)CH2CO-TFP; peak 5, mTHPC-(CH2COOH),CH2CO-TFP, and peak 6, mTHPC-(CH2COHH)CH2CO-TFP; and D, identical to C, corresponding [131I]-labeled compounds. Conjugations were performed with mixtures: E, peak 3/4, two isomers of mTHPC-(CH2COOH)CH2CO-TFP; peak 5, mTHPC-(CH2COOH)CH2CO-TFP, and peak 6, mTHPC-(CH2COOH)CH2CO-TFP, and F, identical to E, corresponding [131I]-labeled compounds.

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mMAb 425 is an IgG2a MAb developed and characterized by Murthy et al. (22). The epitope recognized by mMAb 425 is localized on the external domain of the EGFR, which has been shown to be highly expressed by various tumor types including HNSCC, renal cell cancer, gliomas, and carcinoma of the esophagus, bladder, cervix, stomach, lung, and breast (23–30). After binding to this antigen, anti-EGFR MAbs are internalized and catabolized by A431 cells (31). Anti-EGFR MAbs, MAb 425 included, have been studied extensively in clinical trials (32, 33).

**Cell Lines.** Characteristics of the squamous cell carcinoma cell lines UM-SCC-11B, UM-SCC-22A, and A431 and their culturing conditions have been described previously (34).

**Analyses.** HPLC analysis was performed by using an LKB 2150 HPLC-pump (Pharmacia Biotech, Roosendaal, the Netherlands), an LKB 2152 LC controller (Pharmacia Biotech, Roosendaal), and a 25-cm LiChrosorb 10 RP 18 column (Chrompack, Middelburg, the Netherlands) at a flow rate of 2 ml/min. The eluent consisted of a 9:1 (v/v) mixture of MeCN and 0.1% trifluoroacetic acid. Absorption was measured at 230 and 415 nm by a Pharmacia LKB VWM 2141 UV detector. Radioactivity was measured by an Ortec 406A single-channel analyzer connected to a Drew 3040 data collector (Betron Scientific, Rotterdam, the Netherlands).

$^1$H-NMR spectra were recorded in $[2\text{H}_6]\text{Me}_2\text{SO}$ on a Bruker ARX 400 (400.14 MHz) spectrometer and a Bruker AC 200 (200.13 MHz) spectrometer. Chemical shifts (δ) are given in ppm relative to tetramethylsilane as the internal standard. For description of the NMR spectra of mTHPC and its derivatives, see Table 1.

The absorption spectra of mTHPC and mTHPC-MAb conjugates were
measured using a UltraSpec III spectrophotometer (Pharmacia Biochrom). The mTHPC concentration in the conjugate preparations was assessed with the same apparatus at a wavelength of 415 nm. The absorption of a range of dilutions (1–9 μg/ml) of mTHPC in MeCN was measured and graphically depicted using the least square method. The mTHPC concentration in the conjugate preparations was determined using this calibration curve.

The integrity of the mTHPC-MAb conjugates was analyzed by electrophoresis on a Phastgel System (Pharmacia Biotech) using preformed 7.5% SDS-PAGE gels under nonreducing conditions. After running, gels were stained with 0.2% Coomassie Brilliant Blue (Sigma) and exposed to a Phosphor plate for 1–3 h and analyzed with a Phosphor Imager (B&L -Isogen SD). SDS-PAGE gels under nonreducing conditions. After running, gels were cut into protein bands. Quantitative information was obtained by cutting the lanes into pieces and dual label counting.

Dual Label Counting of 125I and 131I. The amounts of 125I (Ee, 35 keV) and 131I (Ee, 364 keV) were measured simultaneously in a gamma counter in the corresponding window settings (channels 35–102 and 155–185, respectively) with automatic correction for the 131I-comptongammas in the 125I-window setting; in our case, this correction corresponded to 15.2% of the 131I-phoetopeak counts present in the sample.

125I-Labeling of MAbs. Radiiodination of cMAb U36 and mMAb 425 with 125I was performed using Iodogen (Brunschwig Chemie, Amsterdam, the Netherlands) as described by Huisma et al. (35). One to 2 mg of MAb dissolved in 500 μl of PBS (pH 7.4) and 1 mCi of 125I (100 mCi/ml; Amersham, Aylesbury, England) were mixed in a vial coated with 50 μg of Iodogen. After 5 min incubation at room temperature, the reaction mixture was filtered through a 0.22 μm Acrodisc filter (Gelman Sciences, Inc., Ann Arbor, MI), and Unbound 125I was removed using a PD-10 column (Pharmacia Biotech, Woerden) with 0.9% NaCl as eluent. After removal of unbound 125I, the radiochemical purity always exceeded 98%.

131I-Labeling of mTHPC. To facilitate the analysis of the stability of the mTHPC-MAb conjugates in vitro and in vivo and their pharmacokinetic behavior, in most of the experiments mTHPC was trace-labeled with 131I. This labeling and subsequent reaction steps with mTHPC were carried out in the dark and under a N2 atmosphere to prevent unwanted photochemical reactions during the synthesis of the mTHPC-MAb conjugates.

131I-Labeling of mTHPC was performed using Iodo-beads (Brunswwich Chemie) as follows. The appropriate amount of 131I was added to 50 μl of 1 m NaOH containing 10 μg of Na2SO3. This 131I solution was added to 4 Iodo-beads covered with 450 μl of a MeCN/H2O mixture (10:1, v/v), followed by 100 μl (734 nmol) of a 131I solution (5 mg/ml in MeCN). After labeling during 30 min, the reaction mixture was diluted with 400 μl of H2O, loaded on a conditioned Sep-pak C18 cartridge (Waters, Millipore, MA), and washed with 50 ml of H2O. The 131I-labeled mTHPC (actually consisting of a small proportion of 131I-mTHPC and an excess of unlabeled mTHPC) was eluted with 3 ml of MeCN. The solvent was evaporated under a stream of N2.

The radiochemical purity of 131I-mTHPC was determined by HPLC analysis. The HPLC retention times were 9.8 min for 131I-mTHPC, between 5–9 min for 131I-labeled minor impurities, and 9.6 min for mTHPC (for the 1H-NMR data of mTHPC, see Table 1).

Preparation of the TFP Ester. Preparation of the ester (either in labeled or unlabeled form) was performed in two steps. The first step was tetracarboxymethylation of 125I-mTHPC/mTHPC. To 125I-mTHPC/mTHPC dissolved in 600 μl of a DMF:H2O mixture (5:1; v/v), 150 mg (3.7 mmol) of powdered NaOH were added, and the mixture was stirred until the solution was green (2–3 min). Hereafter, 70 mg (380 μmol) of iodooctic acid (Janssen Chimica, Beerse, Belgium) were added, and stirring was continued for another 90 min. The pH was adjusted to 5.0 with 3 ml of 1 N HCl, and the tetracarboxymethylated product was isolated by extraction with four portions of 0.5 ml of CH2 Cl2. The HPLC retention times were 7.1 min for 131I-mTHPC-(CH2COOH)4 and 7.3 min for mTHPC-(CH2COOH)4 [1H-NMR data of mTHPC-(CH2COOH)4 are given in Table 1].

In the next reaction step, the four carboxylic acid groups were esterified with an excess of TFP (Janssen Chimica). To the tetracarboxymethylated product in CH2Cl2, 150 μl of a TFP solution (100 mg/ml in DMF) and 50 mg of solid EDC (Janssen Chimica) were added. The pH was adjusted to 5.7–5.9 with 1 N Na2CO3. After reaction for 30 min, column chromatography was performed to remove all impurities. This purification was performed with a 24-cm LiChroprep Si 60 (40–63 μm) column (Merck, Darmstadt, Germany).

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using CH₂Cl₂/MeCN (97:3, v/v) as the eluent (flow rate of 1 ml/min). Fractions of 0.5 ml were collected and analyzed by HPLC with absorption measurement at 230 and 415 nm. The pure tetraester fractions (under our conditions, fractions 20–23) were pooled, and the solvent was evaporated. The HPLC retention times were 17.2 min for ¹³¹I-THPC-(CH₂CO-TFP) (Fig. 1B) and 5.9 min for ¹³¹I-THPC-(CH₂CO-TPP) (Fig. 1A). H-NMR data of ¹³¹I-THPC-(CH₂CO-TPP) are given in Table 1.

Preparation of ¹³¹I-THPC-(CH₂COOH)₃CH₂CONH¹²⁵I-MAB Conjugates. For the coupling reaction with the ¹²⁵I-labeled MAB, the ¹³¹I-THPC-(CH₂CO-TPP) (Fig. 1, A and B) was partly hydrolyzed to leave one reactive ester function, thus preventing cross-linking of MABs during conjugation. The partial hydrolysis was performed by dissolving the tetraester in 300 μl of MeCN and by stepwise addition of 10–25 μl of 10 mM Na₂CO₃. The degree of hydrolysis was monitored by radio-HPLC analysis with simultaneous absorption measurement at 415 nm (Fig. 1, C and D). When the percentage of monoester was optimal for conjugation (no tetra- and triester, <5% diester, 45% monoester together with 50% completely hydrolyzed ¹³¹I-THPC-(CH₂COOH)₃) (Fig. 1, E and F), this mixture was added to 2 mg of ¹²⁵I-labeled MAB in 1 ml of 0.9% NaCl at pH 9.5. After 30 min of incubation, the ¹³¹I-THPC-(CH₂COOH)₃CH₂CONH¹²⁵I-MAB conjugate was purified by gel filtration using a PD-10 column with 0.9% NaCl as eluent.

The conjugation efficiency was determined from the ¹²⁵I:¹³¹I ratio before and after PD-10 purification using dual labeling in a gamma counter. The ¹³¹I:¹²⁵I-THPC-MAB molar ratio was determined by measuring the absorbance at 415 nm to calculate ¹³¹I-THPC concentration and ¹²⁵I measurement for MAB quantitation. The integrity of the conjugate was checked by gel electrophoresis.

In Vitro Stability and Immunoreactivity of ¹³¹I-THPC-¹²⁵I-MAB Conjugates.® For measurement of the serum stability of the ¹³¹I-THPC:¹²⁵I-MAB conjugates, 0.5 μg of conjugate in 10 μl of 0.9% NaCl was added to 40 μl of serum. Stability was measured in mouse and human serum, whereas 0.9% NaCl served as a control. After a 20-h incubation in the dark at 37°C, samples were analyzed with SDS-PAGE. Quantitative information was obtained by cutting the lanes into pieces and dual label counting.

In vitro binding characteristics of ¹³¹I-THPC:¹²⁵I-MAB conjugates were determined in an immunoreactivity assay as described by Lindmo et al. (36) and compared with those of the unconjugated ¹²⁵I-MAB. UM-SCC-11B cells were used for ¹²⁵I-MAB U36 and A431 cells for ¹²⁵I-MAB 425.

Biodistribution Studies. The biodistribution of ¹²⁵I-MAB U36 and ¹³¹I-THPC:¹²⁵I-MAB U36 conjugate was studied in nude mice bearing s.c. xenografts of the HNSCC cell line HNX-OE, with a tumor size ranging from 50 to 200 mm³. For a comparison the distribution of ¹³¹I-THPC:¹²⁵I-MAB conjugates is given in Table 1 [1].

For the weak β-emitter ¹⁴C, the blood, urine, and organs were treated as follows. After complete decay of ¹³¹I, tissue samples were placed in counting vials, and 1 ml of Soluene-350 (Packard Instrument Company, Groningen, the Netherlands) was added to dissolve the organs. The vials were subsequently heated at 50°C for 24 h, after which 250 μl of a 1:1 (v/v) mixture of 30% H₂O₂ and acetic acid were added for decolorization of the solutions. After 1 h, Ultima Gold liquid scintillation mixture (15 ml; Packard Instrument Company) was added to the samples prior to counting in an LKB-Wallac 1410 Liquid Scintillation Counter (Pharmacia, Woerden). Radioactivity uptake in the tissues was expressed as %ID/g.

Photodynamic therapy in vitro. Phototoxicity of the ¹³¹I-THPC-MAB U36 conjugates and the unconjugated ¹³¹I-THPC was assessed in UM-SCC-22A cells using the SRB (Sigma) assay, which measures the cellular protein content (39). Cells were plated in 96-well plates (2500/well) and grown for 3 days before incubating with ¹³¹I-THPC or ¹³¹I-THPC-MAB U36 conjugates (0.1 nM to 1.0 nM ¹³¹I-THPC equivalent) in DMEM supplemented with 2 mM l-glutamine, 5% FCS, and 25 μM HEPES at 37°C. After 20 h, remaining free ¹³¹I-THPC-MAB U36 and ¹³¹I-THPC were removed by washing twice with medium. Fresh medium was added, and cells were illuminated at 652 nm with a 6 W Diode Laser (AOC Medical Systems) at a dose of 25 J/cm². Three days after illumination, growth was assessed by staining the cellular proteins with SRB and spectrophotometric measurement of the absorption at 540 nm with a microplate reader. IC₅₀ were estimated based on the absorption values and defined as the concentration that corresponded to a reduction in growth of 50% compared with values for control cells (no ¹³¹I-THPC-MAB conjugates or ¹³¹I-THPC added but illuminated in the same way). Phototoxicity of the ¹³¹I-THPC-MAB 425 conjugates was assessed in A431 cells (2000 cells/well) in a similar way.

RESULTS

Iodination of mTHPC. The first step in the synthesis of ¹³¹I-THPC-(CH₂COOH)₂CH₂CONH¹²⁵I-MAB conjugates (Fig. 2, scheme 5) was trace labeling of ¹³¹I-THPC with ¹³¹I using lodo-beads. After 30 min incubation at room temperature, HPLC analysis revealed 70–75% ¹³¹I-THPC (Fig. 2, scheme 1), <10% ¹³¹I-labeled impurities, and about 20% unreacted ¹³¹I. After purification on a Sep-pak cartridge, the final preparation contained >94% ¹³¹I-mTHPC, <4% impurities, and about 2% unbound ¹³¹I.

Synthesis of ¹³¹I-THPC-(CH₂CO-TPF)₄. In the next step, ¹³¹I-THPC was tetracarboxymethylated. The reaction, with an excess of iodoacetic acid at pH 13, followed by extraction with CH₂Cl₂, gave 95% ± 5% (HPLC analysis) tetracarboxymethylated product (Fig. 2, scheme 2). Esterification was performed with TFP, and purification of the crude product with a LiChrorep column gave the desired product (Fig. 2, scheme 3) with >95% purity, also containing <5% ¹³¹I-THPC-(CH₂CO-TPF)₃.

The purity of the fractions (0.5 ml) that were recovered from the LiChrorep column was analyzed using HPLC analysis at 415 nm for detection of ¹³¹I-THPC-(CH₂CO-TPF)₄ and at 230 nm for detection of ¹³¹I-THPC-(CH₂CO-TPF)₃, formed as a sideproduct. On the LiChrorep column, this latter ester had a retention time slightly longer than ¹³¹I-THPC-(CH₂CO-TPF)₄. The fractions that only contained ¹³¹I-THPC-(CH₂CO-TPF)₄ (under our conditions, fractions 20–23) were, after collection,
evaporated under a stream of N₂ and stored in the dark at 4°C until use. The LiChroprep purification also removed unbound ¹³¹I and any unreacted TFP, EDC, or ICH₂COOH. As a result, the mTHPC-ester was dissolved in 300 µl of MeCN before starting of the HPLC-monitored hydrolysis with 10 mM Na₂CO₃-buffer. This base was added in portions of 10–25 µl with intervals of 10 min. Approximately 125–150 µl of this buffer were used to reach the optimum mixture for conjugation (Fig. 2, scheme 4).

For conjugation, the mixture was added to a solution of ¹²⁵I-MAb in 0.9% NaCl at pH 9.5. After 30 min at room temperature, the ¹³¹I-mTHPC-(CH₂COOH)₄CH₂CONH-¹²⁵I-MAb conjugate (Fig. 2, scheme 5) was purified on a PD 10 column. When 2 mg of ¹²⁵I-MAb in a conjugation volume of 1.8 ml were used, the ¹³¹I-mTHPC-¹²⁵I-MAb molar ratio was ∼2.0–2.5. The conjugation efficiency was 60% ± 10% (corrected for completely hydrolyzed ester, which is unable to couple), whereas the recovery of the MAb always exceeded 95% (measured by ¹²⁵I activity).

By adapting the ester concentration during conjugation, conjugates with a ratio >4 could be obtained. However, under these conditions, the recovery of the MAb from the PD-10 column dropped significantly, indicating an impairment of the solubility of the MAb.

**SDS-PAGE Analysis of ¹³¹I-mTHPC-¹²⁵I-MAb Conjugates.** SDS-PAGE and subsequent Coomassie Brilliant Blue staining and Phosphor Imager analysis (Fig. 3) of the conjugate revealed one major protein band and a minor band, probably consisting of high molecular weight complexes. Cutting of the gel and dual label counting of the gel pieces showed >90% of the ¹²⁵I-MAb and >80% of the ¹³¹I-mTHPC to be localized in the main band, when conjugates with a ratio of up to 4 were analyzed. The remaining radioactivity was predominantly localized in the high molecular weight band. A typical example is shown by Fig. 3.

**In Vitro Stability and Immunoreactivity of ¹³¹I-mTHPC-¹²⁵I-MAb Conjugates.** After 24 h incubation in serum, the ¹³¹I-mTHPC-¹²⁵I-Mab conjugates were analyzed by SDS-PAGE. Cutting of the gel and subsequent dual label counting showed that the ¹²⁵I:¹³¹I ratio of the IgG peak after incubation in mouse and human serum did not differ from that of the control incubation in 0.9% NaCl. Therefore, ¹³¹I-mTHPC-¹²⁵I-MAb conjugates were fully stable in both serum sources.

Lindmo assays were performed to determine whether coupling of mTHPC to cMAb U36 or mMAb 425 influenced the immunoreactivity of the MAb. For conjugates with a mTHPC:MAb ratio of up to 4, no effect on the immunoreactivity was seen in comparison to the unconjugated MAb. Immunoreactivity was >93% in all cases, irrespective of whether assessed by ¹²⁵I or ¹³¹I counting.

**Biodistribution of ¹³¹I-mTHPC-¹²⁵I-cMAb U36 Conjugates.** Dual label experiments were performed to determine whether coupling of ¹³¹I-mTHPC-(CH₂COOH)₄ to ¹²⁵I-cMAb U36 resulted in improved targeting of the sensitizers to the tumor. To this end, the biodistribution of unconjugated ¹²⁵I-mTHPC-U36 and ¹³¹I-mTHPC-(CH₂COOH)₄ were first determined. For evaluation of ¹²⁵I-cMAb U36, 5 µCi of ¹²⁵I-cMAb U36 (100 µg) were injected in five HNX-OE xenograft-bearing nude mice. The mice were sacrificed 48 h after injection, and the biodistribution was determined. The mean uptake in tumor tissue was 19.5%ID/g, whereas the mean blood level was 13.9%ID/g. Uptake in all other organs was <4%ID/g (Fig. 4A). For evaluation of mTHPC-(CH₂COOH)₄, 2.5 µCi (5 µg) of ¹³¹I-mTHPC-(CH₂COOH)₄ were injected in five HNX-OE xenograft-bearing nude mice. Fig. 4B shows the biodistribution after 24 h. The compound was cleared very rapidly from the circulation. The mean blood level was 1.5%ID/g, whereas the uptake in the tumor was...
therapy with mTHPC-(CH$_2$COOH)$_3$ to cMAb U36 resulted in selective targeting of the sensitizer to the tumor (Fig. 4, D and D). However, tumor uptake levels of the sensitizer appeared to be lower than could be expected on the basis of the biodistribution of the unconjugated MAb (compare Fig. 4, A and D). The fact that tumor uptake of the transporter of the sensitizer, i.e., the conjugated MAb, was also lower than expected indicated that a proportion of the conjugate became rapidly eliminated from the blood (compare blood levels Fig. 4, A and C). This elimination was more pronounced for conjugates with the higher mTHPC:MAb ratio. For both the free mTHPC-(CH$_3$COOH)$_3$ and the conjugate, high mTHPC levels were found in the liver (Fig. 4, B and D).

To establish the overall efficiency of sensitizer targeting by the MAb, the biodistribution of unmodified mTHPC was assessed in the same model as $^{131}$I-mTHPC-(CH$_3$COOH)$_3$. Externally labeled $^{125}$I-mTHPC (5.0 μCi/mouse; specific activity, 11.3 Ci/mmol) and internally labeled $^{14}$C-mTHPC (5.0 μCi/mouse; specific activity, 74 mCi/mmol) were coinjected in six HNX-OE-bearing nude mice. As found previously by others in BALB/c mice (37), the free sensitizer showed a random distribution in the organs and no selective tumor uptake (Fig. 5). For both mTHPC compounds, the highest accumulation was observed in liver, spleen, and lung, and the lowest uptake was observed in muscle. Besides this, small differences were observed in the distribution pattern of the compounds, partly originating from the difficulty to assess the $^{14}$C radioactivity in solid/colored tissue.

**Photoinmunotherapy in Vitro.** The efficacy of photoinmunotherapy with mTHPC-cMAb U36 conjugates was tested in 22A cells using the SRB assay. After exposure to the relatively high concentration of 1 μM conjugated mTHPC, ~25% growth inhibition was observed (Fig. 6A). In the same assay, unconjugated mTHPC showed an IC$_{50}$ of 0.75 mM. Conjugated and free mTHPC appeared to be nontoxic without illumination.

To investigate the possibility that the sensitizer must be internalized for phototoxicity to occur, we coupled mTHPC to mMAb 425, an internalizing MAb directed against EGFR. Internalization of the mTHPC-MAb 425 conjugate was confirmed according to a method described before (40, 41). The efficacy of these conjugates was tested in A431 cells. The IC$_{50}$ using mTHPC-mMAb 425 conjugates was 7.3 nM, whereas in this cell line, the IC$_{50}$ for free mTHPC was 2.0 nM (Fig. 6B). Once again, conjugated and free mTHPC were nontoxic without illumination. Unconjugated cMAb U36 or mMAb 425 did not result in growth inhibition with or without illumination (data not shown).

**DISCUSSION**

Several attempts to use MAbs for selective delivery of photosensitizers to tumors have been made. However, none of these has led to conjugates suitable for therapeutic use. In 1983, Mew et al. (42) described the synthesis of hematoporphyrin-MAb conjugates, but the in vivo efficacy of these conjugates appeared to be minimal. The same research group developed benzoporphyrin derivative monocoid ring A (BPD)-MAb conjugates, using polyvinyl alcohol as a linker (43), but no data on the therapeutic applicability of these conjugates have been reported. The photosensitizer chlorin e$_6$ was conjugated to MAbs by Goff et al. (44) using polyglutamic acid as a linker. Preliminary results of PDT after i.p. injection of these conjugates in a murine i.p. ovarian cancer model showed an improved survival (44). No data on the i.v. use of these conjugates or for chlorin e$_6$-MAb conjugates using a dextran polymer linker (45) have been published thus far.

Although mTHPC is one of the most promising photosensitizers available for clinical use, no reports on mTHPC-MAb conjugates have been published. In this report, a reproducible procedure for conjugation of mTHPC to MAbs is provided in detail. Of major importance is that all reactions, including the modification of mTHPC, conjugation, and subsequent purification, are performed in the dark, and that solvents used are saturated with nitrogen. Fig. 7 illustrates the phototoxic effect of free mTHPC, when these precautions are not taken. In this case, the integrity of the MAb was impaired in such a way that it could not penetrate a 7.5% SDS-PAGE gel.

As anticipated, the development of this procedure was hampered by the necessity for modification of mTHPC and its hydrophobicity. Because mTHPC lacks a functional moiety for direct coupling to a MAb, mTHPC was carboxymethylated. In our initial attempts to produce mTHPC-MAb conjugates, monocarboxymethylated mTHPC was synthesized. With such a derivative, after esterification no cross-linking can occur during conjugation. However, the TFP ester of this compound did not chemically conjugate to the MAb. The reason was found to be that, after esterification, the compound is poorly water soluble. The ester, once brought in aqueous medium, immediately adheres to the MAb without forming covalent bonds.

To obtain a water-soluble mTHPC-TFP ester derivative, mTHPC was tetracarboxymethylated. Attempts were made to form the monooester of this compound directly, which would be the optimal compound for conjugation. However, the synthesis of this monoester, implying the combination of 1 molar equivalent TFP and EDC, led to an intractable mixture of products, which were not identified further.

The final route to the reproducible production of mTHPC-(CH$_3$COOH)$_3$CH$_2$CO-TFP was the synthesis of the tetraesterified compound, followed by partial hydrolysis to leave the monoester. The esterification of mTHPC-(CH$_3$COOH)$_3$ using an excess of TFP and EDC gave, after column chromatography, pure mTHPC-(CH$_2$CO-TFP)$_4$ in a reasonable yield (60%). During the following partial hydrolysis procedure, the formation of fully hydrolyzed mTHPC-(CH$_3$COOH)$_3$ is unavoidable. When hydrolysis was performed until no diester was left, only 20% of the mixture consisted of monoester. In our experiments, we hydrolyzed until 45% of the mixture consisted of monoester. Under these conditions, less than 5% diester was left, which was judged acceptable for conjugation. As a result, the overall amount of mTHPC available for conjugation was about 30%, owing to the loss during the modification, esterification, and subsequent hydrolysis.

It was possible to couple four mTHPC molecules to one MAb molecule without impairment of its solubility. When conjugates with a higher ratio were made, the MAbs formed insoluble aggregates during the conjugation. These aggregates were not recovered from the PD-10 column. Aggregate formation was also observed by the group of Mach when making indocyanin-MAb conjugates at a ratio of 2 (14). After the synthesis and purification of these conjugates, a small proportion of the MAbs aggregated during a subsequent 24-h storage period.

mTHPC-MAb conjugates prepared according to the method described herein showed a minimal impairment of the integrity on SDS-PAGE (<10% aggregate formation), full stability in serum in vitro, and an optimal immunoreactivity, provided that not more than four mTHPC molecules were coupled to the MAb. Nevertheless, the pharmacokinetics of mTHPC-MAb conjugates in xenograft-bearing nude mice differed from that of unconjugated MAb. For conjugates...
with a mean ratio of 0.9 and 1.8, the $^{125}\text{I}$ levels of the $^{131}\text{I}$-mTHPC-$^{125}\text{I}$-MAb in the blood at 4 h.p.i. were 69 and 52%, respectively, of that of an unconjugated $^{125}\text{I}$-MAb. In addition, the $^{131}\text{I}$ levels decreased more extensively than the $^{125}\text{I}$ levels. These data indicate that conjugates with a higher ratio are more susceptible for removal from the blood. Our biodistribution data, therefore, indicate hepatic extraction with retention of the sensitizer in this organ after catabolism. Rapid blood clearance and extensive liver accumulation have also been observed for MAbs coupled with other chemical groups to their lysine residues (12, 46–48).

$^{131}\text{I}$-mTHPC-(CH$_2$COOH)$_2$ was cleared more rapidly from the circulation than the unmodified mTHPC. The tumor selectivity of MAb-conjugated mTHPC was increased in comparison with both of these, despite the more rapid elimination of the conjugates with a higher ratio. For the conjugates with a ratio of 0.9 and 1.8, the tumor levels of $^{131}\text{I}$-mTHPC were 5.5 and 4.4%ID/g, respectively. In absolute amounts, this corresponds with 23 and 36 ng/g tumor, respectively. Given the fact that increasing the MAb dose to 400 $\mu$g/mouse does not result in antigen saturation, this implies that about 150 ng of mTHPC per g of tumor can be delivered.

Another aspect of evaluation is the uptake in the skin, in view of the problem of skin photosensitization. At 48 h after injection, in the levels of the MAb-conjugated mTHPC in the skin were much lower than in the tumor (skin:skin ratios were 3.5; Fig. 4D). For the unconjugated $^{131}\text{I}$-mTHPC and the reference compound $[^{14}\text{C}]\text{mTHPC}$, the levels in the skin and tumor were almost the same 24 h after injection (tumor:skin ratios were 0.8 and 0.9, respectively; Fig. 5). This is in agreement with data of Whelpton et al. (37), who showed that tumor:skin ratios remained about 1 between 1 and 4 days after administration of $[^{14}\text{C}]\text{mTHPC}$ to C6o-26-bearing mice. Westermann et al. (49) showed that in colon carcinoma-bearing nude mice, these ratios were improved by using $^{125}\text{I}$-mTHPC-PEG conjugates instead of $^{131}\text{I}$-mTHPC.

The improved selectivity of mTHPC directed by the MAb does not guarantee improved efficacy. Therefore, in vitro studies were performed to compare the phototoxicity of internalizing and noninternalizing MAb-conjugated mTHPC with that of free mTHPC at equimolar doses. Our data on the photodynamic efficacy of the mTHPC-MAb conjugates revealed a remarkable difference between internalizing and noninternalizing MAbs. When coupled to mMAb 425, which was internalized by the cell after conjugation, mTHPC exhibited more phototoxicity than when coupled to the noninternalizing cMAB U36. Sobolev et al. (50) also reported that the photosensitizer chlorin e$_6$ was more effective when localized intracellularly. For BDP-MAB conjugates, produced by Ji et al. (51), internalization enhanced the cell killing by 10-fold. These data strongly suggest that the critical target for photodynamic damage is localized intracellularly. When this is true, it is clear that the kinetics of cellular uptake of free mTHPC versus mMAb 425-conjugated mTHPC are crucial parameters and might have influenced the relative efficacy as observed in our SRB experiments (Fig. 6). For the same reason, at this moment it is difficult to speculate about the relative efficacy in vivo. Therapy experiments with the use of mTHPC-mMAb 425 conjugates in HNSCC-bearing nude mice will be started shortly.

REFERENCES


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DEVELOPMENT OF αTHPC-MAB CONJUGATES


Development of meta-Tetrahydroxyphenylchlorin-Monoclonal Antibody Conjugates for Photoimmunotherapy
