Epidermal Growth Factor-mediated Targeting of Chlorin e₆ Selectively Potentiates Its Photodynamic Activity

Antoon Gijsens, Ludwig Missiaen, Wilfried Merlevede, and Peter de Witte


ABSTRACT

Certain tumor cells, such as squamous carcinoma cells, express an increased number of epidermal growth factor (EGF) receptors. Therefore, we studied the targeted delivery of the phototoxic photosensitizer Sn(IV)chlorin e₆ monoethylenediamine [SnCe₆(ED)] conjugated to the epidermal growth factor (EGF) receptor. EGF was conjugated to SnCe₆(ED) through a carrier, such as dextran (Dex) and human serum albumin (HSA), and the phototoxicity on the EGF receptor-overexpressing MDA-MB-468 breast adenocarcinoma cell line was evaluated. The photobiological activities of these EGF conjugates, of the conjugates of the photosensitizer to HSA or Dex, or of the photosensitizer alone were compared. The affinity of EGF for its receptor was substantially impaired when conjugated in EGF-Dex-SnCe₆(ED), in contrast to EGF-HSA-SnCe₆(ED). In corresponding results, EGF-HSA-SnCe₆(ED) displayed a high phototoxicity (IC₅₀, 63 nm) on MDA-MB-468 cells at a light dose of 27 kJ/m², whereas EGF-Dex-SnCe₆(ED) showed very limited phototoxicity. EGF-HSA-SnCe₆(ED) was no longer phototoxic in the presence of a competing EGF concentration. The high phototoxicity of EGF-HSA-SnCe₆(ED) was shown to be the result of a high intracellular concentration in MDA-MB-468 cells, which could be lowered dramatically by incubating the conjugate with a competing EGF concentration. In contrast, EGF-Dex-SnCe₆(ED) accumulated poorly in MDA-MB-468 cells, in agreement with its low EGF receptor affinity and phototoxicity. EGF-HSA-SnCe₆(ED) produced much more intracellular reactive oxygen species on light irradiation than EGF-Dex-SnCe₆(ED). It is concluded that the photodynamic activity of the EGF-HSA conjugate of SnCe₆(ED) on MDA-MB-468 breast adenocarcinoma cells is EGF specific and is much more potent than EGF-Dex-SnCe₆(ED) or free SnCe₆.

INTRODUCTION

During the past two decades, there has been a large increase in research on photosensitizers as a tool for PDT. Activation of a photosensitizer by light at specific wavelengths leads to the production of singlet oxygen and radical species, resulting in direct tumor cell killing, immune inflammatory responses, and damage to the microvasculature of the tumor (1). Since 1993, regulatory approval for PDT involving use of a partially purified hematoporphyrine derivative (Photofrin) in patients with cancer of the lung, digestive tract, and genitourinary tract has been obtained in several countries (2). The efficiency of HPD and other photosensitizers depends, among other factors, on tumor selectivity and the degree of accumulation. Most photosensitizers tested accumulate with some selectivity in tumors, but they also concentrate in normal tissues, including the skin (3). As a result of this, prolonged cutaneous phototoxicity after exposure to sunlight has been reported (4). Targeted delivery of the photosensitizer could solve these problems through an enhanced phototoxicity as a result of higher and more selective accumulation in the tumor cells. Targeting implies conjugation of the photo-active compound to a tumor-seeking molecule, either directly or by the use of a carrier.

Several photosensitizers have already been conjugated with antibodies directed against tumor-associated antigens (5–7). Ligands such as low-density lipoprotein (8, 9), insulin (10), and transferrin (11) have all been used for ligand-based targeting of photosensitizers to cells overexpressing the receptors for these ligands.

Because a high EGF receptor expression frequently accompanies several tumor types, such as squamous carcinomas (12, 13), its natural ligand EGF is an attractive candidate for the conception of a targeting strategy. This approach might be promising for the targeted delivery of photosensitizers because most of the above-mentioned tissues or organs are easily accessible with laser light guided through an optical fiber.

On binding to its receptor, EGF is internalized in the cell through receptor-mediated endocytosis, enabling the intracellular accumulation of photosensitizers. EGF covalently linked with cysteine-containing proteins such as 131I-tyrosine (14), 10B-labeled starburst dendrimers (15), Pseudomonas toxin A (16), and diptheria toxin (17) has already been used successfully. In these and several other studies (18–20), it was shown that EGF of murine origin could be conjugated through its NH₂ terminus without losing biological activity. In contrast, human EGF contains two additional primary ε-amino groups in lysyl residues that might impair its biological activity upon modification. The affinities of mouse EGF and human EGF for the human EGF receptor are comparable (21), and direct conjugation of mouse EGF with photo-active hematoporphyrin (1:1 ratio) has been described previously (22).

However, this preparation failed to load cancer cells efficiently within a reasonable time period. Chlorin e₆ is a second-generation photosensitizer that can be easily modified through its carboxyl groups (23, 24), which makes the compound suitable for conjugation. In this study, we describe the photodynamic action of chlorin e₆ conjugated to a carrier such as Dex or HSA substituted with EGF or not, using MDA-MB-468 cells stably overexpressing the EGF receptor (1–2 × 10⁶ EGF receptors/cell; Ref. 25).

MATERIALS AND METHODS

Isolation of EGF. EGF was isolated from male mouse submaxillary glands according to the procedure of Savage and Cohen (26). Eleven mg of EGF were obtained from 100 male Swiss Webstar mice. SDS-PAGE (Phastgel 20% homogeneous; Pharmacia, Uppsala, Sweden) showed one band on the same position as tissue culture-grade EGF obtained from Sigma (catalogue number E-4127; St. Louis, MO). The identity was confirmed by protein sequencing (LF 3000, Beckman; Fullerton, CA) and mass spectroscopy (Tofspec, Micromass; Manchester, United Kingdom).

Derivatization of Chlorin e₆. Chlorin e₆ was purchased from Porphyrin Products (Logan, UT). Its monoethylenediamine derivative, SnCe₆(ED), was prepared as described by Lu et al. (27).
Preparation of EGF-Dex-SnCe₆(ED) and Dex-SnCe₆(ED) Conjugates. Activation of Dex towards the primary amino groups of SnCe₆(ED) and EGF was achieved using CDAP (Sigma; Fig. 1A) as described by Andersson et al. (28). Therefore, 10 mg of CDAP (42.6 μmol) were mixed for 10 s with 5 mg of Dex (15–20 kDa; approximately 0.29 μmol; catalogue number 31387; Fluka, Buchs, Switzerland) in 1 ml of distilled water, and then triethylamine (6.1 μl; 44 μmol) was added. The total activation time was 130 s. An aliquot (10 μl) of the mixture, corresponding to 2.86 nmol of Dex, was transferred to 0.4 ml of PBS (pH 7.4) containing 0.4 mg/ml EGF (26.5 nmol of EGF) to which 0.2 ml of a 1 M NaHCO₃ solution was added. After 8 min, 0.2 ml of dimethyl formamide containing 20 mg/ml SnCe₆(ED) (6.26 μmol) was added to make the photosensitizer react with the residual active sites in the carrier. The total coupling time was 4 h at room temperature. The preparation of Dex-SnCe₆(ED) was similar to the preparation of the EGF conjugate, except that the PBS used did not contain EGF.

After coupling, the conjugates were purified immediately on a Sephadex G-50 (Pharmacia) gel filtration column (1 × 40 cm). The column was equilibrated with PBS. The separation was monitored with a Pharmacia UV-1 280 (3). After coupling, the conjugates were collected (total volume, 16 ml) and concentrated by ultrafiltration (Centricon-3; Amicon, Beverly, MA) up to a volume of 0.5 ml.

Preparation of EGF-HSA-SnCe₆(ED) and HSA-SnCe₆(ED) Conjugates. The homobifunctional cross-linker glutaraldehyde (catalogue number G-7526; Sigma) was used to link the primary amino groups of HSA to those of SnCe₆(ED) and EGF (Fig. 1B). Therefore, 2 ml of PBS containing 5 mg/ml HSA (0.15 μmol; catalogue number A-8763; Sigma) were mixed with 0.55 ml of the mixture, corresponding to 2.86 nmol of Dex, was transferred to 0.4 ml of PBS (pH 7.4) containing 0.4 mg/ml EGF (26.5 nmol of EGF) to which 0.2 ml of a 1 M NaHCO₃ solution was added. After 8 min, 0.2 ml of dimethyl formamide containing 20 mg/ml SnCe₆(ED) (6.26 μmol) was added to make the photosensitizer react with the residual active sites in the carrier. The total coupling time was 4 h at room temperature. The preparation of Dex-SnCe₆(ED) was similar to the preparation of the EGF conjugate, except that the PBS used did not contain EGF.

After coupling, the conjugates were purified immediately on a Sephadex G-100 (Pharmacia) gel filtration column (1 × 40 cm). The column was equilibrated with PBS. The separation was monitored at 280 nm. Fractions (2 ml) were collected with a Pharmacia UV-1 280 (3). After coupling, the conjugates were collected (total volume, 4 ml) and concentrated by ultrafiltration (Centricon-3; Amicon, Beverly, MA) up to a volume of 0.5 ml.

Preparation of EGF-Dex-SnCe₆(ED) and Dex-SnCe₆(ED) Conjugates. At neutral or slightly alkaline pH values, isourea derivatives (ii) are formed. These react with primary amino groups forming stable secondary carbamates (iii). HSA was derivatized with EGF and SnCe₆(ED) (B) by the homobifunctional cross-linker glutaraldehyde (43). At neutral or slightly alkaline pH values, ω,ω-unsaturated aldehyde polymers are formed that cross-link amino groups. Therefore, 10 mg of CDAP (42.6 μmol; catalogue number 31387; Fluka, Buchs, Switzerland) were mixed for 10 s with 5 mg of Dex (15–20 kDa; approximately 0.29 μmol; catalogue number 31387; Fluka, Buchs, Switzerland) in 1 ml of distilled water, and then triethylamine (6.1 μl; 44 μmol) was added. The total activation time was 130 s. An aliquot (10 μl) of the mixture, corresponding to 2.86 nmol of Dex, was transferred to 0.4 ml of PBS (pH 7.4) containing 0.4 mg/ml EGF (26.5 nmol of EGF) to which 0.2 ml of a 1 M NaHCO₃ solution was added. After 8 min, 0.2 ml of dimethyl formamide containing 20 mg/ml SnCe₆(ED) (6.26 μmol) was added to make the photosensitizer react with the residual active sites in the carrier. The total coupling time was 4 h at room temperature. The preparation of Dex-SnCe₆(ED) was similar to the preparation of the EGF conjugate, except that the PBS used did not contain EGF.

After coupling, the conjugates were purified immediately on a Sephadex G-50 (Pharmacia) gel filtration column (1 × 40 cm). The column was equilibrated with PBS. The separation was monitored with a Pharmacia UV-1 280 (3). After coupling, the conjugates were collected (total volume, 16 ml) and concentrated by ultrafiltration (Centricon-3; Amicon, Beverly, MA) up to a volume of 0.5 ml.

Preparation of EGF-HSA-SnCe₆(ED) and HSA-SnCe₆(ED) Conjugates. The homobifunctional cross-linker glutaraldehyde (catalogue number G-7526; Sigma) was used to link the primary amino groups of HSA to those of SnCe₆(ED) and EGF (Fig. 1B). Therefore, 2 ml of PBS containing 5 mg/ml HSA (0.15 μmol; catalogue number A-8763; Sigma) were mixed with 0.55 ml of the mixture, corresponding to 2.86 nmol of Dex, was transferred to 0.4 ml of PBS (pH 7.4) containing 0.4 mg/ml EGF (26.5 nmol of EGF) to which 0.2 ml of a 1 M NaHCO₃ solution was added. After 8 min, 0.2 ml of dimethyl formamide containing 20 mg/ml SnCe₆(ED) (6.26 μmol) was added to make the photosensitizer react with the residual active sites in the carrier. The total coupling time was 4 h at room temperature. The preparation of Dex-SnCe₆(ED) was similar to the preparation of the EGF conjugate, except that the PBS used did not contain EGF.

After coupling, the conjugates were purified immediately on a Sephadex G-100 (Pharmacia) gel filtration column (1 × 40 cm). The column was equilibrated with PBS. The separation was monitored at 280 nm. Fractions (2 ml) were collected with a Pharmacia UV-1 280 (3). After coupling, the conjugates were collected (total volume, 4 ml) and concentrated by ultrafiltration (Centricon-3; Amicon, Beverly, MA) up to a volume of 0.5 ml.

Cell Culture. MDA-MB-468 (human breast adenocarcinoma) and A431 (human skin carcinoma) cells were obtained from the American Type Culture Collection (Manassas, VA). MDA-MB-468 cells were grown at 37°C in a humidified 100% air atmosphere in Leibovitz medium supplemented with nonessential amino acids (100×), penicillin (100 IU/ml), streptomycin (100 μg/ml), tylocine (60 μg/ml), amphotericin B (0.25 μg/ml), and 10% fetal bovine serum. A431 cells were cultured as described previously (29). Leibovitz medium, MEM, L-glutamine, nonessential amino acids, penicillin, streptomycin, tylocine, amphotericin B, fetal bovine serum, and PBS were obtained from Life Technologies, Inc. (Paisley, Scotland).

Light Source. Cultured cells were exposed to laser light (636 nm; corresponding to the absorption maximum of SnCe₆(ED)) emitted by a fiberoptic laser diffusing device connected to a rhodamine 6G dye laser (375B; Spectra-Physics, Mountain View, CA) pumped by a 4 W argon laser (Stableite 2071; Spectra-Physics). At the surface of the 96-well plate, the uniform fluence rate was 3 mW/cm², as measured with an IL 1400 radiometer (International Light, Newburyport, MA). During irradiation, the temperature never exceeded 25°C. This temperature did not influence the viability of the cells.

Receptor Binding Assay. Under strictly subdued light conditions, the ability of the EGF conjugates to compete with 125I-EGF (ICN, Costa Mesa, CA) for EGF receptor binding was assayed to determine their affinity to the EGF receptor, as described by Rousseau et al. (30). Briefly, A431 cells grown to confluency in 24-well plates (Costar, Cambridge, MA), were washed twice with 1 ml of prewarmed Hank’s balanced salt solution (HBSS). One ml of HBSS was then added, and the plates were cooled on ice for 20 min. At the start of the assay, the third wash of HBSS was removed, and 0.5 ml of binding medium (MEM supplemented with 0.2% BSA) containing 100 ng/ml 125I-EGF (9000 cpm/ng) and varying concentrations of nonradioactive EGF was added. In parallel experiments, nonradioactive EGF was replaced by EGF-Dex-SnCe₆(ED) or EGF-HSA-SnCe₆(ED). Plates were incubated for 2 h at 4°C, and then the cells were washed four times with 1 ml of ice-cold HBSS supplemented with 0.1% BSA. After the final wash, the contents of the well were solubilized by the addition of 1 ml of 1 N NaOH and incubation at 37°C for 1 h. Contents of each well were transferred into counting vials and counted with a Berthold BF5300 gamma counter (Berthold, Wildbad, Germany). Nonspecific binding was determined by preblocking binding sites with a 100-fold excess of unlabeled EGF.

Antiproliferative Assay. MDA-MB-468 cells were seeded onto transparent 96-well tissue culture plates (Costar) at 5 × 10⁴ cells/well and incubated for 16 h at 37°C. Under strictly subdued light conditions, the medium was replaced with fresh medium containing different concentrations of the photoactive compounds or vehicle, in the absence or presence of an excess of EGF (10 μg/ml or 60.4 μg/ml). Subsequently, the cells were incubated under dark conditions.
at 37°C for 4 h. The drug-containing medium was then replaced with drug-free medium under subdued light conditions, and the cells were immediately exposed to light (or not exposed to light) for 15 min (fluence rate, 3 mW/cm²). Afterward, the cells were incubated under dark conditions for 3 days. Cell proliferation was determined by quantification of the cellular protein content using naphthol blue-black (Acros, Geel, Belgium), as described by Palombarilla and Vilcek (31). The amount of dye was measured at 620 nm using a microtitrator plate reader (SLT; Gödide, Munich, Germany). After curve fitting using nonlinear regression (Prism, San Diego, CA), the IC₅₀ values were determined separately for each experiment. The average of the IC₅₀ values was calculated from four replicates.

Accumulation Assay. MDA-MB-468 cells were seeded onto black-sided, clear-bottomed, 96-well tissue culture plates (Costar) at 3 × 10⁴ cells/well and incubated for 16 h at 37°C. Under strictly subdued light conditions, the medium was then replaced with fresh medium containing 1 μM [expressed as a function of SnCe₆(ED) concentration] photo-active compounds or vehicle. In the case of EGF conjugates, incubations were carried out in the presence or absence of EGF (10 μM). After an incubation period of 4 h in the dark, the cells were washed four times with PBS and solubilized with 0.2% SDS solution. The fluorescence of the released SnCe₆(ED) was then measured by using a Biotek FL600 microplate fluorescence reader (Winooski, VT), and the concentration was calculated from a calibration curve. In parallel experiments, cells incubated under the same conditions were washed, trypsin was added, and the cell number was quantified using a Z1 Coulter Counter (Coulter, Luton, United Kingdom). The intracellular concentration was estimated assuming a mean volume of 3 μl for 10⁴ cells, as reported previously (32).

Visualization of Intracellular ROS Production. MDA-MB-468 cells were seeded onto Lab-Tek coverglass 8-chamber slides for tissue culture (25 × 56 mm; Nunc, Naperville, IL) at 3 × 10⁴ cells/chamber and incubated for 24 h at 37°C. Under strictly subdued light conditions, the medium was then replaced with fresh medium containing 1 μM [expressed as a function of SnCe₆(ED) concentration] of the photo-active compounds. In the case of EGF conjugates, incubations were carried out in the presence or absence of EGF (10 μM). After an incubation period of 4 h in the dark, the cells were selectively illuminated. After an incubation period of 4 h in the dark, the cells were washed three times under strictly subdued light conditions with PBS, followed by a 15-min incubation in the dark with PBS containing 10 μg/ml carboxy-H₂DCFDA (Molecular Probes, Eugene, OR), a fluorescent probe for detecting oxidative activity in cells (33). After irradiation of the cells with a light dose of 0.9 J/cm² (3 min, 500 mW/cm²), the cells were washed three times with PBS. The chambers were then mounted in a laser-scanning confocal fluorescence microscope (MRC-1024; Bio-Rad, Hertfordshire, United Kingdom) coupled to an inverted epifluorescence microscope with a chromatic aberration-free fluor (coherent Innova Enterprise laser, and the fluorescence was recorded at 530 nm.

RESULTS

Analysis of Conjugates. After purification and concentration of the Dex conjugates, the amount of EGF and SnCe₆(ED) covalently bound to Dex was measured. The ratio of EGF-Dex-SnCe₆(ED) was 3:1:4 as determined by the ratio of absorbances at 636 nm [SnCe₆(ED)] and 280 nm (EGF; Ultrospec 2000 spectrophotometer; Pharmacia) in three different batches. Purification of the HSA conjugates by gel filtration revealed that more than 95% of EGF-HSA-SnCe₆(ED) existed in a monomeric form. SDs-PAGE (Phastgel 10–15% Gradient; Pharmacia) showed one band of approximately 85 kDa as compared to a set of broad range (6.5–200 kDa) SDs-PAGE standards (Bio-Rad, Hercules, CA). The ratio of the EGF-HSA-SnCe₆(ED) conjugate was 3:1:3 (as found in three different batches), as determined by the ratio of absorbances at 636 nm [SnCe₆(ED)] and 280 nm. The absorbance at 280 nm, corrected for the absorbance of SnCe₆(ED) and the absorbance of the calculated amount of HSA present, was used to estimate the amount of EGF.

Receptor Binding Assay. To obtain a selective and efficient loading of cancer cells, preservation of receptor affinity is a prerequisite in the construction of ligand-based delivery systems. Therefore, the ability of the EGF conjugates to bind to the EGF receptor was assayed using A431 cells, which are widely used in EGF receptor binding studies (30, 34, 35). Approximately five times more EGF-HSA-SnCe₆ was needed to obtain 50% displacement of 125I-EGF as compared with EGF (Fig. 2). The highest concentration of EGF-HSA-SnCe₆(ED) (4 μg EGF/ml) was able to displace about 50% of the 125I-EGF binding. Conversely, the EGF-Dex-SnCe₆(ED) conjugate showed little affinity. Nonspecific binding of 125I-EGF was always less than 10%.

Antiproliferative Assay. Targeting SnCe₆(ED) through EGF receptor-mediated endocytosis should enhance its antiproliferative action in an EGF-dependent manner. This principle was examined by comparing the photocytotoxic activity of the compounds by means of an antiproliferative assay, using MDA-MB-468 cells overexpressing the EGF receptor (1–2 × 10⁶ receptors/cell). Preliminary experimental work showed that the cellular accumulation (see accumulation assay) of EGF-HSA-SnCe₆(ED) reached about 70% of the maximum level after 4 h. Therefore, the cells were incubated with the different compounds for 4 h, and then they were light-irradiated.

The nontargeting compounds SnCe₆(ED), Dex-SnCe₆(ED), and HSA-SnCe₆(ED) showed no antiproliferative action on MDA-MB-468 cells at a light dose of 27 kJ/m² (Fig. 3A). As one could anticipate from the receptor affinity of the targeting conjugates, EGF-HSA-SnCe₆(ED) displayed a potent photo-dependent antiproliferative effect (IC₅₀ value, 63 nM) whereas EGF-Dex-SnCe₆(ED) exhibited only very limited photocytotoxicity. When cells incubated with EGF-HSA-SnCe₆(ED) were simultaneously exposed to a competing concentration of EGF (10 μM), the photocytotoxic effect of the EGF conjugate was completely abolished (Fig. 3B). None of the tested compounds showed cytotoxicity in the dark on MDA-MB-468 cells (data not shown).

Accumulation Assay. The large differences in photocytotoxicity strongly suggested a dissimilar accumulation of the photo-active compounds in MDA-MB-468 cells. Therefore, the cellular accumulation of the photo-active compounds was investigated using MDA-MB-468 cells, which were incubated in the dark for 4 h with 1 μM of the compounds. In the case of EGF-HSA-SnCe₆(ED), the average intracellular concentration of SnCe₆(ED) was 38.9 ± 2.2 μM. Hence, the accumulation of EGF-HSA-SnCe₆(ED) was substantially higher than the cellular uptake of the other conjugates or free SnCe₆(ED) (Table 1). In the presence of a competing concentration of EGF, the accumulation of EGF-HSA-SnCe₆(ED) dropped dramatically, reaching a level similar to that seen for the other compounds. The cellular concentration of EGF-Dex-SnCe₆(ED) after a 4-h incubation was somewhat higher than the concentration of Dex-SnCe₆(ED) but 5-fold lower than the concentration of EGF-HSA-SnCe₆(ED). The cellular uptake of EGF-Dex-SnCe₆(ED) was also influenced by the presence of a competing concentration of EGF in the incubation medium.

Fig. 2. Displacement of 100 ng/ml (16.5 nM) 125I-EGF by different concentrations of EGF (○), EGF-Dex-SnCe₆(ED) ( ), or EGF-HSA-SnCe₆(ED) ( ) using A431 cells. Results are the mean ± SE of triplicate wells.
higher intracellular photosensitizer concentration could be obtained, thereby enhancing the cytotoxic effect after photo-activation. For the purpose of comparison, two water-soluble carriers of different chemical composition, i.e., a polysaccharide (Dex) and a protein (HSA), were used. Although the molecular masses of both structures are somewhat different (15–20 versus 68 kDa), both macromolecules became substituted with approximately the same amount of EGF and photosensitizer under the experimental conditions used (3:1:4 and 3:1:3 in the case of EGF-Dex-SnCe6(ED) and EGF-HSA-SnCe6(ED), respectively). It is probable that the use of a carrier is also essential for a potential in vivo activity of these compounds because the short human plasma half-life of EGF (1.5 min, Ref. 36) could be increased substantially by a protective effect of the carrier, as demonstrated for other ligands (37). By expressing a large number of EGF receptors, it is anticipated that liver cells in an in vivo condition will also tend to accumulate a large amount of EGF conjugate. However, hepatic toxicity is not necessarily to be expected because these PDT tools feature a dual specificity: they selectively accumulate in cells overexpressing the EGF receptor, but they only become cytotoxic when irradiated. Therefore, hepatic damage can simply be avoided by preventing light irradiation of the liver.

The data show that EGF incorporated in EGF-Dex-SnCe6(ED) had apparently lost most of its affinity for the EGF receptor, as evidenced by the receptor binding assay. The restricted affinity of EGF-Dex-SnCe6(ED) for the EGF receptor resulted in a poor cellular accumulation that proved to be too low for a significantly increased photocytotoxic effect over Dex-SnCe6(ED). Moreover, at the given light dose and incubation time, the photocytotoxicity, accumulation, and ROS generation capacity of EGF-Dex-SnCe6(ED) was similar to the corresponding profile of SnCe6(ED). Conversely, in case of EGF-HSA-SnCe6(ED), only a moderate decrease of affinity for the EGF receptor was seen. This characteristic resulted in a potent EGF-dependent photocytotoxicity (IC50, 63 nm). The actual IC50 value of EGF-HSA-SnCe6(ED) is therefore in the same range as that observed for other cytotoxic principles used on MDA-MB-468 cells, e.g., (E)-2′-deoxy-2′-(fluoromethylene)cytidine (IC50, 15–26 nm; Ref. 38) and paclitaxel (growth inhibition for concentrations ≥ 10 nm; Ref. 39). However, because the activity of a photosensitizer depends on the fluence used, it is stressed that the photocytotoxic effect of EGF-HSA-SnCe6(ED) observed in this study using a low fluence (27 kJ/m²) could be even further enhanced by using higher fluence rates and longer irradiation times.

The photodynamic effect of EGF-HSA-SnCe6(ED) was a direct result of an EGF receptor-dependent intracellular accumulation because all parameters investigated (photocytotoxicity, cellular accumulation, and ROS generation) could be dramatically affected when the compound was incubated together with an excess of competing EGF. HSA-SnCe6(ED) showed no improved characteristics over SnCe6(ED), an additional proof that the higher intracellular concentration could be obtained, thereby enhancing the cytotoxic effect after photo-activation. For the purpose of comparison, two water-soluble carriers of different chemical composition, i.e., a polysaccharide (Dex) and a protein (HSA), were used. Although the molecular masses of both structures are somewhat different (15–20 versus 68 kDa), both macromolecules became substituted with approximately the same amount of EGF and photosensitizer under the experimental conditions used (3:1:4 and 3:1:3 in the case of EGF-Dex-SnCe6(ED) and EGF-HSA-SnCe6(ED), respectively). It is probable that the use of a carrier is also essential for a potential in vivo activity of these compounds because the short human plasma half-life of EGF (1.5 min, Ref. 36) could be increased substantially by a protective effect of the carrier, as demonstrated for other ligands (37). By expressing a large number of EGF receptors, it is anticipated that liver cells in an in vivo condition will also tend to accumulate a large amount of EGF conjugate. However, hepatic toxicity is not necessarily to be expected because these PDT tools feature a dual specificity: they selectively accumulate in cells overexpressing the EGF receptor, but they only become cytotoxic when irradiated. Therefore, hepatic damage can simply be avoided by preventing light irradiation of the liver.

The data show that EGF incorporated in EGF-Dex-SnCe6(ED) had apparently lost most of its affinity for the EGF receptor, as evidenced by the receptor binding assay. The restricted affinity of EGF-Dex-SnCe6(ED) for the EGF receptor resulted in a poor cellular accumulation that proved to be too low for a significantly increased photocytotoxic effect over Dex-SnCe6(ED). Moreover, at the given light dose and incubation time, the photocytotoxicity, accumulation, and ROS generation capacity of EGF-Dex-SnCe6(ED) was similar to the corresponding profile of SnCe6(ED). Conversely, in case of EGF-HSA-SnCe6(ED), only a moderate decrease of affinity for the EGF receptor was seen. This characteristic resulted in a potent EGF-dependent photocytotoxicity (IC50, 63 nm). The actual IC50 value of EGF-HSA-SnCe6(ED) is therefore in the same range as that observed for other cytotoxic principles used on MDA-MB-468 cells, e.g., (E)-2′-deoxy-2′-(fluoromethylene)cytidine (IC50, 15–26 nm; Ref. 38) and paclitaxel (growth inhibition for concentrations ≥ 10 nm; Ref. 39). However, because the activity of a photosensitizer depends on the fluence used, it is stressed that the photocytotoxic effect of EGF-HSA-SnCe6(ED) observed in this study using a low fluence (27 kJ/m²) could be even further enhanced by using higher fluence rates and longer irradiation times.

The photodynamic effect of EGF-HSA-SnCe6(ED) was a direct result of an EGF receptor-dependent intracellular accumulation because all parameters investigated (photocytotoxicity, cellular accumulation, and ROS generation) could be dramatically affected when the compound was incubated together with an excess of competing EGF. HSA-SnCe6(ED) showed no improved characteristics over SnCe6(ED), an additional proof that the higher intracellular concentration, as seen in the case of EGF-HSA-SnCe6(ED), was due to an EGF-dependent mechanism. The combined data on photocytotoxicity

**DISCUSSION**

In the present study, carriers were used to increase the molar ratio between EGF and SnCe6(ED). It was anticipated that by doing so, a
and accumulation show that at least \(70.3 \times 10^6\) molecules SnCe\(_6\) (ED) must be delivered to each tumor cell (equal to 38.9 \(\mu\)m intracellular concentration) to reach a pronounced photocytotoxic effect when a light dose of 27 kJ/m\(^2\) is used. This accumulation was reached when cells were exposed to an extracellular concentration of 1 \(\mu\)m SnCe\(_6\) (ED) as present in EGF-HSA-SnCe\(_6\) (ED) (Fig. 3A; Table 1). EGF-Dex-SnCe\(_6\) (ED) showed a lower accumulation (12.8 \(\times\) 10\(^6\) molecules SnCe\(_6\) (ED)/cell), which was insufficient to produce cell death.

The presence of intracellular fluorescence due to the local conversion of nonfluorescent dichlorodihydrofluorescein into its oxidized fluorescent analogue, as revealed by confocal laser microscopy, implies the intracellular presence of the different compounds used. It is well known that on ligand binding, EGF/receptor complexes are rapidly clustering in coated pit areas of the cell surface and are further internalized in clathrin-coated vesicles. After uncoating, the vesicles are targeted to endosomes where EGF dissociates from its receptor. EGF is then delivered to the lysosomes, where it becomes degraded (40). At present, no information is available regarding the influence of the HSA-SnCe\(_6\) (ED) moiety on the complete EGF construct on its cellular internalization. However, the fact that all parameters investigated (photocytotoxicity, cellular accumulation, and ROS generation) could be dramatically influenced by competing EGF, combined with the finding that ROS were generated intracellularly, suggests that the molecule follows a regular ligand/receptor trafficking pattern.

Taken together, our data indicate that the intracellular accumulation of an EGF conjugate is affected extensively by the nature of the carrier and/or the conjugation chemistry used. In a previous work (29), we have shown that the intracellular accumulation of SnCe\(_6\) (ED) was not enhanced by the covalent linkage to Dex, as confirmed in this study. Of importance, HSA also showed the neutral behavior of Dex with respect to this aspect. As a matter of fact, our results showing intracellular ROS production after exposing the cells to the different photo-active compounds suggest a limited inhibitory effect of the carriers (without EGF substitution) on the cellular uptake of the photosensitizer. In contrast, other carriers (e.g., polyvinyl alcohol) dramatically influenced a nonselective accumulation of SnCe\(_6\) (ED), significantly compromising a selective receptor-mediated endocytosis (29). Although Dex therefore offers an advantage in an EGF receptor targeting strategy, our results clearly show that conjugation of the ligand to Dex dramatically affects the affinity of the EGF construct for its receptor. These findings are in line with the results of Andersson et al. (14), who found that EGF conjugated with Dex and \(^{131}\)I-tyrosine by means of CDAP chemistry (as used in this study) showed a substantial loss of affinity for the EGF receptor as well. The reason why the ligand affinity for the EGF receptor became impaired on coupling with Dex is not clear at present. The low affinity of the EGF-Dex conjugate could be ascribed to a deleterious effect of Dex on the conformation of EGF by interactive forces, especially because EGF and the carrier are positioned closely. The lipophilic photosensitizer bound to Dex could have a similar effect, and a harmful effect of the cyanylating agent CDAP on specific amino acids (e.g., Ser and Thr) present in EGF-Dex (41) present in EGF cannot be completely ruled out. However, the latter situation is unlikely because excess CDAP, which is not consumed in the activation of Dex, is rapidly hydrolyzed at the high alkaline pH used in the step before adding EGF (41). A simple steric hindrance by Dex could also explain the pronounced low affinity. Significantly, the CDAP method introduces only one extra carbon atom between Dex and the NH\(_2\) terminus of EGF, whereas glutaraldehyde introduces a bridge of five carbon atoms. Although the molecular mass of HSA
is about 4-fold higher than the average mass of Dex molecules, it is inferred that the spacer could dramatically improve the binding ability of the ligand to its receptor. It is likely that an appropriate spacer also lowers the destructive interaction between EGF and the carrier or photosensitizer. Future works exploring the impact of spacers of different sizes on the affinity of EGF constructs for the EGF receptor might therefore lead to the development of improved EGF-mediated targeting of photosensitizers.

In conclusion, our results demonstrate that a photosensitizer linked to an appropriate carrier such as HSA covalently bound to EGF can efficiently photosensitize cancer cells overexpressing the EGF receptor in a selective EGF-dependent manner. The present EGF-HSA-SnC_5E_6(ED) carries three SnC_5E_6(ED) molecules per albumin. It is assumed that by altering the chemical conditions used, an increased ratio of photosensitizer:albumin could be obtained. This would further improve the photocytotoxic potency of the compounds. Another possibility includes the use of carriers that are easier to modify to reach a higher photosensitizer:carrier ratio. For this purpose, starburst dendrimers seem to be suitable carriers because they have been used successfully to attach a high number of ^10 B atoms to EGF for neutron capture therapy (15). It is believed that further investigation of these constructs could be very promising for a selective in vivo PDT of cells overexpressing the EGF receptor.

REFERENCES