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Targeting of Aluminum (III) Phthalocyanine Tetrasulfonate by Use of Internalizing Monoclonal Antibodies: Improved Efficacy in Photodynamic Therapy

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ABSTRACT

The use of monoclonal antibodies (MAbs) directed against tumor-associated antigens for targeting of photosensitizers is an interesting option to improve the selectivity of photodynamic therapy (PDT). Hydrophilic photosensitizers are most suitable for conjugation to MAbs because of their water solubility. The photosensitizer aluminum (III) phthalocyanine tetrasulfonate [$AlPc(SO_3H)_4$] has many ideal photochemical properties; however, because of its hydrophilicity, the free form of this sensitizer does not readily reach the critical intracellular target and, therefore, is ineffective in PDT. On the basis of our previous studies, we hypothesized that $AlPc(SO_3H)_4$ might be suitable for PDT when coupled to internalizing tumor-selective MAbs. In this study, a reproducible procedure is presented for coupling of $AlPc(SO_3H)_4$ to MAbs via the tetra-glycine derivative $AlPc(SO_2N_{gly})_4$. Conjugation was performed to chimeric MAb (cMAb) U36 and murine MAbs (mMAb) E48 and 425 using a labile ester. Conjugates showed preservation of integrity and immunoreactivity and full stability in serum *in vitro.* At molar ratios >4, the solubility of the conjugates decreased. Data on the *in vitro* efficacy of PDT showed that in the chosen experimental setup the internalizing $AlPc(SO_2N_{gly})_4$-mMAb 425 conjugate was about 7500 times more toxic to A431 cells than the free sensitizer (IC_{50}s, 0.12 nM *versus* 900 nM). The $AlPc(SO_2N_{gly})_4$-mMAb 425 conjugate was also more toxic than *meta*-tetrahydroxyphenylchlorin-mMAb 425 conjugates and free *meta*-tetrahydroxyphenylchlorin that had been tested previously (M. B. Vrouenraets *et al.*, Cancer Res., *59:* 1505–1513, 1999) in the same system (IC_{50}s, 7.3 nM and 2.0 nM, respectively). Biodistribution analysis of $AlPc(SO_2N_{gly})_4$-^{125}I-labeled cMAb U36 conjugates with different sensitizer:MAb ratios in squamous cell carcinoma-bearing nude mice revealed selective accumulation in the tumor, although to a lesser extent than for the unconjugated ^{125}I-labeled cMAb U36, whereas tumor:blood ratios were similar. These findings indicate that $AlPc(SO_3H)_4$ has high potential for use in PDT when coupled to internalizing tumor-selective MAbs.

INTRODUCTION

The use of MAbs[2] directed against tumor-associated antigens for selective targeting of photosensitizers is an interesting option. This approach should selectively increase the photosensitizer concentration in tumors. If this also translates to an increased photodynamic effect, this could be a major advantage for PDT of large surface areas where normal tissue toxicity becomes dose limiting. Expression of tumor-associated antigens on normal tissues is limited; therefore, it can be anticipated that these tissues will be spared when using MAb-conjugated photosensitizers.

In a series of studies on photoimmunoconjugates, we started with the development of *m*THPC-MAb conjugates for PDT of SCC (1). *m*THPC was selected because in free form it is considered to be one of the most potent and promising photosensitizers for clinical use. Biodistribution analysis in tumor-bearing nude mice showed that the tumor selectivity of *m*THPC was improved by coupling to tumor-selective MAbs. Furthermore, *m*THPC-MAb conjugates were effective in *in vitro* photoimmunotherapy of A431 cells, although less effective than the free sensitizer (IC_{50}, 7.3 *versus* 2 nM). Importantly, efficacy was only observed when *m*THPC-MAb conjugates were internalized, which was a strong indication that the critical target for photodynamic damage is localized intracellularly. A serious problem in the development of these conjugates was the poor water solubility of *m*THPC.

For coupling to MAbs, photosensitizers more hydrophilic than *m*THPC would be much more suitable. As free compounds, such photosensitizers are ineffective because of their inability to enter the tumor cell, but, coupled to internalizing MAbs, phototoxicity can be enhanced, as we recently described in our study on the hydrophilic sensitizer TrisMPyP-ΦCO_2H (2). This sensitizer was selected as a conceptual model compound because of its hydrophilicity. Its photochemical properties make the photosensitizer of limited value for clinical photoimmunotherapy. It is excited with light of 595 nm (with a very low ϵ of 7.0×10^3 $M^{-1}cm^{-1}$), and light of this short wavelength is not suitable for treatment of larger tumors because it hardly penetrates into tissue.

In the present study, the concept of using internalizing MAbs for photoimmunotherapy with hydrophilic sensitizers is further investigated by using a more suitable photosensitizer, aluminum (III) phthalocyanine tetrasulfonate [$AlPc(SO_3H)_4$]. Within the family of sulfonated phthalocyanines, which can have a sulfonation degree from 0 up to 4, this compound is the most hydrophilic member. Because phthalocyanines have a strong absorption maximum at about 675 nm ($\epsilon = 1.7 \times 10^5$ $M^{-1}cm^{-1}$), they can be used for treatment of larger tumors.

Several conjugation procedures for phthalocyanine-MAb conjugates have been described previously. In 1994, Morgan *et al.* (3) described the coupling of $AlPc(SO_3H)_4$ to MAb E7 via $AlPc(SO_2Cl)$. Very recently, Carcenac *et al.* (4) reported on the preparation of $AlPc(SO_3H)_4$-MAb 35A7 conjugates via a mono five-carbon spacer chain. Both groups observed limited phototoxicity of their conjugates in PDT *in vitro*.

In this study, we describe a reproducible procedure for conjugation of $AlPc(SO_3H)_4$ as the tetra-glycine derivative. The modified sensitizer was coupled to MAbs selectively reactive with SCC. The *in vitro* photodynamic efficacy of the photosensitizer, both in free form and coupled to internalizing SCC-selective MAbs, was studied. Biodistribution analysis of the conjugates was performed in SCC-bearing nude mice.

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[2] The abbreviations used are: MAb, monoclonal antibody; PDT, photodynamic therapy; *m*THPC, *meta*-tetrahydroxyphenylchlorin; SCC, squamous cell carcinoma; mMAb, murine monoclonal antibody; cMAb, chimeric monoclonal antibody; HPLC, high-performance liquid chromatography; HNX-OE, head and neck xenograft line OE; TrisMPyP-ΦCO_2H, 5-{4-[5-(carboxyl)-1-butoxy]phenyl}-10,15,20-tris-(4-methylpyridiniumyl)porphyrin iodide; BTA, N,*O*-bis(trimethylsilyl)acetamide; TFP, 2,3,5,6-tetrafluorophenol; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide solution; SRB, sulforhodamine B; p.i., after injection; %ID/g, percentage of the injected dose/g; DMF, *N*,*N*-dimethylformamide.

MATERIALS AND METHODS

Sensitizer. Aluminum (III) phthalocyanine tetrasulfonate chloride [$AlPc(SO_3H)_4$; Fig. 1, *Scheme* 1; M_r 895.19] was obtained from Porphyrin Products (Logan, UT).

MAbs. Selection, production, and characterization of mMAb U36 directed against CD44v6 (5), its chimeric (mouse/human) IgG1 derivative (cMAb U36), the IgG2a mMAb 425 directed against the epidermal growth factor receptor (6, 7), and the IgG1 mMAb E48 directed against a M_r 16,000–22,000 glycosylphosphatidylinositol-anchored surface antigen (8) have been described before.

Cell Lines. The head and neck SCC cell lines UM-SCC-11B and UM-SCC-22A (kindly provided by Dr. T. E. Carey, University of Michigan, Ann Arbor, MI) were cultured under 5% CO_2 at 37°C in DMEM (BioWhittaker, Alkmaar, the Netherlands) supplemented with 2 mM L-glutamine, 5% FCS (BioWhittaker), and 25 mM HEPES. The vulvar SCC cell line A431 was cultured under the same conditions.

Analyses. The absorption of free and MAb-conjugated $AlPc(SO_3H)_4$ was measured using an Ultrospec III spectrophotometer (Pharmacia Biotech, Roosendaal, the Netherlands). The sensitizer concentration in the conjugate preparations was assessed with the same apparatus at a wavelength of 351 nm. The absorption of a range of dilutions (1–9 μg/ml) of $AlPc(SO_3H)_4$ in H_2O was measured and graphically depicted using the least square method. The sensitizer concentration in the conjugate preparations was determined using this calibration curve.

HPLC analysis during the several modification reactions of the starting compound $AlPc(SO_3H)_4$ was performed using a LKB 2150 HPLC-pump (Pharmacia Biotech), a LKB 2152 LC controller (Pharmacia Biotech), and a 25-cm Lichrosorb 10 RP 18 column (Chrompack, Middelburg, the Netherlands). Two eluentia were used: eluent A, consisting of a 5:95 (v/v) mixture of ethanol and 0.01 M sodium phosphate buffer (pH 6), and eluent B, consisting of a 9:1 (v/v) mixture of methanol and H_2O. A gradient was used in which eluent A was gradually replaced by eluent B. The gradient (flow rate, 1 ml/min) was as follows: 5 min, 100% eluent A; linear increase of eluent B to 100% during 10 min; 10 min, 100% eluent B. Absorption was measured at 210 and 351 nm by a Pharmacia LKB VWM 2141 UV detector.

For HPLC analysis of phthalocyanine-^{125}I-labeled MAb conjugates, an LKB 2150 HPLC-pump, an LKB 2152 LC controller, and a 10 × 300-mm Superdex 200 HR 10/30 column (Pharmacia Biotech) were used. The eluent consisted of 0.05 M sodium phosphate/0.15 M sodium chloride (pH 6.8), and the flow rate was 0.5 ml/min. A Pharmacia LKB VWM 2141 UV detector was used at 351 nm for detection of the sensitizer, whereas radioactivity of the ^{125}I-labeled MAb was measured by an Ortec 406A single-channel analyzer connected to a Merck-Hitachi D2000 integrator (Merck, Darmstadt, Germany).

The integrity of the phthalocyanine-^{125}I-labeled 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 exposed to a Phosphor plate for 1–3 h and analyzed with a Phosphor Imager (B&L-Isogen Service Laboratory, Amsterdam, the Netherlands) for quantitation of the radiolabeled protein bands.

^{125}I-labeling of MAbs. Labeling of MAbs with ^{125}I was performed under mild conditions using Iodogen (9). One mg of MAb dissolved in 500 μl of PBS (pH 7.4) and 1–2 mCi ^{125}I (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 Science, Inc., Ann Arbor, MI) and unbound ^{125}I was removed using a PD-10 column (Pharmacia Biotech) with 0.9% NaCl as eluent. After removal of unbound ^{125}I, the radiochemical purity always exceeded 98% (HPLC analysis).

Preparation of $AlPc(SO_2NHCH_2CO\text{-}TFP)_4$ Ester. The synthesis of the phthalocyanine derivatives and subsequent conjugation reaction were carried out in the dark and under N_2 to prevent any unwanted photochemical reactions.

Preparation of the ester was performed in three steps. The first step was the synthesis of the tetrasulfonylchloride $AlPc(SO_2Cl)_4$ (Fig. 1, *Scheme 2*; Ref. 10). For this, 250 mg (0.28 mmol) of $AlPc(SO_3H)_4$ were stirred together with 3.0 ml (41 mmol) of thionylchloride ($SOCl_2$; Sigma-Aldrich, Zwijndrecht, the Netherlands) and 100 μl of DMF for 2 h at 80°C. The solution was then cooled to 0°C and added to 7 ml of ice water (3.8% NaCl). The temperature was kept at 0°C. The precipitated $AlPc(SO_2Cl)_4$ was filtered off and washed with ice water. The product was dried in a vacuum over P_2O_5 at room temperature.

In the next step, the tetracarboxylic acid $AlPc(SO_2NHCH_2COOH)_4$ (Fig. 1, *Scheme 3*) was prepared *in situ* as follows. To 7.0 mg (7.2 μmol) of $AlPc(SO_2Cl)_4$, dissolved in 1 ml of DMF, 14.4 mg (0.19 mmol) of glycine and 65 μl (0.26 mmol) of BTA (Sigma-Aldrich) were added (11). The mixture was stirred at room temperature for 48 h before adding 500 μl of water to quench all of the reactive intermediates and stop the reaction.

Hereafter, the four carboxylic acid groups were esterified using an excess of TFP (Janssen Chimica, Beerse, Belgium). To 100 μl of the crude $AlPc(SO_2NHCH_2COOH)_4$ solution (containing 460 nmol), 700 μl of water, 200 μl (0.12 mmol) of a TFP solution (100 mg/ml in MeCN/H_2O 9/1, v/v), and 50 mg (0.26 mmol) of solid EDC (Janssen Chimica) were added. During 30-min stirring, the $AlPc(SO_2NHCH_2CO\text{-}TFP)_4$ ester (Fig. 1, *Scheme 4*) precipitated. After centrifugation, the supernatant was removed, and the product was washed twice with 5 ml H_2O at 4°C, followed by drying in a vacuum over P_2O_5. The ester was dissolved in 250 μl of MeCN, analyzed by HPLC, and the concentration was determined by absorption measurement.

Preparation of $AlPc(SO_2NHCH_2COOH)_3SO_2NHCH_2CONH\text{-}^{125}I$-labeled MAb Conjugates. For conjugation, chosen ester aliquots (containing 10 to 45 nmol ester) in MeCN were added to 1 mg of ^{125}I-labeled MAb dissolved in 1 ml 0.9% NaCl (pH 9.5). After 30-min incubation, the $AlPc(SO_2NHCH_2COOH)_3SO_2NHCH_2CONH\text{-}^{125}I$-labeled MAb conjugate (Fig. 1, *Scheme 5*) was purified on a PD-10 column with 0.9% NaCl as the eluent. The integrity of the conjugate was analyzed by HPLC and gel electrophoresis as described above.

***In Vitro* Stability of $AlPc(SO_2N_{gly})_4\text{-}^{125}I$-labeled MAb Conjugates.** For measurement of the serum stability of the $AlPc(SO_2N_{gly})_4\text{-}^{125}I$-labeled MAb

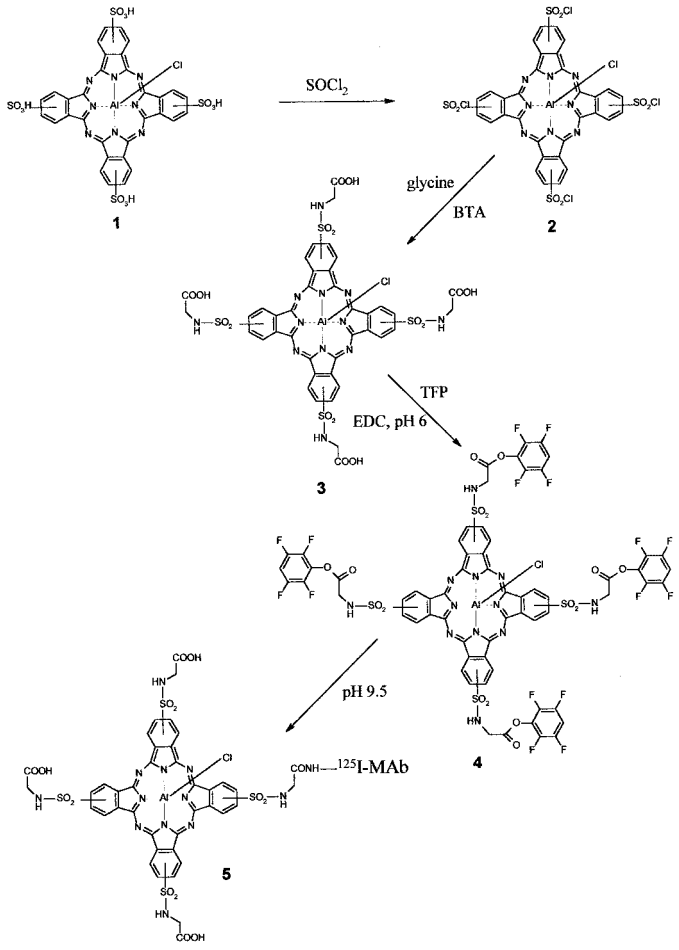

Fig. 1. Schematic representation of the synthesis of $AlPc(SO_2NCH_2COOH)_4$ via $AlPc(SO_2Cl)_4$, its esterification, and conjugation to a ^{125}I-labeled MAb.

conjugates,[3] 15 μg of conjugate in 25 μl 0.9% NaCl were added to 25 μl of human serum. After incubation for 24 h at 37°C, samples were analyzed with HPLC at 280 and 351 nm.

Immunoreactivity of AlPc(SO_2N_{gly})$_4$-^{125}I-labeled MAb Conjugates. *In vitro* binding characteristics of AlPc(SO_2N_{gly})$_4$-^{125}I-labeled MAb conjugates were determined in an immunoreactivity assay as described previously (1) and compared with those of the unconjugated ^{125}I-labeled MAb. UM-SCC-11B cells were used for binding assays with cMAb U36, A431 cells for mMAb 425, and UM-SCC-22A cells for mMAb E48.

Internalization of AlPc(SO_2N_{gly})$_4$-^{125}I-labeled MAb Conjugates. *In vitro* experiments to determine the internalization of AlPc(SO_2N_{gly})$_4$-^{125}I-labeled cMAb U36, AlPc(SO_2N_{gly})$_4$-^{125}I-labeled mMAb 425, and AlPc(SO_2N_{gly})$_4$-^{125}I-labeled mMAb E48 conjugates by A431 cells were performed exactly as described previously (2). For this purpose, the MAbs were labeled with 2 mCi ^{125}I/mg MAb, and conjugates with a sensitizer:MAb ratio of 2:1 were synthesized.

Photoimmunotherapy *in Vitro*. *In vitro* PDT experiments were performed to determine the phototoxicity of free AlPc(SO_3H)$_4$ and cMAb U36-conjugated, mMAb 425-conjugated, and mMAb E48-conjugated AlPc(SO_3H)$_4$. The toxicity was determined using the SRB (Sigma Chemical Co.) assay, which measures the cellular protein content, as follows (12). A431 cells were plated in 96-well plates (750 cells/well) and grown for 3 days before incubating with AlPc(SO_3H)$_4$ or AlPc(SO_2N_{gly})$_4$-MAb conjugates [range, 0.1 nM-1.0 μM AlPc(SO_3H)$_4$ equivalents] in DMEM supplemented with 2 mM L-glutamine, 5% FCS, and 25 mM HEPES at 37°C. After 20 h, remaining unbound AlPc(SO_3H)$_4$ and AlPc(SO_2N_{gly})$_4$-MAb conjugates were removed by washing twice with medium. Fresh medium was added, and cells were illuminated at 675 nm with a Spectra Physics dye laser (model 373) pumped by a 12-W argon laser (Spectra Physics model 171) at a dose of 25 J/cm^2. 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. As a control, cells were illuminated in the absence of AlPc(SO_3H)$_4$ or MAb-conjugated AlPc(SO_3H)$_4$.

Biodistribution Studies. The biodistribution of unconjugated ^{125}I-labeled cMAb U36 and AlPc(SO_2N_{gly})$_4$-^{125}I-labeled cMAb U36 conjugates with different sensitizer:MAb ratios was determined in nude mice bearing s.c. xenografts of the HNSCC cell line HNX-OE. Tumor size ranged from 50 to 100 mm^3. The ^{125}I-labeled MAb preparations were injected i.v. in 100 μl of 0.9% NaCl. At 48 h p.i., mice were anesthetized, bled, killed, and dissected. The organs were removed and weighed. The amount of γ-emitting radioactivity in organs and blood was measured in a gamma counter (LKB-Wallac, 1282 CompuGamma; Pharmacia, Woerden, the Netherlands). Radioactivity uptake in the tissues was expressed as the %ID/g of tissue. Tumor:nontumor ratios were also calculated.

RESULTS

Preparation of AlPc(SO_2NHCH_2CO-TFP)$_4$ Ester. The first step in the synthesis of the ester was the preparation of the tetrasulfonylchloride AlPc(SO_2Cl)$_4$ (Fig. 1, *Scheme 2*) by stirring the starting compound AlPc(SO_3H)$_4$ (Fig. 1, *Scheme 1*) in ~150-fold excess of liquid $SOCl_2$ for 2 h at 80°C. After work-up, the product was isolated in a yield of about 80%.

In the next step, the AlPc(SO_2Cl)$_4$ was converted to its tetra-glycine derivative AlPc(SO_2NHCH_2COOH)$_4$ (Fig. 1, *Scheme 3*) in DMF using a large excess of glycine and BTA. The use of BTA to dissolve glycine in DMF by means of conversion into the corresponding disilylated intermediate has been described by Dressman *et al.* (11). After addition of water, the crude reaction mixture was esterified during 30 min using a large excess of TFP and EDC at pH 5.8. After thorough washing of the resulting precipitate with H_2O (which removed acetamide, trimethylsilylhydroxide, EDC, TFP, glycine-TFP ester, and the partially hydrolyzed sensitizer derivatives), the AlPc(SO_2NHCH_2CO-TFP)$_4$ ester (Fig. 1, *Scheme 4*) was isolated in a yield of 45% ± 5%. This tetra-ester was found to hydrolyze relatively easily. HPLC analysis at 351 nm (Fig. 2) revealed that hydrolysis had occurred during the washing step, resulting in a product mixture consisting of 80% AlPc(SO_2NHCH_2CO-TFP)$_4$ (HPLC retention time, 16.3 min), 20% AlPc(SO_2NHCH_2CO-TFP)$_3SO_2NHCH_2COOH$ (retention time, 14.4 min), and the presence of a small amount of TFP, detected at 210 nm (retention time, 5.4 min).

Conjugation. The AlPc(SO_2NHCH_2CO-TFP)$_4$ ester was dissolved in 250 μl of MeCN before conjugation. When 35 nmol of ester (in about 50 μl of MeCN) were added to 6.6 nmol (1.0 mg) of ^{125}I-labeled MAb in 1 ml of 0.9% NaCl, followed by incubation for 30 min at pH 9.5 and PD-10 column purification, a AlPc(SO_2NHCH_2COOH)$_3SO_2NHCH_2CONH$-^{125}I-labeled MAb conjugate (Fig. 1, *Scheme 5*) was obtained with a molar ratio of about 3.0, corresponding with a conjugation efficiency of about 55%. Purification of the conjugates on a PD-10 column also removed MeCN and TFP (13).

Under the conditions that would lead to conjugates with a ratio >4, the recovery of the ^{125}I-labeled MAb from the PD-10 column dropped significantly, because the solubility of the MAb became impaired. Therefore, conjugates with a ratio above 4 were not further evaluated.

Analyses and Quality Control of the Conjugates. The quality of the AlPc(SO_2N_{gly})$_4$-MAb conjugates was analyzed by HPLC and SDS-PAGE analysis. HPLC analysis of a AlPc(SO_2N_{gly})$_4$-^{125}I-labeled cMAb U36 conjugate, with UV detection at 351 nm for detection of the sensitizer and radioactivity measurement for detection of the ^{125}I-labeled MAb, showed the conjugate to be eluted as a monomeric peak. All of the sensitizer was confined to the MAb, whereas the recovery of the radioactivity from the column was >95%.

Fig. 3 shows the results of the SDS-PAGE and subsequent Phosphor Imager quantitation of an AlPc(SO_2N_{gly})$_4$-^{125}I-labeled cMAb U36 conjugate (Fig. 3, *A* and *C*) with unconjugated ^{125}I-labeled cMAb U36 (Fig. 3, *B* and *D*) as a control. In both cases, a single radiolabeled protein band was observed with an apparent molecular weight of ±150,000 (deduced from SDS-PAGE analysis; data not shown).

The AlPc(SO_2N_{gly})$_4$-cMAb U36 conjugate was incubated in human serum for 24 h at 37°C for determination of the serum stability of the SO_2-N_{gly} bond. HPLC analysis at 280 and 351 nm revealed that the HPLC profiles were identical to that at the start of the incubation, indicating that the conjugate was fully stable in human serum.

Cell-binding assays were performed to determine whether the coupling of AlPc(SO_3H)$_4$ influenced the immunoreactivity of cMAb U36, mMAb 425, or mMAb E48. For all of the conjugates studied (sensitizer:MAb ratios of 1:1 to 4:1), the immunoreactivity was the same as for their corresponding ^{125}I-labeled MAbs (85–90%).

Internalization of AlPc(SO_2N_{gly})$_4$-^{125}I-labeled MAb Conjugates. Internalization experiments were performed to determine whether the AlPc(SO_2N_{gly})$_4$-^{125}I-labeled cMAb U36, AlPc(SO_2N_{gly})$_4$-^{125}I-labeled mMAb 425, and AlPc(SO_2N_{gly})$_4$-^{125}I-labeled mMAb E48 conjugates were internalized by the A431 cells. Within our experimental setup, 54.3 ± 2.3% (1.0×10^6 sensitizer molecules/cell), 36.6 ± 0.9% (1.1×10^6 sensitizer molecules/cell), and 31.6 ± 0.5% (mean ± SD) internalization (0.65×10^6 sensitizer molecules/cell) was observed for ^{125}I-labeled cMAb U36, ^{125}I-labeled mMAb 425, and ^{125}I-labeled mMAb E48 conjugates, respectively. The addition of excess naked MAb totally blocked binding.

Photoimmunotherapy *in Vitro*. The phototoxicity of unconjugated AlPc(SO_3H)$_4$ and cMAb U36-conjugated, mMAb 425-conjugated, and mMAb E48-conjugated AlPc(SO_3H)$_4$, with a molar ratio of 2, was assessed in A431 cells using the SRB assay. The results are depicted in Fig. 4. The IC_{50} of the free sensitizer was 900 nM. When coupled to the MAbs U36, 425, or E48, the sensitizer showed an

[3] AlPc(SO_2NHCH_2COOH)$_3SO_2NHCH_2CONH$-MAb conjugates are designated as AlPc(SO_2N_{gly})$_4$-MAb conjugates if the modification of AlPc(SO_3H)$_4$ is not relevant for understanding.

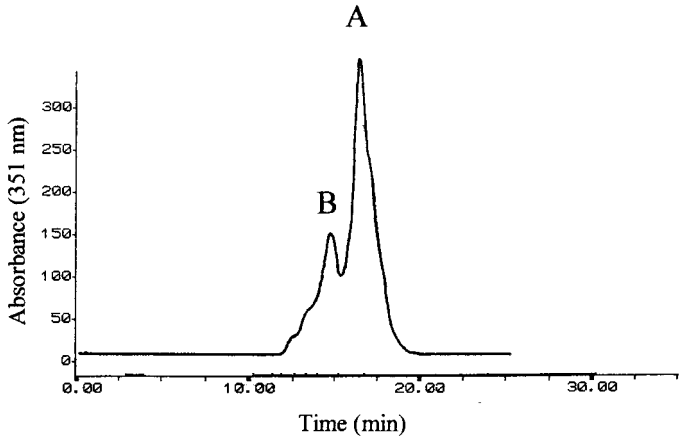

Fig. 2. Hydrolysis profile of the TFP-ester of the modified tetrasulfonate phthalocyanine in MeCN/H_2O by HPLC analysis (absorbance measurement at 351 nm). *A*, $AlPc(SO_2NHCH_2CO\text{-}TFP)_4$; *B*, $AlPc(SO_2NHCH_2CO\text{-}TFP)_3SO_2NHCH_2COOH$.

increased photodynamic efficacy. The mMAb 425-conjugated $AlPc(SO_2N_{gly})_4$ showed the highest phototoxicity with an IC_{50} of 0.12 nM. At sensitizer concentrations from 1 nM up to 1 μM, PDT resulted in cell killing [lower absorption than at the day of illumination (day 0)]. The efficacies of PDT with cMAb U36-conjugated (IC_{50}, 1.6 nM) and mMAb E48-conjugated (IC_{50}, 32 nM) $AlPc(SO_3H)_4$ were less than with mMAb 425-conjugated $AlPc(SO_3H)_4$ but were still much greater than for the free sensitizer.

Conjugated and free $AlPc(SO_3H)_4$ appeared to be nontoxic without illumination. The unconjugated MAbs did not result in growth inhibition with or without illumination (data not shown).

Biodistribution Studies. Biodistribution analysis was performed in HNX-OE xenograft-bearing nude mice. Fig. 5 shows the biodistribution data (Fig. 5*A*) and tumor to nontumor values (Fig. 5*B*) of ^{125}I-labeled cMAb U36 and $AlPc(SO_2N_{gly})_4$-^{125}I-labeled cMAb U36 conjugates with ratios of 1.2 and 2.4. The unconjugated ^{125}I-labeled MAb (100 μg; 10 μCi of ^{125}I) and both conjugates (100 μg; 10 μCi of ^{125}I) were injected in three groups of six mice, and the mice were killed 48 h after injection.

The results depicted in Fig. 5*A* show that the $AlPc(SO_2N_{gly})_4$-conjugated ^{125}I-labeled MAbs accumulate selectively in the tumor, but the uptake is lower than for the unconjugated ^{125}I-labeled MAb. The conjugate with the highest ratio shows the lowest tumor accumulation. The tumor uptake was 12.6, 9.6, and 6.8%ID/g for the unconjugated ^{125}I-labeled cMAb U36 and the conjugates with a ratio of 1.2 and 2.4, respectively. The blood values were 13.1, 10.3, and 7.7%ID/g, respectively. Therefore, coupling of $AlPc(SO_2N_{gly})_4$ decreases the half-life of the ^{125}I-labeled MAb in blood, resulting in a lower tumor accumulation.

Tumor:blood ratios 2 days p.i. were about 0.9 for both the unconjugated ^{125}I-labeled cMAb U36 and for the conjugates (Fig. 5*B*). For most organs, tumor:normal tissue ratios slightly decreased for conjugates with increasing sensitizer:MAb ratio.

DISCUSSION

The photosensitizer $AlPc(SO_3H)_4$ in its free form is not clinically effective because of its hydrophilicity, which hampers uptake in the tumor cells. In the present study, we showed that, because of its water solubility, $AlPc(SO_3H)_4$ could easily be coupled to MAbs. When coupled to internalizing MAbs, conjugates were obtained that were highly effective for *in vitro* photoimmunotherapy and that resulted in selective tumor targeting in nude mice. mMAb 425-conjugated $AlPc(SO_2N_{gly})_4$ was about 7500 times more effective than the free sensitizer *in vitro* (IC_{50}s, 0.12 nM *versus* 900 nM). These data indicate that the sensitizer $AlPc(SO_3H)_4$, although ineffective in free form, becomes highly effective in photoimmunotherapy when coupled to an internalizing tumor-selective MAb. Ineffectiveness of $AlPc(SO_3H)_4$ in free form can be explained by its limited capacity to enter the cell and by the chosen experimental setup in which a washing step was performed just before illumination. In the same system, we previously tested *m*THPC-mMAb 425 conjugates and free *m*THPC. IC_{50}s for these compounds were 7.3 and 2.0 nM, respectively (1). In this case, the free sensitizer was more effective than the conjugate.

Although several attempts have been described to develop photosensitizer-MAb conjugates, clinically effective conjugates have not been produced thus far. Duska *et al.* (14) developed several conjugation procedures for the photosensitizer $chlorin_{e6}$. Conjugates produced with poly-L-lysine as a linker appeared to be most promising. Biodistribution data (14) and phototoxicity studies (15) after i.p. administration of $chlorin_{e6}$-mMAb OC125 $F(ab')_2$ in a xenograft nude mouse model of ovarian cancer revealed that the conjugate was more tumor selective and phototoxic than the free sensitizer. Because complete eradication of tumor cells was not consistently found, additional refinement investigations are ongoing.

$AlPc(SO_3H)_4$ lacks a functional moiety suitable for direct conjugation to MAbs; therefore, the sensitizer required prior modification.

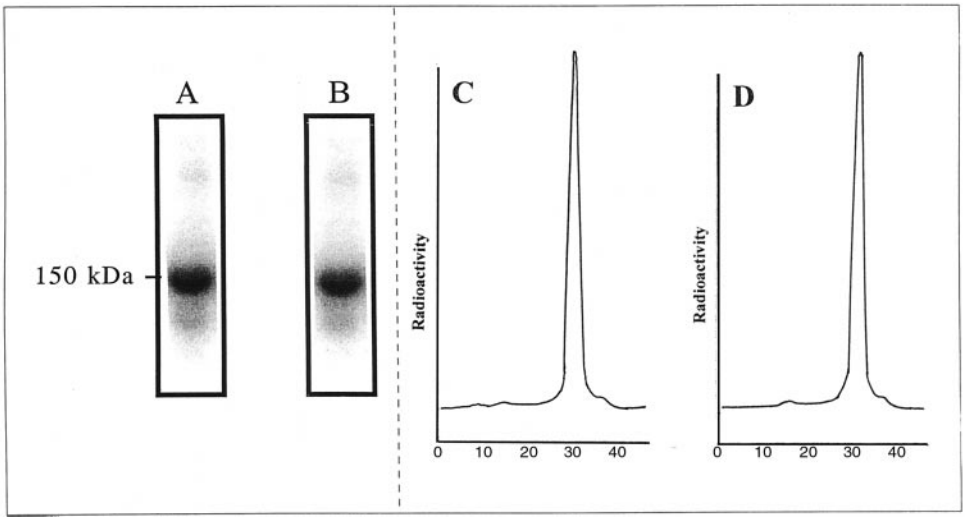

Fig. 3. SDS-PAGE and Phosphor Imager analysis of a $AlPc(SO_2N_{gly})_4$-^{125}I-labeled cMAb U36 conjugate with sensitizer. MAb ratio 2 (*A* and *C*) and unconjugated ^{125}I-labeled cMAb U36 as a control (*B* and *D*). The M_r 150,000-band contained 93% (*C*) and 94% (*D*) of the total amount of radioactivity.

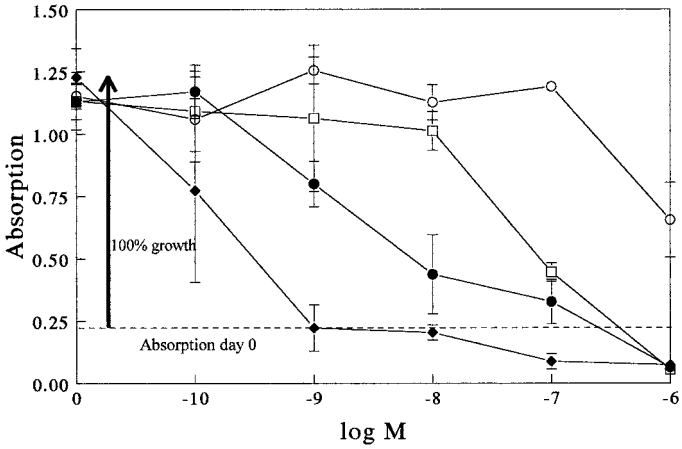

Fig. 4. The antiproliferative effect of $AlPc(SO_3H)_4$ and $AlPc(SO_2N_{gly})_4$-MAb conjugates with sensitizer. MAb ratio 2 on A431 cells upon illumination with 25 J/cm^2 (SRB assay). ○, $AlPc(SO_3H)_4$; ●, $AlPc(SO_2N_{gly})_4$-cMAb U36; ◆, $AlPc(SO_2N_{gly})_4$-mMAb 425; □, $AlPc(SO_2N_{gly})_4$-mMAb E48. Results of triplicate experiments are indicated (means ± SD). The molarity (*M*; *X* axis) of the free or conjugated $AlPc(SO_3H)_4$ is indicated logarithmically.

To obtain a good yield (80%), the tetrasulfonylchloride $AlPc(SO_2Cl)_4$ was precipitated in ice water. The water temperature was a critical parameter in this process because sulfonylchlorides are labile in water, and hydrolysis of the SO_2-Cl bond readily takes place at higher temperatures. Morgan *et al.* (3) reported on the synthesis of conjugates by direct addition of $AlPc(SO_2Cl)_4$ to MAbs. In our hands, this approach resulted in the formation of unstable conjugates.

In the second step, therefore, the SO_2Cl group was converted to SO_2NHCH_2COOH so that the carboxylic acid moiety could be converted into the active TFP-ester. This TFP-ester approach was used previously for *m*THPC-MAb (1) and TrisMPyP-ΦCO-NH-MAb photoimmunoconjugates (2), and it is routinely applied in ongoing clinical radioimmunotherapy studies with ^{186}Re-labeled MAG3-MAb conjugates. The chemistry was most straightforward when all of the four SO_2Cl groups were converted. Because glycine did not dissolve in DMF, BTA was used. The two $(CH_3)_3Si$-groups of BTA bind to the NH_2 and COOH group of glycine (to form $NHSi(CH_3)_3$ and $COOSi(CH_3)_3$, respectively), which renders the compound soluble in DMF, whereas the silylated nitrogen is more nucleophilic, increasing the product yield.

After this reaction, the resulting product was esterified with TFP in a one-pot reaction. $AlPc(SO_2NHCH_2CO\text{-}TFP)_4$ precipitated, thus providing an easy and convenient purification and isolation. This tetra-ester was found to be susceptible to hydrolysis, even under neutral conditions, but for conjugation this hydrolysis was not a problem (see below). The overall yield of these two subsequent reaction steps (45%) was reasonable.

In a previous study (1) on the development of *m*THPC-MAb conjugates, we analyzed conjugate formation as a function of the number of ester groups/sensitizer molecule. When tetra-esterified *m*THPC-$(TFP)_4$ was used for conjugation, the sensitizer immediately adhered to the MAb because of its poor water solubility without forming covalent bonds. To deal with this problem, before conjugation *m*THPC-$(TFP)_4$ was partially hydrolyzed to leave a conjugation mixture mainly consisting of mono-ester and completely hydrolyzed sensitizer. In the present study, we intended to follow the same strategy for conjugation with the tetra-ester $AlPc(SO_2N_{gly}\text{-}TFP)_4$, also because di-, tri-, and tetra-esters are theoretically able to cross-link MAbs. Partial hydrolysis of the $AlPc(SO_2N_{gly}\text{-}TFP)_4$ ester before conjugation resulted in phthalocyanine-MAb conjugates with a conjugation efficiency of only 20%. When the more polar $AlPc(SO_2N_{gly}\text{-}TFP)_4$ tetra-ester [compared with *m*THPC-$(TFP)_4$] was used without prior hydrolysis, an immediate noncovalent adherence to MAbs did not take place. Moreover, cross-linking of the MAbs did not occur as assessed by SDS-PAGE and HPLC analysis, whereas the conjugation efficiency was 55%. Apparently, with the MAb concentration used (1 mg/ml), the remaining ester groups were hydrolyzed before a second MAb molecule could bind to the sensitizer. On the basis of these results, partial hydrolysis of the ester before conjugation to the MAb was not performed.

It was possible to couple four $AlPc(SO_2N_{gly})_4$ molecules to one MAb molecule without impairment of the solubility of the resulting MAbs. Under the chemical conditions that would lead to conjugates with a higher phthalocyanine:MAb ratio, the MAbs precipitated during the conjugation. This precipitation of MAbs after conjugation of photosensitizers to lysine residues is consistent with previous observations. *m*THPC-MAb conjugates with a ratio >4, TrisMPyP-ΦCO-NH-MAb conjugates with a ratio >3, and indocyanin-MAb conjugates with a ratio >2 showed the same phenomenon (1, 2, 16).

Although $AlPc(SO_2N_{gly})_4$-MAb conjugates with a molar ratio of ≤4 showed preservation of immunoreactivity, and HPLC and SDS-PAGE analysis indicated that there was no aggregate formation, the

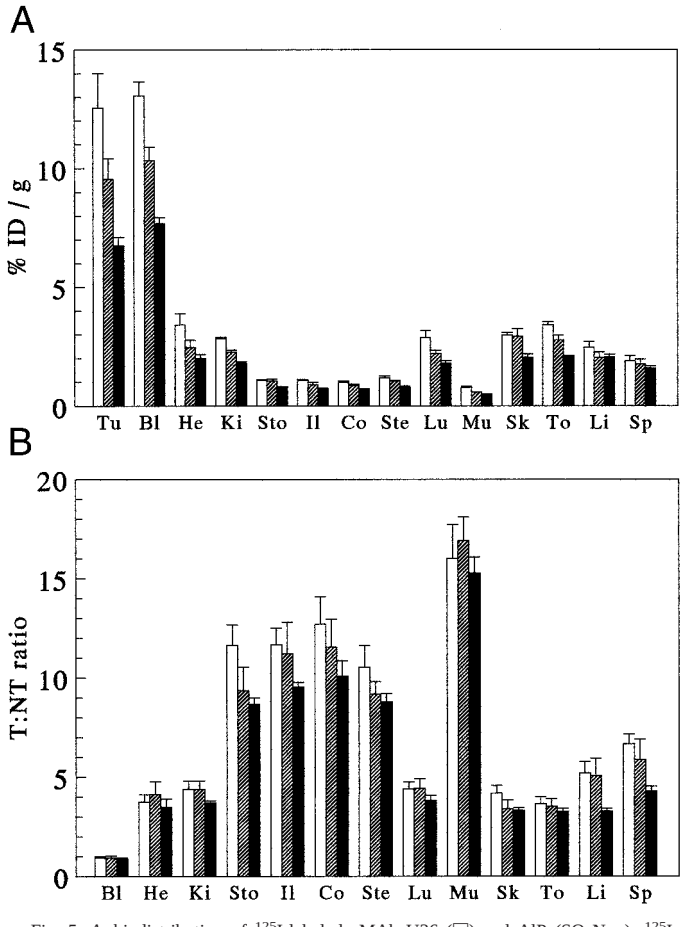

Fig. 5. *A*, biodistribution of ^{125}I-labeled cMAb U36 (□) and $AlPc(SO_2N_{gly})_4$-^{125}I-labeled cMAb U36 conjugates with a sensitizer:MAb ratio of 1.2 (▨) and 2.4 (■). Each preparation (100 μg of MAb; 10 μCi of ^{125}I) was i.v. injected in six HNX-OE-bearing nude mice; 48 h p.i., mice were bled, sacrificed, and dissected, and the radioactivity levels (%ID/g + SE) of blood, tumor, and several organs were assessed. *Tu*, tumor; *Bl*, blood; *He*, heart; *Ki*, kidney; *Sto*, stomach; *Il*, ileum; *Co*, colon; *Ste*, sternum; *Lu*, lung; *Mu*, muscle; *Sk*, skin; *To*, tongue; *Li*, liver; *Sp*, spleen; *B*, tumor:nontumor ratios for ^{125}I-labeled cMAb U36 (□) and $AlPc(SO_2N_{gly})_4$-^{125}I-labeled cMAb U36 conjugates with a ratio of 1.2 (▨) and 2.4 (■), 48 h p.i.

radiopharmacokinetic behavior of these conjugates in xenograft-bearing nude mice differed from that of unconjugated ^{125}I-labeled MAb. For conjugates with a mean ratio of 1.2 and 2.4, the ^{125}I levels in the blood at 48 h p.i. were 79 and 59%, respectively, of that of the unconjugated ^{125}I-labeled MAb. We observed a similar ratio-dependent blood clearance for *m*THPC-MAb, TrisMPyP-ΦCO-NH-MAb, ^{99m}Tc/^{99}Tc-labeled MAG3-MAb, and ^{186}Re-labeled MAG3-MAb conjugates (1, 2, 17). Other groups have also described this phenomenon (18, 19). In view of these data, the recent results of Carcenac *et al.* (4), published during our ongoing studies, are remarkable. They reported on the coupling of $AlPc(SO_3H)_4$ to MAb 35A7 via a mono five-carbon spacer chain and produced conjugates with a ratio as high as 16 in this way. These conjugates had neither impaired solubility nor impaired biodistribution characteristics.

Our data on the photodynamic efficacy of MAb- and unconjugated $AlPc(SO_3H)_4$, assessed by using the SRB assay, confirmed the hypothesis that the phototoxicity of the sensitizer was increased by coupling to internalizing MAbs. The mMAb 425-conjugated compound, in particular, showed a superior phototoxicity (IC_{50}, 0.12 nM). For $(AlPcS_4A_1)_{12}$-MAb 35A7 conjugates, Carcenac *et al.* (4) used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay for phototoxicity measurement and found, while using a double light dose, an IC_{50} of about 350 nM. The low toxicity might be attributable to the fact that their conjugate did not internalize, which might also explain why the conjugate was only about five times more effective than the free sensitizer.

In conclusion, our data show that hydrophilic photosensitizers, although ineffective in free form, can be transformed into very potent antitumor agents when coupled to internalizing MAbs.

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