Transferrin-Conjugated Liposome Targeting of Photosensitizer AlPcS4 to Rat Bladder Carcinoma Cells

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Background: The efficacy and safety of photodynamic therapy for superficial bladder cancer depend on tumor-selective accumulation of the photosensitizer. Bladder transitional-cell carcinoma cells overexpress the transferrin receptor on their surface. We examined whether transferrin-mediated liposomal targeting of the photosensitizer aluminum phthalocyanine tetrasulfonate (AlPcS4) is an effective strategy to attain tumor-selective accumulation of this compound when applied intravesically. Methods: AlPcS4 was stably encapsulated in unconjugated liposomes (Lip–AlPcS4) or transferrin-conjugated liposomes (Tf–Lip–AlPcS4). The accumulation of free AlPcS4, Lip–AlPcS4, and Tf–Lip–AlPcS4 in human AY-27 transitional-cell carcinoma cells and in an orthotopic rat bladder tumor model was visualized by fluorescence microscopy. In vitro AlPcS4 accumulation was quantified by fluorescence measurements following drug extraction, and the photodynamic efficacy of AlPcS4 was measured in a clonogenic assay. All statistical tests were two-sided. Results: AY-27 cells incubated with Tf–Lip–AlPcS4 had much higher intracellular AlPcS4 levels than AY-27 cells incubated with Lip–AlPcS4 (384.1 versus 3.7 µM; difference = 380.4 µM, 95% CI = 219.4 to 541.3; P = .0095). Among rats bearing AY-27 cell–derived bladder tumors, intravesical instillation with Tf–Lip–AlPcS4 resulted in mean AlPcS4 fluorescence in tumoral tissue, normal urothelium, and submucosa/muscle of 77.9 fluorescence units (fu) (95% CI = 69.1 to 86.8 fu), 4.3 fu (95% CI = 4.0 to 4.5 fu), and 1.0 (95% CI = 0.1 to 1.9 fu), respectively, whereas instillation of free AlPcS4 resulted in nonselective accumulation throughout the whole bladder wall, and Lip–AlPcS4 instillation resulted in no tissue accumulation. Photodynamic therapy of AY-27 cells incubated with Lip–AlPcS4 resulted in cell viabilities greater than 90% for all concentrations and incubation times tested; photodynamic therapy of cells incubated with 1 µM Tf-Lip–AlPcS4 or AlPcS4 resulted in cell viabilities of 0.19% (95% CI = 0.02% to 0.36%) and 1.32% (95% CI = 0.46% to 2.19%), respectively. Higher concentrations of either AlPcS4 or Tf-Lip–AlPcS4 resulted in cell kills of more than 3 logs. Conclusions: Transferrin-mediated liposomal targeting of photosensitizing drugs is a promising potential tool for photodynamic therapy of superficial bladder tumors. [J Natl Cancer Inst 2004;96:1620–30]

Photodynamic therapy is an antitumor modality for the treatment of some types of superficial carcinomas and sarcomas. This technique involves the systemic or topical administration of a photosensitizer that, after a specific interval that allows its maximal accumulation in the neoplastic tissue, is irradiated with laser light of a particular wavelength. Upon exposure to and excitation by light, the photosensitizer reacts with molecular oxygen, generating reactive oxygen species. As a result of this photodynamic therapy–induced oxidative stress, cellular organelles and membranes become damaged, ultimately leading to apoptosis or necrosis of the treated tumor cells. In addition to killing tumor cells directly, photodynamic therapy can also lead to indirect tumor ablation by causing tumor ischemia and nutritional starvation, because vascular leakage, blood flow stasis, and vascular collapse have been observed after photodynamic therapy (1,2). However, the limited tissue penetration of the wavelength that must be used to excite the photosensitizer restricts the application of photodynamic therapy to superficial tumors.

Most bladder cancers (~98%) occur within the transitional epithelium as superficial transitional-cell carcinomas. Bladder cancer is ideally suited to treatment with photodynamic therapy because the lumen of the bladder is easily accessible by endoscopy and bladder tissue is more translucent than other human tissues (3). Photodynamic therapy of urothelial malignancies initially involved focal light delivery to one area, but the frequent multifocal and occult character of the disease requires irradiation of the whole bladder (4). The safety of whole-bladder photodynamic therapy depends on the selective uptake and retention of the photosensitizer by the malignant tissue to avoid major side effects, such as fibrosis and contraction of the bladder with upper tract obstruction due to oxidative damage of the muscularis (5,6).

One way to target the photosensitizer to malignant tissue is to take advantage of iron transport systems. Iron is transported within the body between sites of absorption, storage, and use by transferrin. Cells that require iron express transferrin receptors on their cell surface that mediate the cellular uptake of iron from the plasma transferrin. Because iron is an essential element for cell proliferation and metabolism, and because the increased proliferation of malignant cells is accompanied by a higher...
demand for iron, many tumor cells overexpress transferrin receptors on the cell surface and have a higher turnover of these receptors compared with normal cells (7). The magnitude of transferrin receptor expression and turnover is proportional to the proliferative ability of the tumor tissue, because a higher proliferation rate requires more iron (8). For example, bladder transitional-cell carcinoma cells overexpress transferrin receptors compared with normal bladder mucosa, in which—except for the proliferating cells of the basal layer—transferrin receptors are undetectable (9,11). Moreover, the degree of transferrin receptor expression is associated with the histologic grade and pathologic stage of a tumor (10). In addition, superficial bladder tumors that overexpressed transferrin receptors compared with their normal cell counterparts had a higher recurrence rate than tumors that did not (11,12).

These data raise the possibility that transferrin can be used to target therapeutic compounds to bladder transitional-cell carcinoma cells. Previous investigations using a transferrin conjugate to target an HT29 human colon cancer growing in nude mice resulted in a poor tumor accumulation after systemic administration of the compound (16), probably because of the large amount of competing transferrin present in blood (13). However, whole-bladder photodynamic therapy provides an ideal situation in which transferrin conjugates can be administered directly to the bladder through catheterization and intravesical instillation. Intravesical instillation, unlike systemic administration, allows direct exposure of bladder tumor cells to transferrin conjugates without interference by competing transferrin.

We examined whether transferrin-mediated targeting of the photosensitizer aluminum phthalocyanine tetratsulfonate (AlPcS₄) is an effective strategy to attain a tumor-selective behavior of this compound when applied intravesically. For that purpose, the photosensitizer was stably encapsulated in polyethylene glycol (PEG) liposomes, and transferrin was conjugated to the functionalized terminal ends of the PEG chains. An advantage of liposomal delivery is that it provides the opportunity to increase the ratio of the entrapped compound to the targeting molecule, resulting in enhanced cellular uptake and therapeutic efficacy of the liposome-associated drug. We chose AlPcS₄ over other photosensitizers because its hydrophilic character permits stable encapsulation in the aqueous internal compartment of the liposome. Moreover, AlPcS₄ exhibits high molar absorption at 672 nm, a wavelength that is not absorbed or dispersed by endogenous tissue components (15). We prepared sterically stabilized liposomes by incorporating a fraction of PEG-derivatized phospholipids into the liposomal membranes. These highly hydrophilic polymers form a water shell at the liposome surface, repelling the absorption of opsonins (proteins or peptides that label targets for phagocytosis) and resulting in a reduced clearance of the liposomes by the mononuclear phagocyte system (16). This steric barrier allows the liposomes to be applied intravenously and enables them to transit more easily across tissue when applied topically because of its lubricating properties (17). We analyzed the accumulation of free AlPcS₄ and AlPcS₄ in transferrin-conjugated and unconjugated liposomes in both human transitional-cell carcinoma cells in vitro and in a rat orthotopic model system in vivo.

**Materials and Methods**

**Liposome Preparation**

PEG liposomes were prepared as described previously by Huwyler et al. (18) by dissolving 5.2 μmol of distearoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL), 0.3 μmol of distearoyl phosphatidylethanolamine–PEG [2000 d] (Shearwater Polymers, Huntsville, AL), 0.17 μmol of distearoyl phosphatidylethanolamine–PEG [3400 d]–maleimide (custom synthesized by Shearwater Polymers), and 4.5 μmol of cholesterol (Sigma, St. Louis, MO) into 5 mL of chloroform. The chloroform was evaporated over 1 hour with the use of a rotavapor, forming a thin lipid film that was then hydrated for 15 minutes at 40 °C with 1 mL of a 10 mM solution of AlPcS₄ (Porphyrin Products, Logan, UT) in phosphate-buffered saline (PBS [pH 7.4]; Gibco-BRL, Paisley, Scotland). The hydrated phospholipids were mixed with 20 3-mm glass beads and resuspended by rotating on a rotavapor (180 rpm) without applying vacuum for 15 minutes at 65 °C. The resulting PEG liposomes were subjected to five freeze–thaw cycles (at −80 °C and 60 °C, respectively) and homogenized by extrusion through a 100-nm–pore size polycarbonate membrane (Avestin, Ottawa, Ontario, Canada). The PEG liposome suspension was then divided into two aliquots: One was used to prepare transferrin-conjugated PEG liposomes, and the other was used without further modification as nonconjugated PEG liposomes (Lip-AlPcS₄).

All manipulations involving the photosensitizer AlPcS₄ (preparation, purification, and analysis of liposomes) were performed under low-light conditions (i.e., <1 μW/cm²).

**Transferrin Conjugation**

Holo-transferrin (Sigma) was thiolated by adding 10 nmol of the compound to a fresh solution of 400 nmol of Traut’s reagent (2-iminothiolane; Pierce, Rockford, IL) in 2 mL of borate–EDTA buffer, pH 8.5 (0.15 M sodium borate, 0.1 mM EDTA). The mixture was shaken in the dark for 1 hour. The thiolated transferrin was then concentrated by ultrafiltration (Centricon-30; Amicon, Beverly, MA) to a volume of 0.2 mL, washed with 2 mL of PBS, pH 8.0 (0.15 M sodium chloride, 0.1 M Na₂HPO₄/2H₂O), and reconstituted by ultrafiltration to a volume of 0.2 mL. The thiolated transferrin was immediately added to one aliquot of PEG liposomes, and the mixture was incubated for 24 hours at 4 °C to allow reaction with the maleimide linkers of the PEG liposomes to generate transferrin-conjugated AlPcS₄-containing PEG liposomes (Tf-Lip–AlPcS₄).

**Purification of Liposome Constructs**

Lip-AlPcS₄ and Tf-Lip–AlPcS₄ were purified on Sephacryl S-500 HR gel filtration columns (1.6 × 16 cm; Pharmacia, Uppsala, Sweden) at 4 °C. The columns were equilibrated and eluted with PBS (pH 7.4). This method allowed us to separate AlPcS₄-containing PEG liposomes from free AlPcS₄ and transferrin-conjugated PEG liposomes from nonconjugated transferrin.

**Analysis of Liposome Constructs**

Average liposome diameter was determined by dynamic light scattering (λ = 632.8 nm [HeNe laser], θ = 173°, T = 26 °C) with the use of a noninvasive back-scattering high-performance
particle size (ALV Laser, Langen, Germany). After incubation of freshly prepared liposomes at 37 °C or 4 °C (i.e., the temperatures of the experimental and storage conditions) for 24 hours or 1 month, respectively, the average diameters of the liposomes were reanalyzed.

The number of liposomes per preparation was determined from the average number of phospholipids per liposome and the amount of initially added phospholipid that was effectively incorporated into liposomes. The average number of phospholipids per liposome was based on the area (per polar head group) of the phospholipids and cholesterol, taking into account their molar ratios, and the total surface area of a liposome (19). The amount of initially added phospholipid that was effectively incorporated into liposomes was determined by complexation of the phospholipids with ferrocyanate as previously described (20).

To determine the average number of AlPcS₄ molecules per liposome, we diluted an aliquot of the liposome suspension 100-fold in methanol:water (80:20 [vol/vol]) and measured the fluorescence of the released AlPcS₄ at 360 nm (excitation wavelength) and 645 nm (emission wavelength) with the use of a microplate fluorescence reader (FL600; Bio-Tek Instruments, Winooski, VT). The concentration of AlPcS₄ was calculated by using a calibration curve and converted to the number of AlPcS₄ molecules per liposome.

The average number of transferrin molecules conjugated to a PEG liposome was quantified by the bicinchoninic acid assay as described by Wessel et al. (21). To test for leakage of AlPcS₄ from the liposomes, we incubated a 10 μM suspension of liposomes (liposome concentration is expressed as a function of the AlPcS₄ concentration) in cell culture medium (see below) at 37 °C for 24 hours or 1 week. The mixtures were then subjected to Sephacryl S-500 HR gel filtration, and fractions containing AlPcS₄ were collected at 37 °C for 24 hours or 1 week. The mixtures were then subjected to microplate fluorescence reader (FL600; Bio-Tek Instruments, Winooski, VT). The concentration of AlPcS₄ was calculated by using a calibration curve and converted to the number of AlPcS₄ molecules per liposome.

Tumor Cells and Culture Conditions

We used AY-27 rat bladder carcinoma cells, which originated from an N-[4-(5-nitro-2-furyl)-2-thiazolyl]-formamide–induced bladder transitional-cell carcinoma of Fischer CDF rats (cells originally developed by Dr. S. Selman and Dr. J. Hampton, Medical College of Ohio, Toledo, OH) and obtained from Dr. F. Guillemine, University of Nancy, Nancy, France). J-82 and T-24 cells (transitional-cell carcinoma, urinary bladder, human) and RT-4 cells (transitional-cell papillary carcinoma, urinary bladder, human) were obtained from the American Type Culture Collection (Manassas, VA). RT-112 cells (transitional-cell papillary carcinoma, urinary bladder, human) were obtained from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH [DSMZ], Braunschweig, Germany). Cells were grown as monolayers at 37 °C in a humidified atmosphere with 5% CO₂ in minimum essential medium containing Earle’s salts and 2 mM l-glutamine supplemented with penicillin (100 IU/mL), streptomycin (100 μg/mL), amphotericin B (0.25 μg/mL), tylocin (60 μg/mL), 1% (vol/vol) nonessential amino acids, and 10% (vol/vol) fetal calf serum. The medium and all cell culture ingredients were obtained from Gibco-BRL. All cell manipulations involving AlPcS₄ were performed under low-light conditions (<1 μW/cm²).

Flow Cytometry

AY-27 cells (0.5 × 10⁶ cells) were fixed for 5 minutes in 1 mL of 0.5% paraformaldehyde (Sigma) in PBS (pH 7.4) at 4 °C and then washed with 1.7 mL of PBS and labeled with 3 μM (100 μL) transferrin–polylysine–fluorescein isothiocyanate conjugate (TF–polylysine–FITC; conjugate concentration expressed as a function of the FITC concentration; Sigma) in PBS for 30 minutes at 4 °C. The cell suspension was washed with 1.7 mL of PBS, and the resulting cell pellet was resuspended in 300 μL of 1% paraformaldehyde in PBS and subjected to flow cytometry. As a control for nonspecific binding of the fluorescent dye, AY-27 cells were similarly fixed, labeled with 3 μM fluorescein (Sigma), and subjected to flow cytometry. We used a FACSort flow cytometer (Becton Dickinson, Mountain View, CA) utilizing an argon laser with an excitation wavelength of 488 nm. The fluorescence was measured after passage through a 530-nm band-pass filter. A total of 5 × 10⁵ events were analyzed per sample. Three replicate experiments were performed, and each sample was assayed in triplicate. The data were analyzed using CellQuest software (Becton Dickinson).

In Vitro Quantification of Intracellular AlPcS₄ Accumulation

AY-27 cells were seeded onto transparent 24-well tissue culture plates (Costar, Cambridge, MA) at 3 × 10⁴ cells per well and incubated for 24 hours at 37 °C. Under low-light conditions, the culture medium was replaced with fresh medium containing 10 μM free AlPcS₄, 10 μM Lip-AlPcS₄, or 10 μM Tf-Lip–AlPcS₄ (photoactive compound concentration expressed as a function of the AlPcS₄ concentration). The cells were incubated for 4 hours or 24 hours, after which the cellular accumulation of the photoactive compounds was determined as previously described (22). Three replicate experiments were performed.

We conducted two additional experiments for AY-27 cells incubated with Tf-Lip–AlPcS₄. In the first experiment, AY-27 cells were incubated for 4 hours with Tf-Lip–AlPcS₄ free AlPcS₄ in the presence of 50 μM free transferrin. In the second experiment, AY-27 cells were first incubated for 2 hours in a 10 μM solution of colchicine or cytochalasin B (Sigma) in medium supplemented with 1% fetal calf serum, as described previously (23–25), and then immediately incubated for an additional 2 hours in medium supplemented with 10% fetal calf serum and 10 μM Tf-Lip–AlPcS₄ (concentration expressed as a function of AlPcS₄ concentration). The cellular accumulation of the photoactive compounds was then determined as previously described (22). Three replicate experiments were performed.

In Vitro Fluorescence Microscopy

AY-27 cells were seeded onto Lab-Tek two-chamber glass slides for tissue culture (Nunc, Naperville, IL) at 6 × 10⁴ cells per chamber and incubated for 24 hours at 37 °C. Under low-light conditions, the culture medium was then replaced with fresh medium containing 10 μM free AlPcS₄, 10 μM Lip-
AlPcS₄, or 10 μM Tf-Lip–AlPcS₄ (photoactive compound concentration expressed as a function of the AlPcS₄ concentration). The cells were incubated for 2, 4, or 24 hours, and then the medium was removed and the cells were washed three times with PBS (pH 7.4). Fluorescence microscopy was used to localize the intracellular accumulation of AlPcS₄. We used an AxioSkop 2+ fluorescence microscope (Carl Zeiss, Göttingen, Germany) equipped with a 100-W mercury lamp. The filter set used for fluorescence imaging included a 575–625-nm band-pass excitation filter and a 660–710-nm band-pass emission filter. Fluorescence images were acquired with a light-sensitive charge-coupled device digital camera (AxioCam HR; Carl Zeiss). Rapid observation and electronic image storage were used to prevent photobleaching of the AlPcS₄ fluorescence. For uniformity, all parameters pertaining to fluorescence excitation and detection (e.g., exposure time and gain) were held constant throughout the study. Three replicate experiments were performed.

**In Vitro Photodynamic Therapy**

AY-27 cells were seeded onto transparent six-well tissue culture plates (Costar) at 9 × 10⁵ cells per well and incubated for 24 hours at 37 °C. For each six-well plate, we used only a single well, the size of which corresponded to the diameter of the light beam used for photodynamic therapy (see below). Under low-light conditions, the culture medium was then replaced with fresh medium containing 1, 5, or 10 μM free AlPcS₄, Lip-AIPcS₄, or Tf-Lip–AlPcS₄ (photoactive compound concentration expressed as a function of the AlPcS₄ concentration). The cells were incubated for 2 or 4 hours, the medium was removed, and the cells were washed two times with PBS (pH 7.4) and then irradiated for 10 minutes in 2.5 mL of PBS. For irradiation, the six-well plate was mounted 10 cm above a water-cooled 1000-W halogen lamp equipped with a filter (center wavelength = 651 nm; band width = 85 nm at 50% transmission; Melles Griot, Irvine, CA). The output at the absorption maximum of AlPcS₄ (672 nm) was 84% of the peak transmission of the filter. The uniform fluence rate at the surface of the irradiated well was 20 mW/cm², as measured with an IL 1400 radiometer (International Light, Newburyport, MA). During irradiation, the temperature of the cell culture medium never exceeded 25 °C. Immediately after irradiation, the cells were harvested by trypsinization and pelleted by centrifugation (5 minutes, 500g), and the cell pellets were resuspended in fresh cell culture medium lacking photoactive compounds. The number of cells per well was quantified by using a Coulter Z1 particle counter (Coulter Electronic, Luton, U.K.). An appropriate number of cells sufficient to yield 50–100 colonies was plated onto a 100-mm Petri dish in 15 mL of cell culture medium lacking photoactive compounds and incubated for 9 days. The cells were then fixed and stained for 2 minutes with 1% (wt/vol) methylene blue in methanol.

Colonies with more than 50 cells were counted during viewing with a dissection microscope. Because photodynamic therapy can lead to an immediate and complete fragmentation of cells, a fraction of the cells originally present in the irradiated wells are not quantified by the Coulter counter and therefore are not processed in the clonogenic assay. This fraction (R₁) was quantified from the ratio of the number of cells harvested immediately after photodynamic therapy to the number of untreated, control cells (as assessed by using the Coulter counter).

The final survival fraction (SF) was then calculated according to SF = R₁ × R₂, in which R₂ is the ratio of the cloning efficiency of treated cells to the cloning efficiency of untreated, control cells (as assessed by the clonogenic assay).

Three replicate experiments were performed.

**Rat Orthotopic Superficial Bladder Tumor Model**

Ten- to fifteen-week-old female Fischer CDF (F-344)/CrlBR rats weighing 150–175 g (Charles River Laboratories, Sulzfeld, Germany) were used to establish a superficial urothelial orthotopic bladder tumor model as previously described (26,27). The rats were anesthetized with intraperitoneal injections of sodium pentobarbital (45 mg/kg of body weight) and placed in a supine position on a homeothermic blanket to maintain their body temperature. We next inserted a catheter into the bladder via the urethra with the use of an 18-gauge plastic intravenous cannula and rinsed the bladder for 15 seconds with 0.4 mL of 0.1 N hydrochloric acid, followed by a 15-second rinse with 0.4 mL of 0.1 N sodium hydroxide. The bladder was then drained and washed five times with PBS (pH 7.4). A suspension of AY-27 cells (10⁵ cells in 0.5 mL of medium) was then instilled and maintained in the bladder for 1 hour. The rats were turned 90° laterally every 15 minutes to ensure homogeneous exposure of the entire bladder wall to the tumor cells. The catheters were removed, and the rats were allowed to void spontaneously. The rats were then used for intravesical administration of the photoactive compounds 6 days after tumor cell instillation. All animal procedures were carried out in compliance with national and European regulations and were approved by the Animal Care and Use Committee of the Katholieke Universiteit Leuven.

**Intravesical Administration of Photoactive Compounds**

We used intravesical instillation to introduce Lip-AlPcS₄, Tf-Lip–AlPcS₄, and free AlPcS₄ into the bladders of rats that either had or had not been instilled with AY-27 cells. To allow the liposomes to penetrate the bladder wall, the glycolalyx of the bladder transitional epithelium was partially removed by pre-treating the bladder with *Proteus vulgaris* chondroitinase ABC (Sigma), an enzyme that selectively digests chondroitin and dermatan sulfates (28). Rats were anesthetized and catheterized, and 3 U of chondroitinase ABC in 0.5 mL of PBS or 0.5 mL of PBS alone was instilled into the bladder through the catheter and maintained in the bladder for 1 hour. The rats were turned 90° laterally every 15 minutes to ensure homogeneous exposure of the bladder wall to the enzyme solution. The bladder was then drained, and 0.5 mL of AlPcS₄, Lip-AlPcS₄, or Tf-Lip–AlPcS₄ in PBS (at 10, 100, or 400 μM; concentration expressed as a function of AlPcS₄ concentration) was instilled into the bladder through the catheter for 2 hours before biodistribution evaluation. Rats were separated into 36 experimental groups (three rats per experiment group; i.e., three photoactive compounds at three different concentrations; tumoral versus nontumoral bladder; with versus without chondroitinase ABC treatment).

**Biodistribution Studies**

After exposure of the bladders to the compounds, rats were killed by pentobarbital overdose. Their bladders were immediately removed, snap-frozen, and stored in liquid nitrogen.

**Immunohistochemical staining for chondroitin.** We performed immunohistochemical staining for chondroitin on 5-μm
immersed in liquid nitrogen. Two consecutive 5-OCT embedding medium (Miles Laboratories, Elkhart, IN) and the catheter. The rats were killed, and their bladders were drained and rinsed twice with PBS (pH 7.4) through after instillation of the liposome constructs or AlPcS4, the bladders from three control rats with tumoral bladders in which PBS did not change appreciably after liposomes were incubated for 24 hours at 37 °C or 1 month at 4 °C (results not shown).

Localization and quantitation of AlPcS4 fluorescence in the bladder wall. Imaging of AlPcS4 fluorescence in sections of bladder tissues from rats that had and had not received AY-27 cells was carried out by fluorescence microscopy. Two hours after instillation of the liposome constructs or AlPcS4, the bladders were drained and rinsed twice with PBS (pH 7.4) through the catheter. The rats were killed, and their bladders were removed, cut open, and immediately embedded in Tissue Tek OCT embedding medium (Miles Laboratories, Elkhart, IN) and immersed in liquid nitrogen. Two consecutive 5-μm tissue sections were cut transversely from each frozen bladder with a cryostat. The first section was stained with hematoxylin–eosin, and the second section was examined by fluorescence microscopy as described above. We used KS imaging software (Carl Zeiss, Hallbergmoos, Germany) to measure the mean AlPcS4 fluorescence in specific areas of the bladder. We measured the fluorescence in arbitrary units of 20 sections (size = 3.8 × 150 μm) taken randomly from regions of interest in the bladders from three rats and corrected those measurements for the autofluorescence measured in the respective tissue layer of bladders from three control rats with tumoral bladders in which PBS (pH 7.4) (no photoactive compounds) was instilled.

Statistical Analysis

The statistical significance of differences was calculated using unpaired Student’s t test (two-sided) that assumed unequal variance (Welch correction) (Instat; GraphPad Software, San Diego, CA).

RESULTS

Characteristics of Liposome Constructs

Dynamic light-scattering measurements demonstrated that the average liposome diameter was 146 nm (95% confidence interval [CI] = 117 to 174 nm; based on data obtained from five different liposomal preparations). The average liposomal diameter did not change appreciably after liposomes were incubated for 24 hours at 37 °C or 1 month at 4 °C (<1% change in diameter; based on data from three independent experiments performed on one batch of liposomes).

Given the area occupied by the polar head groups of the phospholipids and cholesterol, and taking into account their molar ratio versus the total surface of a liposome (19), we estimated that a 146-nm liposome contains approximately 144 000 molecules of phospholipid. Based on an initial phospholipid concentration of 5.67 μmol, the average incorporation efficiency of phospholipids into liposomes was 62.0% (95% CI = 59.8% to 64.1%; data obtained from three different liposomal preparations). We used these data to determine the exact number of liposomes per preparation and to calculate the numbers of AlPcS4 and transferrin molecules per liposome.

Fluorescence measurements revealed that the intraliposomal concentration of AlPcS4 was 29.0 mM (95% CI = 27.0 to 31.0 mM), which corresponded to 22 995 AlPcS4 molecules per liposome (95% CI = 21 532 to 24 458 AlPcS4 molecules per liposome). As quantified by the bicinchoninic acid assay, the coupling of transferrin to the liposomes yielded 112 transferrin molecules per liposome (95% CI = 106 to 118 transferrin molecules per liposome), corresponding to a conjugation efficiency of 13.9% (data obtained from seven different liposomal preparations).

We also examined the stability of AlPcS4-containing liposomes under the conditions used for the later experiments. Tf–Lip–AlPcS4 incubated at 37 °C in cell culture medium for 24 hours and for 1 week retained 100% (95% CI = 94.8% to 105.2%) and 96.7% (95% CI = 93.1% to 100.3%), respectively, of the original amount of AlPcS4. These results were based on data from three independent experiments performed on one batch of liposomes.

Transferrin Receptor Expression on AY-27 Cells

To examine whether rat AY-27 bladder cancer cells express transferrin receptors, cells were stained with Tf–polylysine–FITC, a transferrin conjugate that binds specifically to transferrin receptors, and analyzed by flow cytometry. As shown in Fig. 1, more than 99.7% of AY-27 cells stained with Tf–polylysine–FITC, showing a mean fluorescence intensity (MFI) of 1306 (95% CI = 691 to 2192). The specificity of Tf–polylysine–FITC binding was confirmed by comparing the Tf–polylysine–FITC–stained AY-27 cells with AY-27 cells that were stained with fluorescein alone, which had an MFI of 4.4 (95% CI = 2.1 to 6.9). Moreover, similar experiments performed on several other bladder transitional-cell carcinoma cell lines of human origin (T24, RT4, J82, and RT112) indicated that these cell lines expressed similar levels of transferrin receptors (results not shown).

In Vitro Quantification of Intracellular AlPcS4 Accumulation

A 4-hour incubation of AY-27 cells with free AlPcS4 and Tf–Lip–AlPcS4 resulted in intracellular AlPcS4 concentrations of 52.7 μM (95% CI = 36.8 to 68.6 μM) and 384.1 μM (95% CI = 223.2 to 545.0 μM) (calculated as AlPcS4 concentration), respectively. By contrast, very little intracellular accumulation of the photosensitizer was observed for cells incubated with Lip-AlPcS4 (i.e., 3.7 μM, 95% CI = 1.1 to 6.3 μM) (Fig. 2). The intracellular concentration of AlPcS4 was statistically significantly lower for cells incubated with Lip-AlPcS4 than for cells.
cellular concentration of AlPcS₄ was 7.7 μM, and Tf-Lip–AlPcS₄ exhibited a stronger AlPcS₄-induced fluorescence signal, indicating that almost no AlPcS₄ had accumulated. For AY-27 cells incubated with Tf-Lip–AlPcS₄ in the absence of 50 μM transferrin, the intracellular concentration of AlPcS₄ was 7.7 μM, a statistically significant decline from the intracellular AlPcS₄ concentration for cells incubated with Tf-Lip–AlPcS₄ in the absence of 50 μM transferrin (difference = 376.4 μM, 95% CI = 215.4 to 537.4 μM; P = .0097). Extending the incubation period from 4 to 24 hours did not statistically significantly increase the intracellular AlPcS₄ concentrations of cells exposed to the different photosensitizer preparations (Fig. 2). For cells incubated with Tf-Lip–AlPcS₄, this result suggests that their transferrin receptors had reached saturation during the first 4 hours of incubation.

To address the possibility that the cellular uptake of Tf-Lip–AlPcS₄ is merely the result of a nonspecific interaction of the protein-bearing liposome with the negatively charged cell membrane—an interaction that is opposed by the nonspecific binding of an excess of free transferrin to the cellular membrane—we performed additional experiments in the presence of a 10 μM solution of the microfilament inhibitor cytochalasin B or the microtubule inhibitor colchicine. These compounds specifically inhibit the receptor-mediated uptake of transferrin (23–25). In all cases, preincubating the cells for 2 hours with either inhibitor, followed by a second 2-hour incubation in the presence of the inhibitor and Tf-Lip–AlPcS₄, resulted in intracellular AlPcS₄ concentrations that were below the quantification limit (i.e., <0.1 μM), a concentration that is statistically significantly lower (P < .001) than those observed in the corresponding experiment without inhibitors (data not shown). These results imply that the cellular uptake of Tf-Lip–AlPcS₄ is completely inhibited by these cytoskeleton-disrupting drugs and therefore that it involves the specific activity of transferrin.

**Visualization of Intracellular AlPcS₄ Accumulation**

Consistent with our quantitative results for intracellular AlPcS₄ accumulation, we found that cells incubated with Tf-Lip–AlPcS₄ exhibited a stronger AlPcS₄-induced fluorescence signal, except for cells incubated with Tf-Lip–AlPcS₄. Control cells (i.e., cells incubated in the absence of any photosensitizer) displayed no fluorescence (Fig. 3, A).

**Effect of Photodynamic Therapy on Cell Viability**

We evaluated AY-27 cell killing in response to photodynamic therapy for cells incubated with each of the photosensitizer compounds for two different times: a 2-hour interval, which corresponded to the in vivo incubation time, and a 4-hour interval, which corresponded to the exposure time used for in vitro quantification of the intracellular accumulation. The survival
curves are shown in Fig. 4. Photodynamic therapy of AY-27 cells incubated with Lip-AlPcS₄ resulted in cell viabilities greater than 90% for all concentrations and incubation times tested. By contrast, photodynamic therapy of cells incubated with 1 μM Tf-Lip-AlPcS₄ for 2 and 4 hours resulted in cell viabilities of 1.34% (95% CI = 1.04% to 1.64%) and 0.19% (95% CI = 0.02% to 0.36%), respectively. For AlPcS₄, these conditions led to cell viabilities of 25.49% (95% CI = 22.65% to 28.30%) and 1.32% (95% CI = 0.46% to 2.19%), respectively. Moreover, higher concentrations of either AlPcS₄ or Tf-Lip–AlPcS₄ further decreased the tumor cell viability, resulting in cell kills of more than 3 logs (Fig. 4). For both phototoxic compounds, we observed a similar cell killing profile at these higher concentrations, regardless of the incubation period. Conversely, Lip-AlPcS₄ at 5 μM and 10 μM induced a moderate photocytotoxic effect. For instance, 2-hour incubations with 5 μM AlPcS₄ or Tf-Lip–AlPcS₄ resulted in cell viabilities of 0.012% (95% CI = 0.010% to 0.014%) and 0.021% (95% CI = 0.017% to 0.025%), respectively, whereas a 2-hour incubation with 5 μM Lip-AlPcS₄ resulted in a cell viability of 30.2% (95% CI = 26.3% to 34.1%). Therefore, the difference in cell viabilities recorded after photodynamic therapy with AlPcS₄ and Lip-AlPcS₄ on the one hand (0.012% versus 30.2%; difference = 30.2%, 95% CI = 26.3% to 34.0%; P<.001) and with Tf-Lip–AlPcS₄ and Lip-AlPcS₄ on the other (0.021% versus 30.2%, difference = 30.2%, 95% CI = 26.3% to 34.0%; P<.001), were very similar.

**Localization of AlPcS₄ Fluorescence in Rat Bladder Wall**

We next investigated the in vivo relevance of transferrin-conjugated liposome targeting of AlPcS₄ for photodynamic therapy of superficial bladder carcinoma by instilling each of the photoactive compounds into healthy and tumoral rat bladders and examining the accumulation of AlPcS₄ in the different tissues of the bladder. Preliminary results of in vivo experiments showed that Tf-Lip–AlPcS₄ failed to accumulate in AY-27 cell–derived bladder tumor tissues of rats. We suspected that this lack of accumulation was due to the presence of the glycosalix layer in the bladder, which is important for maintaining the impermeability of the bladder mucosa. We therefore examined accumulation of Tf-Lip–AlPcS₄ in bladders that had been pretreated with chondroitinase ABC, an enzyme that selectively digests chondroitin and dermatan sulfates. In addition, we investigated whether treatment of the bladder wall with this enzyme results in specific digestion of chondroitin present in the glycocalyx of the bladder wall.

**Immunohistochemical staining** showed that sections of bladders from untreated rats had a layer on the epithelial surface that stained positive with a monoclonal antibody for chondroitin sulfate (Fig. 5, A), a ground substance of the bladder surface mucin. By contrast, bladder sections from rats pretreated with chondroitinase ABC showed no such staining (Fig. 5, B). As a negative control for nonspecific staining, we omitted the murine monoclonal anti–chondroitin sulfate antibody from the staining procedure and found that, apart from some staining of blood vessels, there was no staining of the luminal surface of the urethelium in either untreated or chondroitinase ABC–pretreated rat bladders (Fig. 5, C and D). These results indicate that the chondroitinase ABC pretreatment of the bladder results at least in a partial digestion of the mucopolysaccharide layer.

We used fluorescence microscopy to assess the depths to which liposomal or free AlPcS₄ had penetrated the rats’ bladder walls. Lower concentrations of the AlPcS₄ compounds (i.e., 10 and 100 μM) resulted in no apparent or reproducible fluores-
cence in the bladder wall (data not shown). By contrast, the highest concentration of the photoactive compounds tested (i.e., 400 \( \mu M \)) produced different patterns of fluorescence localization in the bladder wall. For example, after intravesical instillation of a solution of 400 \( \mu M \) free AlPcS\(_4\), we observed strong fluorescence throughout the entire wall of both healthy (Fig. 6, A, B) and tumoral rat bladders (Fig. 6, G, H), independent of chondroitinase ABC pretreatment. Although the luminal surface showed an increased fluorescence, probably because it was in direct contact with the instilled AlPcS\(_4\) solution, we observed no gradient of fluorescence intensity between the superficial and the deeper layers of the bladder wall. By contrast, we observed no fluorescence, regardless of bladder enzymatic pretreatment, across the bladder wall when Lip-AlPcS\(_4\) (400 \( \mu M \)) was instilled into healthy (Fig. 6, C, D) or tumoral (Fig. 6, I, J) bladders. Instillation of Tf-Lip–AlPcS\(_4\) (400 \( \mu M \)) into nonpretreated (Fig. 6, E) or pretreated (Fig. 6, F) healthy bladders and into nonpretreated tumoral bladders (Fig. 6, K) also resulted in no fluorescence in any of layer of the bladder wall. By contrast, instillation of Tf-Lip–AlPcS\(_4\) (400 \( \mu M \)) into chondroitinase ABC–pretreated tumoral bladders gave rise to a strong fluorescent signal that was specifically localized to the urothelial tumor tissue (Fig. 6, L, N). The deeper areas of the bladder wall (submucosa and muscle) exhibited no fluorescence. However, when Tf-Lip–AlPcS\(_4\) (400 \( \mu M \)) was administered to chondroitinase ABC–pretreated tumoral bladders in the presence of 50 \( \mu M \) of competing transferrin, no fluorescence was detected in the urothelial tumor (Fig. 6, M).

We quantitated AlPcS\(_4\) fluorescence in healthy and tumoral bladders that were pretreated with chondroitinase ABC and instilled with Tf-Lip–AlPcS\(_4\). Fluorescence measurements within specific regions of the bladder wall revealed that tumoral tissue, normal urothelium, and submucosa/muscle had mean fluorescence units (fu) of 77.9 fu (95% CI = 69.1 to 86.8 fu), 4.3 fu (95% CI = 4.0 to 4.5 fu), and 1.0 (95% CI 0.1 to 1.9 fu), respectively. The mean fluorescence in tumoral tissue was statistically significantly greater than that in normal urothelium (difference = 73.6 fu, 95% CI = 64.8 to 82.4 fu; \( P < .001 \)) and in submucosa/muscle (difference = 76.9 fu, 95% CI = 68.1 to 85.7 fu; \( P < .001 \)). Moreover, the ratio of mean fluorescence intensity in tumor tissue to that in normal urothelium was 18:1, whereas the ratio of mean fluorescence intensity in tumor tissues to that in underlying tissue layers (submucosa and muscle) was 78:1. Comparison of the accumulation of AlPcS\(_4\) in the tumor tissues of bladders pretreated with chondroitinase ABC and instilled with Tf-Lip–AlPcS\(_4\) (mean fluorescence: 77.9 fu [95% CI = 69.1 to 86.8 fu]) versus Lip-AlPcS\(_4\) (mean fluorescence: 1.0 fu [95% CI = 0.4 to 1.5 fu]) [difference: 76.9 fu, 95% CI = 68.0 to 85.7 fu; \( P < .001 \)] resulted in a tumoral tissue fluorescence intensity ratio of 78:1.

These results indicate that Tf-Lip–AlPcS\(_4\) accumulates selectively in bladder tumor tissues but not in the underlying tissue layers of the tumoral bladder or the normal urothelium. Moreover, the nontargeted liposomes (i.e., Lip-AlPcS\(_4\)) do not show such a pattern of accumulation.

**DISCUSSION**

Given that the recurrence rate for bladder cancer is between 50% and 70%, transurethral resection is presently the primary, but rarely definitive, treatment modality for superficial bladder lesions. This high recurrence rate arises from the failure to resect barely visible or invisible neoplastic lesions and from the incomplete resection of the primary lesion (29,30). The frequent recurrence and the risk of progression toward invasive tumors often compel patients to undergo cystectomy. Tumor-selective accumulation of a photosensitizer in bladder transitional-cell carcinoma cells would allow photodiagnosis and fluorescence-guided transurethral resection of lesions with a high sensitivity and specificity. Furthermore, whole-bladder photodynamic therapy with a tumor-selective photosensitizer obviates the need for surgical removal of the bladder and permits physiologic bladder function, offering a great comfort to patients.

We examined whether targeting the photosensitizer AlPcS\(_4\) by means of transferrin-conjugated liposomes is an effective strategy to attain the tumor-selective behavior of the compound when applied intravesically. The rationale for this approach is based on the finding that transitional-cell carcinoma cells overexpress transferrin receptors on their cell surface (9–12). Indeed, we found that all of the transitional carcinoma cell lines we examined, including some human cell lines, expressed substantial levels of the transferrin receptor. Results of our in vitro experiments showed that transferrin-targeted liposomes statistically significantly increased (by approximately 100-fold) the cellular uptake of AlPcS\(_4\) compared with that of cells exposed to AlPcS\(_4\) encapsulated in nontargeted (i.e., non–transferrin conjugated) liposomes. The transferrin dependence of the cellular uptake of the liposomes was further corroborated by the observation that the intracellular AlPcS\(_4\) accumulation underwent a statistically significant 50-fold drop, in the presence of a competing concentration of transferrin. From these findings, we deduced that the substantial amount of transferrin in human blood (as much as 50 \( \mu M \) (14)) would compete for transferrin receptors and render the transferrin-targeted liposomes unsuitable for intravenous application.

To investigate the practical applicability of photodynamic therapy using targeted liposomes, we measured the in vitro photocytotoxicity of Tf-Lip–AlPcS\(_4\) as well as its in vivo capacity to selectively accumulate in rat tumoral bladders. We found that transferrin targeting increased the in vitro photocytotoxicity of the encapsulated photosensitizer, resulting in a cell kill of more than 3 logs compared with the nontargeted liposomal photosensitizer, which showed only limited photocytotoxicity (<1 log cell kill). At a 1 \( \mu M \) concentration of the photoactive compound, Tf-Lip–AlPcS\(_4\) was more photocytotoxic than AlPcS\(_4\), consistent with the results obtained in our studies of in vitro intracellular accumulation. The photocytotoxicity of Tf-Lip–AlPcS\(_4\) and AlPcS\(_4\) in AY-27 cells further increased as a function of increasing AlPcS\(_4\) concentration, with the higher concentrations of both compounds resulting in a similar loss of cell viability. However, this result does not imply that Tf-Lip–AlPcS\(_4\) has no advantage over free AlPcS\(_4\) in photodynamic therapy because the therapeutic benefit that can be expected from the targeted liposomes resides mainly in an enhancement of the tumor selectivity, not just in their ability to increase the accumulation or photocytotoxicity of the photosensitizer.

Despite our in vitro results, preliminary results of in vivo experiments showed that Tf-Lip–AlPcS\(_4\) failed to accumulate in AY-27 cell–derived bladder tumor tissues of rats. We suspected that this lack of accumulation was due to the presence of the glycocalyx layer in the bladder, which is important for maintaining the impermeability of the bladder mucosa. The glycoca-
Fig. 6. Localization of AlPcS₄ fluorescence in sections of rat bladders that were exposed to 400 µM Lip-AlPcS₄, Tf-Lip–AlPcS₄, or AlPcS₄ (concentration expressed as a function of AlPcS₄ concentration). Photomicrographs of bladder urothelia from healthy rats not pretreated with chondroitinase ABC and instilled with AlPcS₄ (A, A), Lip-AlPcS₄ (C, C), or Tf-Lip–AlPcS₄ (E, E) and from healthy rats pretreated with chondroitinase ABC for 1 hour prior to instillation of AlPcS₄ (B, B), Lip-AlPcS₄ (D, D), or Tf-Lip–AlPcS₄ (F, F) are shown. In addition, photomicrographs of bladder tumor urothelia from rats bearing AY-27 cell–derived bladder tumors, not enzymatically pretreated, and instilled with AlPcS₄ (G, G), Lip-AlPcS₄ (I, I), or Tf-Lip–AlPcS₄ (K, K) and from rats bearing AY-27 cell–derived bladder tumors that were pretreated with chondroitinase ABC for 1 hour prior to instillation of AlPcS₄ (H, H), Lip-AlPcS₄ (J, J), or Tf-Lip–AlPcS₄ (L, L) are depicted. Microphotographs of bladder tumor urothelia from rats bearing AY-27 cell–derived bladder tumors that were pretreated with chondroitinase ABC for 1 hour prior to instillation of Tf-Lip–AlPcS₄ in the presence (M, M) or absence (N, N) of 50 µM of competing transferrin are shown. Italicized letters represent the hematoxylin-eosin–stained photomicrographs of the respective fluorescence picture (nonitalicized letters). Bars correspond to 50 µM (Lip-AlPcS₄ and Tf-Lip–AlPcS₄) or 100 µM (AlPcS₄). Representative images are shown.
lyx is synthesized mainly by the umbrella cells and composed of a dense layer of glycosaminoglycans, most commonly present as constituents of proteoglycans, and glycoproteins or mucin (28,31). We therefore examined accumulation of Tf-Lip–AlPcS4 in bladders that had been pretreated with chondroitinase ABC, an enzyme that can be produced in large quantities by transconjugates of Flavobacterium heparinum (32) and that permeabilizes the glyocalyx layer without damaging the urothelium. Of importance, the glyocalyx can be resynthesized by the umbrella cells in less than 24 hours and completely replaced within 48 hours (33,34). This short recovery period guarantees that a mild (partial) enzymatic digestion of the glyocalyx could be employed in clinical practice.

We found that, in bladders that had been pretreated with chondroitinase ABC, Tf-Lip–AlPcS4 appeared to concentrate selectively in AY-27 cell–derived tumor tissue. We concluded that this retention was tumor selective because Tf-Lip–AlPcS4 was not retained in normal urothelium of healthy rats whose bladders were pretreated with chondroitinase ABC. Furthermore, because the liposomal diameters of Lip-AlPcS4 and Tf-Lip–AlPcS4 were similar, we expected that both constructs would show similar penetration through the tumor tissue. However, Lip-AlPcS4 was virtually absent from the urothelial tumor tissue, indicating that the observed high and specific retention of Tf-Lip–AlPcS4 in the tumor was due to its specific interaction with transferrin receptors followed by intracellular accumulation. Although ligands such as transferrin typically bind to the transferrin receptor, this “binding-site barrier” (35) obviously did not prevent the transferrin-conjugated liposomes from penetrating into the deeper layers of tumor tissue. Because of its much smaller size, free AlPcS4 showed a higher level of uptake in tumor tissue compared with AlPcS4 delivered by Tf-Lip–AlPcS4. However, as observed previously (36), free AlPcS4 does not accumulate selectively in tumors (fluorescence ratio of bladder tumor to normal bladder = 2:1), precluding its use for photodiagnosis and whole–bladderwall photodynamic therapy.

In summary, our results suggest that transferrin-targeted, sterically stabilized liposomes are promising vehicles to selectively deliver photosensitizers, such as AlPcS4, to tumor cells that overexpress transferrin receptors. These targeted liposomes could also be used to visualize and, as suggested by the results of our in vitro clonogenic assay, selectively eradicate urothelial carcinoma lesions. In addition, these liposomal constructs may be useful for the targeted delivery of other anticancer agents. Thus, the potential of the transferrin–liposomal constructs may be far beyond the application described in this study.

REFERENCES


NOTES

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