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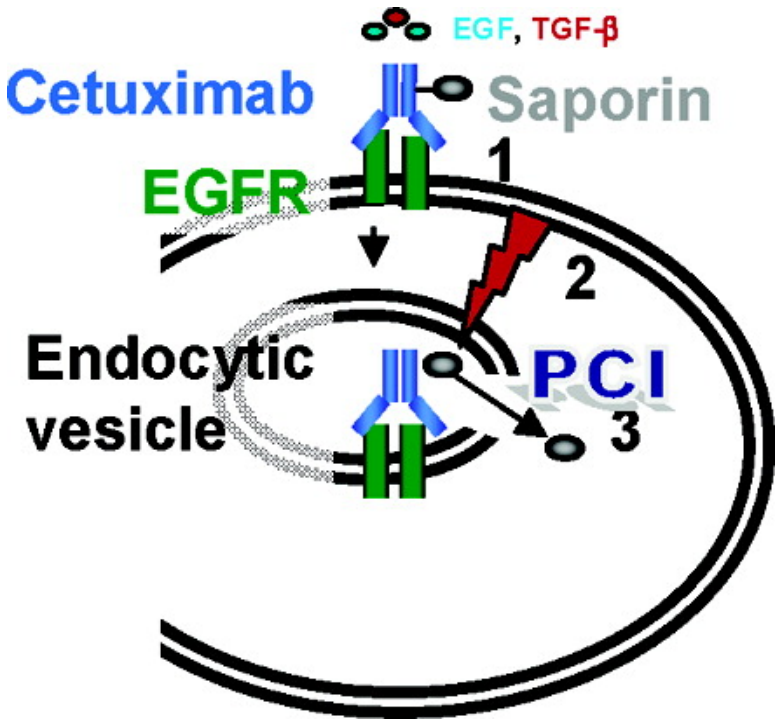

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Targeted Delivery and Enhanced Cytotoxicity of Cetuximab−Saporin by Photochemical Internalization in EGFR-Positive Cancer Cells

Wai Lam Yip,[†] Anette Weyergang,[†] Kristian Berg,[†] Hanne H. Tønnesen,[‡] and Pål K. Selbo*,[†]

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Abstract: Photochemical internalization (PCI) is a novel technology of macromolecular delivery. By PCI, endocytosed membrane-impermeable therapeutic drugs are photochemically released from entrapment in endo-lysosomal compartments to the cytosol of target cells. In the present report, we describe the *in vitro* proof-of-concept for PCI of cetuximab−saporin, an immunotoxin targeting EGFR-expressing cells. This immunotoxin consists of the chimeric murine-human IgG_1 monoclonal antibody cetuximab (C225 or Erbitux) bound to the type I ribosome-inactivating protein toxin saporin by a biotin-streptavidin linkage. The photochemical treatment enhanced the cytotoxicity of the immunotoxin in a synergistic manner in three different EGFR-expressing carcinoma cell lines derived from different tumor tissues (colorectal, HCT-116; prostate, DU-145; and epidermis, A-431). Both cytotoxicity of cetuximab−saporin and epifluorescence of Alexa488−cetuximab were evaluated by competition with cetuximab demonstrating specific binding and uptake of cetuximab−saporin in EGFR positive cells. In the EGFR-negative uterine sarcoma cell line MES-SA, neither binding nor preferential accumulation of Alexa488−cetuximab was detected. In addition, PCI enhanced the cytotoxicity of cetuximab−saporin to the same extent as streptavidin−saporin in the MES-SA cells. In conclusion, PCI enhances selectivity of the cytotoxicity of the immunotoxin cetuximab−saporin in EGFR-expressing cells.

Keywords: Photochemical internalization; photodynamic therapy; drug delivery; immunotoxin; EGFR; saporin; drug targeting; cetuximab

Introduction

The utilization of macromolecules in the therapy of cancer and other diseases is becoming increasingly important. Recent advances in molecular biology and biotechnology have made it possible to improve targeting and design of cytotoxic agents, DNA complexes, and other macromolecules for clinical applications. In many cases the targets of macromolecular therapeutics are intracellular. However, degradation of macromolecules in endocytic vesicles after uptake by endocytosis is a major intracellular barrier for the therapeutic application of macromolecules having intracellular targets of action.

Photochemical internalization (PCI) is a novel technology for the release of endocytosed macromolecules into the cytosol. The technology is based on the activation by light of photosensitizers (PSs) located in endocytic vesicles to induce the release of macromolecules from the endocytic vesicles.[1−3] Thereby, endocytosed molecules can be released to reach their target of action before being degraded in

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lysosomes. PCI has been shown to stimulate intracellular delivery of a large variety of macromolecules and other molecules that do not readily penetrate the plasma membrane, including type I ribosome-inactivating proteins (RIPs), DNA delivered as gene-encoding plasmids or by means of adenovirus or adeno-associated virus, peptide nucleic acids (PNAs) and chemotherapeutic agents such as bleomycin and in some cases doxorubicin. The efficacy and specificity of PCI of macromolecular therapeutics has been improved by combining the macromolecules with targeting moieties, such as the epidermal growth factor.[4] In general, PCI can induce efficient light-directed delivery of macromolecules into the cytosol, indicating that it may have a variety of useful applications for site-specific drug delivery as for example in gene therapy, vaccination, and cancer treatment. Recently, we demonstrated that PCI of the affinity toxin EGF−saporin, using mouse EGF, resulted in enhanced cytotoxicity in two EGFR-positive cancer cell lines.[5] EGF−saporin was efficiently endocytosed, delivery was specific, and the combination between the affinity toxin and photochemical activation of the PS (PCI of the affinity toxin) induced synergistic antiproliferative effects.

In the present study, we wanted to further exploit and improve the concept of PCI-induced drug delivery using an immunotoxin targeting EGFR. The therapeutically approved cetuximab (also known as C225 or Erbitux), which is a chimeric humanized murine mAb recognizing the epidermal growth factor receptor (EGFR),[6] was linked to the type I ribosome-inactivating protein saporin.[7] PCI of cetuximab−saporin was established as a proof-of-concept in three different EGFR-positive human cancer cell lines. In addition, an EGFR-negative cancer cell line was utilized as control. The results reported here indicate that cetuximab−saporin binds specifically to EGFR-positive cells and is subsequently taken up by endocytosis. PCI significantly enhances the cytosolic delivery and the cytotoxicity of cetuximab−saporin. PCI of cetuximab−saporin is a unique combination strategy encompassing three different ways of cell inactivation: (1) receptor blockage and internalization by cetuximab; (2) cytotoxic effect of the photochemical treatment; (3) the RIP activity of saporin.

Experimental Section

Cell Lines and Culture Conditions. All cell lines were purchased from ATCC (Manassas, VA). Three human cancer cell lines expressing EGFR were used: HCT-116 colorectal carcinoma (ATCC No. CCL-247), DU-145 prostate carcinoma (ATCC No. HTB-81), and A-431 epidermoid carcinoma of the skin (ATCC No. CRL-1555). Additionally, the EGFR negative human uterus sarcoma MES-SA cell line (ATCC No. CRL-1976) was selected as control cells. HCT-116 and DU-145 cells were maintained in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO). MES-SA cells were cultured in McCoy's medium (Sigma-Aldrich) while A-431 cells were cultured in DMEM (Bio Whittaker Europe, Velviers, Belgium). All media were supplemented with 10% bovine calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin (Sigma-Aldrich), and 2 mM glutamine (Sigma-Aldrich). All cell lines were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and subcultured three times a week.

Drugs and Chemicals. The PS LumiTrans ($TPPS_{2a}$, mesotetraphenylporphine with two sulfonate groups on adjacent phenyl rings) was a generous gift from PCI Biotech AS (Oslo, Norway). A stock solution of 0.35 mg/mL was kept at −20 °C. All work with $TPPS_{2a}$ was carried out under subdued light. Streptavidin−saporin was purchased from Advanced Targeting Systems (San Diego, CA). The streptavidin−saporin conjugate contains on average two saporin molecules per streptavidin molecule. Biotinylation kit was obtained from Pierce (Rockford, IL). Cetuximab (C225) was purchased from E. Merck AB (Stockholm, Sweden), and the solution of 2 mg/mL was kept at 4 °C. Tyrphostin AG-1478 (Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) to yield a stock solution of 50 mM.

Light Source. Illuminations of cells were performed by using LumiSource (PCI Biotech, Oslo, Norway). This lamp consists of four standard light tubes (18 W/tube, Osram L 18/67), which emit blue light with a main peak at approximately 435 nm. The irradiance varies less than 10% across the whole illumination area (765 cm^2) with an output of 11 mW/cm^2. The light box is air-cooled during light exposure, preventing cells from being exposed to hyperthermia, and keeps the irradiance stable.

Preparation of Cetuximab−Saporin. Cetuximab was biotinylated according to the manufacturer's instructions (Pierce Biotechnology Inc, Rockford, IL). Briefly, a solution of 2 mL of 2 mg/mL cetuximab was mixed with 100 μL of 1 M $NaHCO_3$ to adjust the mAb solution to pH ∼8. Cetuximab was then mixed with 54 μL of 10 mM NHS-

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biotin that was made in 99.9% dimethyl sulfoxide (DMSO from Aldrich, Stenheim, Germany) immediately before, and then mixed at room temperature for 30 min. Sephadex G-25 medium desalting columns (Amersham Biosciences, Amershamplace, U.K.) were used to remove free NHS-biotin from the biotin−cetuximab solution. The concentration of cetuximab−biotin was measured using the DC protein assay kit 2 (BioRad, Laboratories, Philadelphia, PA), and verified spectrophotometrically at 280 nm. It was assumed that all cetuximab was biotinylated. Then, a solution of 10 μM biotinylated cetuximab was mixed with a stock solution of streptavidin−saporin at a molar ratio cetuximab−biotin: streptavidin−saporin of 4:1 for 15 min at room temperature. Due to the very high affinity constant of the binding of streptavidin to biotin (10^{15} M^{-1}),[8] the linking reaction was assumed complete. The immunotoxin solution was then diluted with sterile PBS (pH 7.0) to a concentration of 300 nM and aliquoted and stored at −80 °C.

Photochemical Treatments. Cells were harvested with 500 μg/L trypsin and 200 mg/mL EDTA and seeded out at 3000 cells per well in 96-well plates (Nunclon, Nunc, Roskilde, Denmark) for the MTT assay (as described below) or 1000 cells/well in 6-well plates (Nunclon) for colony-forming ability experiments. Cells were allowed to attach to the bottom of the wells for 24 h prior to the start of experiments. HCT-116 and DU-145 cells were then either incubated with 0.2 μg/mL $TPPS_{2a}$ for PDT only or, for the PCI experiments, coincubated with increasing concentrations of cetuximab−saporin, streptavidin−saporin, or cetuximab for 18 h, unless otherwise stated. For studies with A-431 cells, 0.1 μg/mL $TPPS_{2a}$ was used, since the cell line has higher sensitivity to $TPPS_{2a}$-PDT than the other cell lines used in the present study. The monolayers were then washed twice with drug-free culture medium before it was replaced with fresh drug-free medium and chased for 4 h. Cells were subsequently exposed to increasing doses of LumiSource light. This PCI protocol is referred to as "light after" PCI. Alternatively, "light first" PCI strategy was performed. In these experiments, the cells were first subjected to PDT as described above, and immediately after light exposure cells were incubated with the immunotoxin for 18 h before the cells were washed twice and, subsequently, incubated overnight followed by the MTT viability assay, which was performed 48 h post light exposure (as described below).

Cytotoxicity Assays. The cell viability was measured by the MTT assay 48 h after light exposure, or alternatively, the colony-forming ability of the cells was assessed 7−10 days after light exposure as recently described.[9]

Analysis of EGFR and Phosphorylated EGFR. HCT-116 cells were seeded out in Falcon 3003 dishes (10×10^3 cells/cm^2) overnight. Cells were then incubated with 100 nM cetuximab for 18 h (day 2) or 2 h (day 3) prior to treatment with 100 ng/mL EGF for 2 min before the monolayers were washed once with ice-cold PBS and kept on ice. PBS was aspirated, and 100 μL of lysis buffer containing 62.5 mM Tris-HCl pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% bromphenol blue, 10 μL/mL Protease Inhibitor Cocktail (Sigma-Aldrich), 10 μL/mL Phosphatase Inhibitor Cocktail 1 (Sigma-Aldrich), 10 μL/mL Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich), 1 mM PMSF, 1 mM $NaVO_4$, 1 mM NaF, and 20 mM β-glycerolphosphate was added to the cells. The cell lysates were collected and transferred to 1.8 mL plastic tubes (Eppendorf, Hamburg, Germany). Proteins were then subjected to SDS−PAGE and Western blot followed by antibody-based analysis of total and phosphorylated (p) EGFR as described previously.[5] Primary mouse Ab (Cat. No. 2236) against human p-EGFR and rabbit total EGFR Ab (Cat. No. 2232) was obtained from Cell Signaling Technology (Danvers, MA) and diluted 1:1000 before incubation of secondary HRP conjugated donkey-anti-rabbit Ab (Cat. No. W401B) that was purchased from Promega (Madison, WI) or HRP conjugated sheep-anti mouse (Cat. No. Na 931) from Amersham Biosciences, both diluted 1:5000 prior to incubation of the membrane. Enhanced chemiluminescence (ECL+; Amersham) was used for signal detection.

Preparation and Validation of Alexa488−Cetuximab. Cetuximab was labeled with Alexa Fluor 488 (~0.9 kDa) according to the instructions of the manufacturer (Molecular Probes, Eugene, OR). The Ab−dye conjugate is hereby named Alexa488−cetuximab. Ten micrograms of Alexa488−cetuximab or unconjugated cetuximab was subjected to 4−15% linear gradient SDS−PAGE. Fluorescence intensity of the dye conjugate was detected using the Storm 860 scanner (Amersham/GE Health, London, U.K.). Thereafter, the gel was stained with Coomassie blue for comparing the protein band of Alexa488−cetuximab with cetuximab. Alexa488 labeled goat-anti-mouse mAb (Molecular Probes) were used as a negative control not targeting EGFR.

Fluorescence Microscopy. For cetuximab uptake studies, cells were seeded out in Falcon 3001 dishes (50×10^3 cells per dish, $\sim 5 \times 10^3$ cells/cm^2) overnight. The cells were incubated with 100 nM Alexa488−cetuximab, without or in the presence of 2 μM cetuximab, for 18 h. Then the cells were washed and incubated in drug-free medium for 4 h. The cells were examined by a Zeiss Axioplan epifluorescence and phase contrast microscope equipped with a 63× oil-immersion objective (Zeiss, Oberkochen, Germany). The images were acquired by utilizing a cooled charge-coupled device (CCD) camera (Astromed 3200, Astromed, Cambridge, U.K.). A 450−490 nm band-pass excitation filter, a beam splitter at 510 nm, and a 510−550 nm emission filter were used for the recording of Alexa 488 fluorescence (micrographs were all set with the same sensitivity range). Fluorescence of $TPPS_{2a}$ was recorded using a 395−440 nm band-pass excitation filter, a 460 nm dichroic beam splitter, and a 610 nm long-pass filter. The AnalySIS[D]

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Soft Imaging System (Olympus, Hamburg, Germany) was utilized to process and analyze the digital images.

Results and Discussion

Overexpression of EGFR is a hallmark of numerous cancers and has become a major target for drug delivery and therapy. Despite the anticancer effects of the monoclonal antibody cetuximab and the small molecule tyrosine kinase inhibitors (TKI) gefitinib and erlotinib in clinical trials, they do not appear to be curative as monotherapies. However, improved antitumor effects are obtained when such EGFR-targeting drugs are combined with conventional anticancer drugs[10] or radiotherapy.[11] Therefore, development of more potent treatment strategies is highly needed.

In the present work we aimed to develop and investigate the concept of targeting EGFR-positive cancer cells with the model immunotoxin cetuximab−saporin using PCI as drug delivery method. PCI is based on the principles of photodynamic therapy (PDT), which is approved worldwide for different cancer indications.[12,13] PDT is dependent on three components, all of which are crucial to accomplish successful therapeutic outcome: (1) *Light* of wavelengths that correspond to the absorption properties of a (2) *photosensitizer* (PS). After light activation, the PS utilizes the absorbed energy in (3) *oxygen* dependent reactions to form reactive oxygen species (ROS) of which singlet oxygen ($^1O_2$) has been characterized as the most important.[14] For PCI it is necessary to use a PS which localizes in endo-lysosomal compartments.[1,2]

Selection of EGFR-Positive and EGFR-Negative Control Cells. To demonstrate a general applicability of PCI of EGFR-targeting immunotoxins we used three different human carcinoma cell lines derived from different tumor tissues: (1) HCT-116, colon carcinoma cells; (2) DU-145, prostate carcinoma cells; and (3) A431, epithelial carcinoma cells, all previously shown to be EGFR positive.[5,15−17] The sarcoma cell line MES-SA was included as an EGFR-negative control cell line.[5] As can be seen by Western blots in Figure 1A, the MES-SA cell line was found EGFR negative compared to the EGFR-positive HCT-116 cells.

Selective EGFR Targeting and Cellular Uptake of Cetuximab. Several control experiments to document EGFR selectivity, cellular uptake, and biological function of cetuximab were carried out prior to the PCI experiments. Specific EGFR targeting and cellular uptake of cetuximab was evaluated by labeling with Alexa Fluor 488. Dye labeling of cetuximab was confirmed by fluorescence scanning of the gel after SDS−PAGE as described in the Experimental Section (Figure 1B). Coomassie blue staining of the gel also revealed that the protein band of Alexa488−cetuximab was of approximately the same size as of unconjugated cetuximab. Selective binding and uptake studies of Alexa488−cetuximab were performed using the EGFR-positive HCT-116 cell line and the EGFR-negative MES-SA as a control cell line (Figure 1C−G,I−K). In Figure 1C a weak, diffuse green fluorescence was observed most likely on the plasma membrane of the HCT-116 cells after incubation with 100 nM Alexa488−cetuximab for 30 min at 4 °C. Cells that were further incubated for 3 h in drug-free medium at 37 °C contained a combination of a somewhat stronger diffuse as well as a granular fluorescence, that were most likely due to localization of Alexa488−cetuximab in endocytic vesicles (Figure 1D). Utilizing the same sensitivity range set by the imaging software, HCT-116 cells that were not incubated with Alexa488−cetuximab did not show any autofluorescence (Figure 1E). HCT-116 cells that were incubated with Alexa488−cetuximab for 18 h at 37 °C followed by a chase in drug-free medium for 4 h obtained a bright granular perinuclear fluorescence in addition to a weak diffuse fluorescence, indicating that the dye conjugate was taken up by the cells by endocytosis (Figure 1F). Coincubating the cells with 100 nM Alexa488−cetuximab and 2 μM cetuximab for 18 h, followed by a 4 h chase in drug-free medium, resulted in a strong reduction of fluorescence as can be seen in Figure 1G indicating the EGFR-targeting properties of the dye conjugate in HCT 116 cells. The weak fluorescence observed in Figure 1G is assumed to be due to pinocytosed Alexa488−cetuximab.

Endo-lysosomal localization of the PS is necessary to achieve photochemical rupture of the vesicle membrane and thereby cytosolic release of the immunotoxin. As can be seen in Figure 1H, the intracellular fluorescence of $TPPS_{2a}$ after 18 h of incubation and 4 h chase in drug-free medium was granular and resembled the pattern of typically endocytic vesicle-localizing PSs as previously reported.[2]

EGFR-negative MES-SA cells incubated with Alexa488−cetuximab at 4 °C for 30 min resulted in no observable green fluorescence signal, indicating no unspecific plasma mem-

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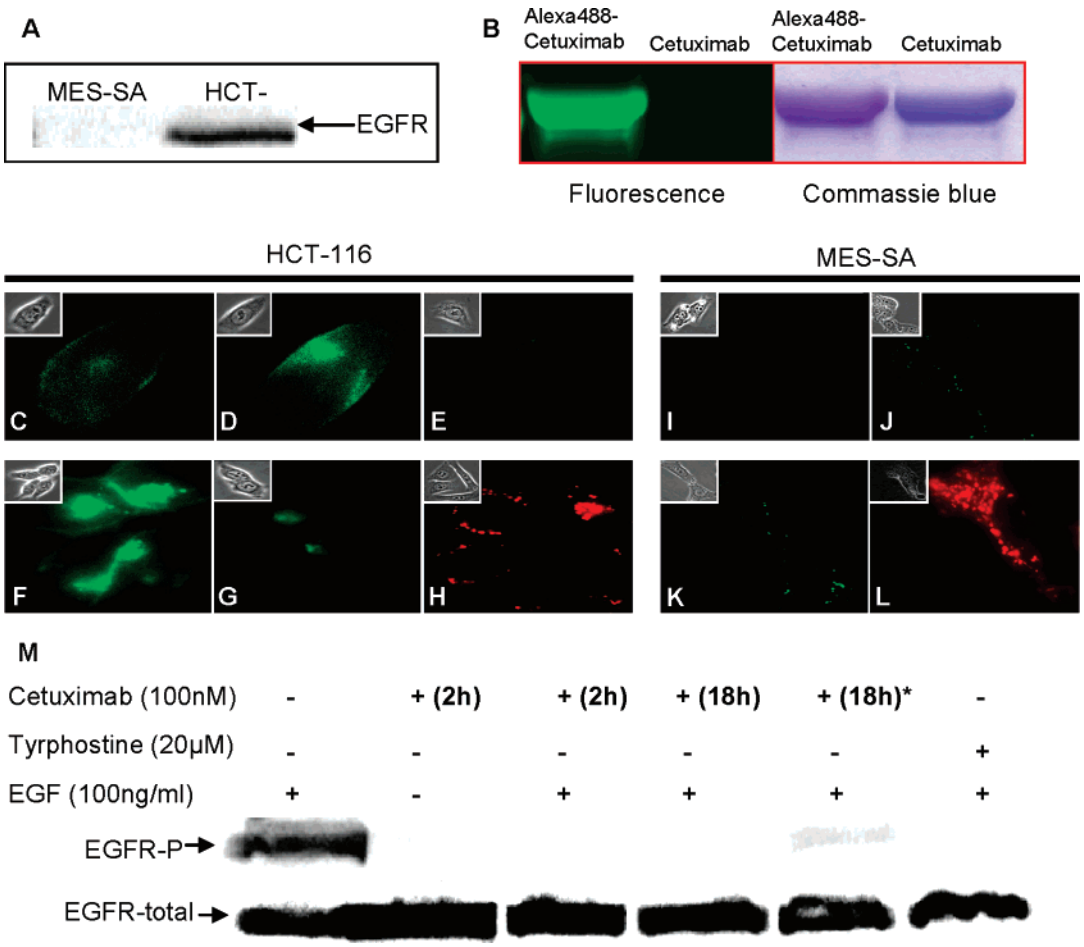

Figure 1. Characterization of cellular binding, uptake, and effect of cetuximab. (A) EGFR expression of HCT-116 and MES-SA cells measured by antibody staining of Western blots. Ponceu staining of membranes confirmed even protein loading (not shown). (B) SDS−PAGE of Alexa488−cetuximab. Quality of labeling was confirmed by using both fluorescence scanning and Coomassie blue staining. (C−H) Fluorescence microscopy of HCT-116 incubated with 100 nM Alexa488−cetuximab. The cells were analyzed after 30 min on ice (C); 30 min on ice, then 3 h chase in drug-free medium for at 37 °C (D); no treatment (E); 18 h incubation at 37 °C, then 4 h chase in drug-free medium at 37 °C (F) or as in F but Alexa488−cetuximab coincubated with 2 μM cetuximab (G). In panel H, HCT-116 cells were incubated with 1 μg/mL $TPPS_{2a}$ for 18 h, then 4 h chase in drug-free medium at 37 °C. (I−L) MES-SA cells were incubated with 100 nM Alexa488−cetuximab after 30 min on ice (I), with Alexa488−cetuximab for 18 h followed by a 4 h chase at 37 °C (J), or as in panel J, but coincubated with 2 μM cetuximab (K). (L) The cells were incubated with 1 μg/mL $TPPS_{2a}$ for 18 h followed by a 4 h chase in drug-free medium at 37 °C. (M) Influence of cetuximab and TKI on EGF induced Tyr1068 phosphorylation of EGFR in HCT-116 cells. One hundred ng/mL EGF was used to activate EGFR when indicated in the figure. The cells were incubated with 100 nM cetuximab for the indicated time and subsequently isolated for analysis or chased in drug-free medium for 4 h before analysis ((18h)*). As a control, 1 h incubation of 20 μM tyrphostin AG1478 was used to block EGF-induced activation of EGFR (lane 6).

brane binding of cetuximab (Figure 1I). MES-SA cells incubated with Alexa488−cetuximab for 18 h, followed by 4 h chase in drug-free medium, showed only a weak granular fluorescence (Figure 1J). The same fluorescence pattern and intensity were observed in MES-SA cells coincubated with Alexa488−cetuximab and 20-fold excess of cetuximab (2 μM) (Figure 1K), indicating that the weak granular fluorescence signal is due to non-receptor mediated endocytosis. In addition, an Alexa488 labeled goat-anti-mouse mAb was included as a non EGFR-targeting control mAb in the HCT-116 cells. There were no differences in the fluorescence intensity or fluorescence pattern between HCT-116 cells incubated with Alexa488 labeled goat-anti-mouse mAb and Alexa488−cetuximab and 20-fold excess of cetuximab (data not shown). The fluorescence pattern of Alexa488 labeled goat-anti-mouse mAb in HCT-116 cells was weak and granular resembling the fluorescence of Alexa488−cetuximab in MES-SA cells, also indicating that mAbs without targeting capabilities are taken up by unspecific endocytosis.

The observations described above demonstrate that cetuximab selectively binds to EGFR and is taken up by receptor-mediated endocytosis in HCT 116 cells. Additionally, the results indicate that cetuximab to a lower extent is taken up by unspecific endocytosis. Unspecific uptake of cetuximab−saporin in normal cells could therefore be a potential limitation causing unspecific toxicity. However, it should be

noted that PCI causes additional specificity by the preferential accumulation of PS in the neoplastic lesions[14] as well as the site-specific light exposure which is required to stimulate PS-mediated IT activation. PCI may therefore reduce the dosage of cancer therapeutic drugs needed.

Inhibition of EGFR Phosphorylation by Cetuximab. Before construction of the immunotoxin we evaluated the ability of cetuximab to antagonize EGF-stimulated activation of EGFR in the HCT-116 cells. As can be seen in Figure 1M, the phosphorylation of Tyr1068 of EGFR by 100 ng/mL (16.7 nM) exogenous EGF is completely inhibited by 100 nM cetuximab administered 2 h or 18 h prior to the EGF treatment. After removal of cetuximab followed by 4 h incubation in drug-free medium, a weak reactivation of EGFR by exogenous EGF was observed. As a control we used tyrphostine AG1478, a selective EGFR TKI, to inhibit phosphorylation of EGFR (p-EGFR). It was found that a 1 h incubation with 20 μM tyrphostine completely blocked EGF-mediated phosphorylation of EGFR. The Western blot data confirmed that cetuximab exerts EGFR-targeting properties in the HCT-116 cells.

PDT in Combination with Cetuximab. The cytotoxicity induced by PDT (PS + light) in HCT-116 cells is shown in Figure 2A. LD_{50} was achieved with ~100 s of light exposure after 18 h of $TPPS_{2a}$ incubation and 4 h of chase in drug-free medium, and was selected as the light dose for treatment of HCT-116 cells if not otherwise described. The cellular cytotoxicity of nonconjugated cetuximab incubated for 72 h was evaluated, either as monotherapy or in combination with PDT. As can be seen in Figure 2B, only subtoxic responses were achieved when treating HCT-116 cells for 72 h with up to 100 nM of cetuximab alone, and only ~10% decrease in viability was observed at the highest concentration of cetuximab. This is in accordance with recent findings reporting that cetuximab as monotherapy of HCT-116 cells results in poor growth inhibition control *in vitro*.[18] In addition, cetuximab alone failed to show any antitumor activity in 7 out of 11 xenografts with EGFR-positive tumor cells, including HCT-116 xenografts.[19]

$TPPS_{2a}$-PDT was combined with cetuximab alone to mimic a PCI protocol. Thus, after 18 h coincubation of $TPPS_{2a}$ and cetuximab, the cells were washed and chased for 4 h in drug-free medium before exposure to light. In these experiments no enhanced cytotoxicity was observed with increasing doses of cetuximab (Figure 2B). It should be noted, as shown in Figure 1M, that EGF activates EGFR of HCT-116 cells 4 h after cetuximab is removed from the medium. Thus, for local control of tumor cells, it is important to inactivate all cells after a single treatment with PDT combined with cetuximab to prevent reactivation of EGFR and regrowth of malignant cells; otherwise repeated treatments are necessary.

Interestingly, del Carmen and co-workers have demonstrated the rational use of a combination of PDT and repeated

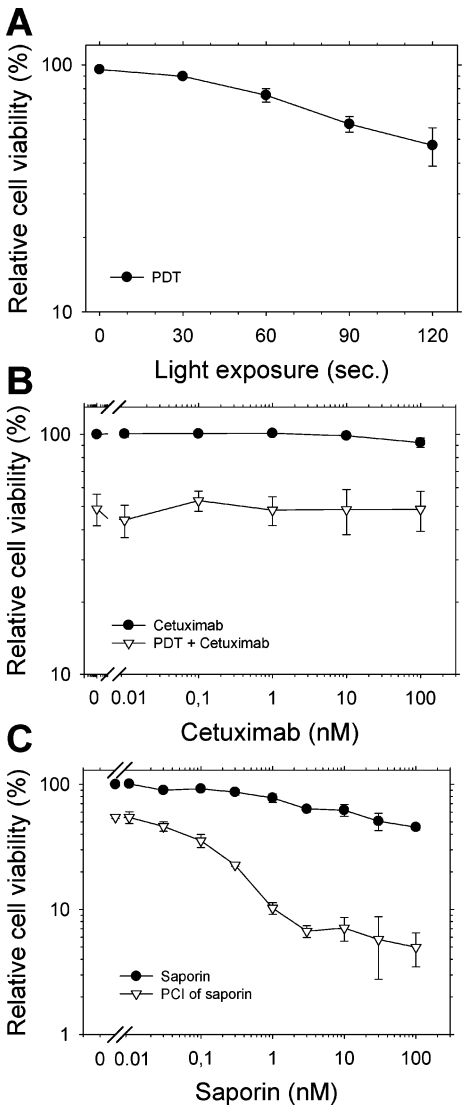

Figure 2. Cytotoxic effects of PDT and PCI of saporin. Relative viability of HCT-116 cells treated with (A) PDT ($TPPS_{2a}$ + increasing light doses), (B) PDT with a fixed light dose (100 s) and increasing concentrations of cetuximab or cetuximab alone, and (C) PCI of saporin. The cells were treated with PDT with a fixed light dose (100 s) and increasing concentrations of saporin or saporin without light exposure. The cells were treated with 0.2 μg/mL $TPPS_{2a}$ and as otherwise described in the Experimental Section. Results are the average of three samples from at least two independent experiments. Cell viability was assessed by the MTT assay 48 h post light exposure, and the values were normalized relative to untreated cells. Bars are standard error (SE).

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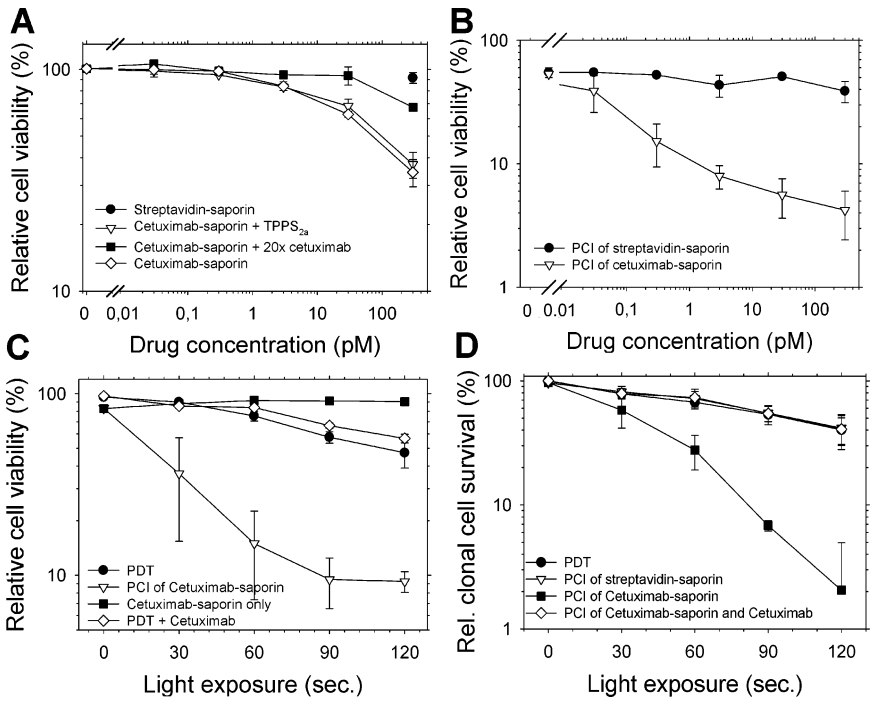

Figure 3. Cytotoxic effects of PCI of cetuximab−saporin in HCT-116 cells. (A) Toxin conjugates incubated with or without $TPPS_{2a}$ or cetuximab in the absence of light exposure as indicated in the figure. (B) PCI of toxin conjugates at different concentrations and a PDT dose demonstrated to kill ∼45% of the cells. (C) Light dose-dependent PDT, PCI of 3 pM cetuximab−saporin, 3 pM cetuximab−saporin only, or PDT combined with 3 pM cetuximab. (D) Relative clonal cell survival of HCT-116 cells treated with PDT at different light doses, PCI of 3 pM streptavidin−saporin, PCI of 3 pM cetuximab−saporin, or PCI of 3 pM cetuximab−saporin combination in the presence of 6 nM cetuximab. Results are the average of three samples from at least two independent experiments. Cytotoxicity as assessed by the MTT assay 48 h post light exposure or by the clonal cell survival assay assessed 10−14 days post light exposure. Values were normalized to nontreated cells; bars, SE.

injections of cetuximab, using the PS benzoporphyrin derivative monoacid A (BPD-MA), which induced a synergistic growth inhibition of advanced and recurrent epithelial ovarian cancer cells *in vivo*.[20] However, in that study cetuximab was delivered 4 times with several days between the injections. Recently, it was demonstrated in a multinational, randomized study that radiotherapy combined with cetuximab enhanced locoregional control and reduced mortality of patients with advanced head and neck cancer,[11] demonstrating the strength of combination treatments.

The effect of saporin alone and PCI of saporin was evaluated in the HCT-116 cells. It has previously been shown that PCI of saporin induces synergistic cytotoxicity *in vitro*.[1] Accordingly, it was found in the HCT 116 cells that a combination of a photochemical treatment and saporin (30 kDa), both inducing a 50% reduction in viability when given separately, caused 95% reduction of cell viability when given together (Figure 2C).

PCI of Cetuximab−Saporin: Cytotoxic Effects in EGFR-Positive Cells. In view of the fact that saporin alone also has a low cytotoxic potential toward whole cells and animals,[5,7] cetuximab was linked to saporin to improve the cellular uptake and specificity. In addition, we used PCI of cetuximab−saporin to increase the cytosolic delivery of the toxin. The IT was formed on the basis of a streptavidin−biotin linkage as described in the Experimental Section. To demonstrate a general applicability of the present IT-based EGFR-targeting strategy, PCI of cetuximab−saporin was performed in three different EGFR-positive cancer cell lines of different tissue origin. The toxicity of streptavidin−saporin and cetuximab−saporin in combination with $TPPS_{2a}$ in subdued light was evaluated in the HCT-116 cells (Figure 3A). The cytotoxicity of cetuximab−saporin (300 pM) in HCT-116 cells was not influenced by the presence of $TPPS_{2a}$ in the absence of light exposure ($P > 0.05$). The cytotoxic effect of the IT cetuximab−saporin was reduced by a 20-fold excess of cetuximab, demonstrating the specificity of the IT. Only a subtoxic response was achieved after incubating the HCT-116 cells with 300 pM streptavidin−saporin. The same pattern of cytotoxicity from these compounds as seen in the HCT-116 cells was also observed in the DU-145 and A431 cell lines (data not shown). The enhanced cytotoxicity of cetuximab−saporin as compared to streptavidin−saporin indicates increased uptake and relocalization of saporin to the cytosol of the cells.

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The impact of PCI of the immunotoxin cetuximab−saporin on cell viability was evaluated in the HCT-116 cells and compared to the treatment of cells that were challenged with PCI of streptavidin−saporin (Figure 3B). As can be seen in the figure, an immunotoxin concentration-dependent cytotoxic response was accomplished with PCI of the immunotoxin, while hardly any cytotoxicity was observed in cells treated with PCI of streptavidin−saporin in this concentration range, confirming the nontargeting ability of streptavidin−saporin as shown recently.[5]

The importance of the dose of light in PCI of cetuximab−saporin was also evaluated (Figure 3C). In these studies the cells were treated with 3 pM of cetuximab−saporin or 3 pM cetuximab. A strong light-dependent synergy by PCI of cetuximab−saporin was demonstrated. Increasing doses of light did not influence the effect of 3 pM cetuximab−saporin in the absence of $TPPS_{2a}$. The highest PDT-dose induced a ∼50% reduction of the cell viability compared to nontreated control cells. Combining PDT with 3 pM free cetuximab did not lead to any additional cytotoxicity as compared to PDT alone, which is in accordance with the results in Figure 2B.

Since 20-fold excess of cetuximab did not completely block the cytotoxic activity of cetuximab−saporin alone (Figure 3A), we performed a flow cytometry based receptor-binding saturation study of Alexa488 labeled cetuximab to find the optimal concentration for blocking cetuximab−saporin binding. HCT-116 cells were incubated on ice for 30 min with increasing concentrations of cetuximab−Alexa488. The results indicate that a concentration above 1 nM Alexa488−cetuximab saturates the EGFR binding site. Thus, 6 nM cetuximab was selected as blocking dose for use in the competition control experiment (data not shown). Measurement of clonal cell survival showed that using 6 nM cetuximab completely blocked the PCI-induced cytotoxic effect of cetuximab−saporin and reduced the effect to the level of PDT only and PCI of 3 pM streptavidin−saporin (Figure 3D). PCI of cetuximab−saporin at 120 s of light reduced the clonogenicity of the HCT-116 cells by more than one log unit, compared to PDT alone. To achieve LD_{50}, a ∼3-fold higher light dose was needed for $TPPS_{2a}$-PDT than for PCI of cetuximab−saporin at a cetuximab−saporin concentration (3 pM) that had no effect on the clonogenicity alone. The clonal cell survival experiment confirmed the synergy of the combination therapy as observed using the MTT assay. Altogether, these findings demonstrate the strength of combining the monotherapies PDT and immunotoxin therapy (i.e., PCI) compared to the monotherapies applied separately.

To further investigate the findings described above we also conducted PCI of the immunotoxin in two other EGFR-positive cell lines: the prostate carcinoma DU-145 cells (Figure 4A,B) and the epidermoid carcinoma A431 cells (Figure 4C). The synergistic effects of PCI of cetuximab−saporin were found in both DU-145 and A431 cells, confirming the efficacy of the present EGFR-targeting PCI technology. A431 cells were more sensitive to both mono-

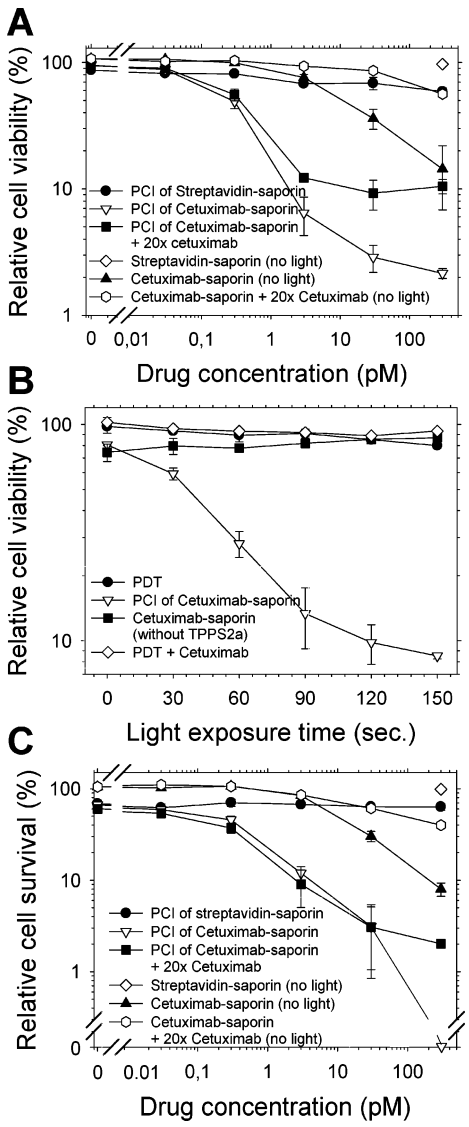

Figure 4. Cytotoxic effects of PCI of cetuximab−saporin in DU-145 and A431 cells. (A) Relative viability of DU-145 cells. Increasing concentrations of toxin conjugates alone or in combination with PCI and in the presence of 20-fold excess of cetuximab as indicated in the figure. The PDT dose alone (120 s of light exposure) killed ∼15% of the cells. (B) Light dose-dependent effects of PDT affecting the relative viability of DU-145 cells, PCI of 3 pM cetuximab−saporin, 3 pM cetuximab−saporin without $TPPS_{2a}$, or 6 nM cetuximab in combination with PDT. (C) Relative viability of A431 cells treated with PDT (100 s of light exposure), demonstrated to kill ∼40% of the cells but otherwise treated as described in panel A. The results are the average of three samples from at least two independent experiments. Cell viability was assessed by the MTT assay 48 h post light exposure. Values were normalized to nontreated cells; bars, SE.

therapies and also to PCI of cetuximab−saporin. The differences in cytotoxic effects after PCI or PDT between cell lines have previously been observed and may be attributable to the variation in the pharmacologic parameters such as cellular uptake and degradation of the drugs or

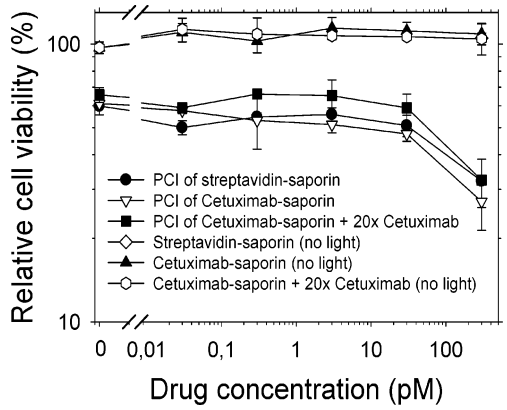

Figure 5. PCI of cetuximab−saporin in EGFR-negative MES-SA cells. MES-SA cells were treated with PCI of increasing concentrations of the toxin conjugates and a PDT dose (60 s of light exposure) killing ∼40% of the cells as indicated in the figure. Cells were also treated with cetuximab−saporin or streptavidin−saporin in the absence of $TPPS_{2a}$. The immunotoxin was also coincubated with 20-fold excess of cetuximab. The results are the average of three samples from at least two independent experiments. Cell viability was assessed by the MTT assay 48 h post light exposure. Values were normalized to nontreated cells; bars, SE.

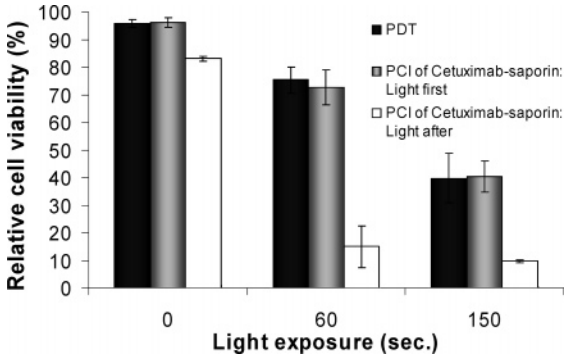

Figure 6. Evaluation of the "light first" versus "light after" principle for the PCI of cetuximab−saporin. Relative viability of HCT-116 cells treated with PDT or PCI of cetuximab−saporin using the "light first" method (first PDT, immediately followed by incubation with cetuximab−saporin). PCI of cetuximab−saporin utilizing the "light after" procedure was performed as described in the Experimental Section (Figure 2-5). The results are the average of three samples from at least two independent experiments. Cell viability was assessed by the MTT assay 48 h post light exposure. Values were normalized to nontreated cells; bars, SE.

differences in the cell survival and cell death signaling. Altogether, these results confirm the targeting concept of PCI that has previously been shown.[5,21,22]

Cytotoxic Effects in EGFR-Negative Cells. The EGFR-negative MES-SA cells (Figure 1A) were included as control cells for evaluation of the specificity of the immunotoxin in the present study (Figure 5). In the absence of light, no cytotoxic responses were detected in MES-SA cells that were challenged with cetuximab, cetuximab−saporin, cetuximab−saporin + 20-fold excess of cetuximab (20 times the molar concentration of the IT), or streptavidin−saporin alone at concentrations up to 300 pM. The selected PDT dose induced a ∼40% reduction of the MES-SA cell viability. However, no difference in cytotoxicity between PCI of cetuximab−saporin, PCI of cetuximab−saporin in combination with 20-fold excess of free cetuximab, or PCI of streptavidin−saporin was detected in the MES-SA cells. Only at the highest dose of the toxin conjugates (300 pM), PCI induced a slight attenuation of the cell viability compared to cells treated with PDT or toxin conjugates only. This indicates that a fraction of the toxin conjugates are internalized due to non-receptor mediated endocytosis, which was also assumed above based on the microscopy fluorescence analysis of MES-SA cells incubated with Alexa488−cetuximab (Figure 1). These experiments confirm that the immunotoxin cetuximab−saporin targets the EGFR receptor and is taken up by receptor-mediated endocytosis.

Light First versus Light After Strategy. The standard PCI procedure has been to incubate the cells with the PS and the macromolecule of interest prior to the light exposure. This protocol may be described as the "light after" PCI procedure since the photochemical treatment is exerted after the macromolecules have been endocytosed by the cells.[1] We have also established "light first" PCI where the cells are first exposed to PDT, leading to photochemical disruption of the endo-lysosomal vesicles before administration of macromolecules.[23] The macromolecule may be delivered up to a few hours after the photochemical treatment. Recently, the "light first" PCI principle was also confirmed *in vivo* using the plant derived RIP gelonin that was administered intratumorally directly after $AlPcS_{2a}$-PDT.[4] In the "light first" PCI procedure we have hypothesized that endocytic vesicles containing the macromolecule of interest will fuse with endosomes with photochemically ruptured membrane. However, there were no significant differences ($P > 0.05$) in cytotoxicity between PDT and PCI "light first" compared to the difference between PDT and cetuximab−saporin delivered by PCI "light after" ($P < 0.001$) (Figure 6). The reason for the lack of a PCI effect of cetuximab−saporin delivered by the "light first" principle is not known. However, the findings are in accordance with previous results in NuTu19 rat ovarian cancer cells where a lower cytotoxic response was observed after the "light first" PCI as compared

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to "light after" PCI for the delivery of EGF−saporin.[5] Studies in progress in our laboratory accordingly show reduced EGF-mediated EGFR phosphorylation after $TPPS_{2a}$-PDT in Nu-Tu19 cells (data not published). The present results may therefore be explained by a PDT-induced damage of the EGF receptor, which may result in a reduced EGFR-mediated endocytosis. Photochemical damage of EGFR utilizing another PS has previously been described *in vitro* and *in vivo*.[24]

Potential *in Vivo* Use of PCI of Cetuximab−Saporin. The streptavidin−biotin linkage used in the present report has been shown to be a simple design for evaluation of new targeting moieties for the treatment of cancer. The results in the present study and in a recent report utilizing EGF−saporin[5] document that the specificity is maintained. However, the immunoconjugate cetuximab−saporin is relatively large (>270 kDa) and, due to diffusion limitations in the tissue, may be regarded as unsuitable for treatment of solid tumors. Therefore, further work should focus on synthesizing immunotoxins with smaller size, e.g., using a disulfide bridge between mAb and toxin or making a recombinant construct where the Fv fragment of a growth factor-targeting mAb is fused with a protein toxin. The use of EGF as a targeting ligand may be beneficial due to its small size and relatively low affinity to EGFR, both of which may stimulate tissue penetration. In addition, EGF stimulates endocytosis of the ligand−toxin complex EGF−saporin. On the other hand, the relatively low affinity of EGF for EGFR indicates a requirement for higher doses of the ligand−toxin complex than when using a high affinity ligand. In addition, EGF is well-known for its stimulatory activity on cell growth, survival, metastasis, and angiogenesis.[25] In comparison, cetuximab binds strongly to EGFR and acts both by inhibiting intracellular signaling and through antibody-dependent cell cytotoxicity. In addition, it is an approved and much used mAb in cancer therapy.

Based on findings reported here, we suggest that PCI of growth-factor receptor directed immunotoxins, as exemplified by targeting EGFR by cetuximab−saporin, will provide a unique drug delivery strategy that specifically kills target cancer cells using at least three different pathways of action (as illustrated in Figure 7): (1) The blocking of the growth receptor by cetuximab antagonizes survival and proliferation signaling (Figure 1M). Cetuximab binds to EGFR with a 2-log higher affinity than EGF and TGF-β.[26] In addition, endocytosis of the cetuximab−EGFR complex leads to an overall downregulation of EGFR expression (Figure 1M), as previously shown with mAb225,[27] the murine version of cetuximab/C225. (2) The ROS-mediated cytotoxic responses after the photochemical exposure (PDT) result in apoptosis or necrosis. (3) The RIP activity of the toxin after PCI-induced cytosolic release results in cell inactivation by means of inhibition of the cellular protein synthesis. In addition, antibody-mediated cellular cytotoxicity could be a fourth way of inactivating tumor cells.[28,29]

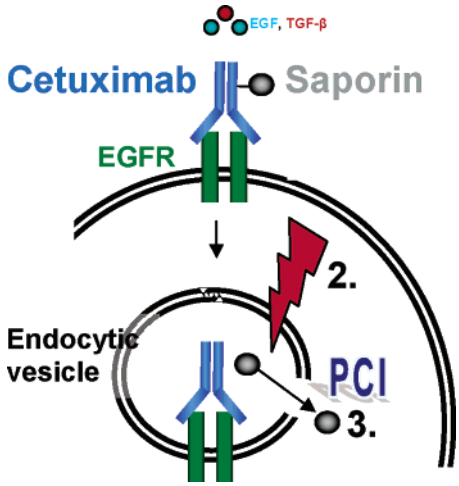

Figure 7. Schematic representation of three postulated pathways of cancer cell death induced by PCI of cetuximab−saporin. (1) Blockage of EGFR signaling by cetuximab resulting in reduced survival and growth signaling. (2) PDT-induced ROS inducing cell death. (3) PCI-triggered release of RIP toxin into the cytosol, where the toxin exerts its enzymatic anti-rRNA activity resulting in protein synthesis shutdown.

A potential limitation in the utilization of light for therapeutic purposes is the penetration of light through tissues. The penetration of light through tissues is limited mainly by heme, especially in hemoglobin, and melanin. Efficient penetration of light therefore requires wavelengths for photoactivation above about 600 nm where absorption of light by these chromophores is low.[30] An upper maximum wavelength for photoactivation is usually set by the energy required for excitation of O_2 to form singlet oxygen, i.e., 23

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kcal/mol, which is equivalent to the excess energy of the long-lived form of singlet oxygen. This requires that the photosensitizer must be excited at wavelengths below about 850 nm. The therapeutic wavelength window is therefore usually defined as 600−800 nm, but shorter wavelengths may be used when thin lesions are to be treated. The treatment of internal organs is generally of no limitations any more due to the development of light sources and light applicators in PDT. Necrosis has been reported up to 3 cm into the treated tissue.[31]

Conclusions

The main goal of the present report was to demonstrate a proof-of-principle concept using PCI of an EGFR-targeting immunotoxin. We have demonstrated in three different EGFR-positive cell lines, and in EGFR-negative control cells, derived from different cancer tissues that PCI of the immunotoxin cetuximab−saporin is a potent and specific anticancer cell therapy. Based on the present study we propose that our PCI concept introduces a unique triple cytotoxicity by (1) cetuximab, (2) PDT, and (3) the photochemically internalized saporin. The efficacy and multiple selectivity of the PCI drug delivery concept documented in this report warrant further preclinical investigations.

Acknowledgment. We thank the Norwegian Cancer Society (Grant C 96138/007) and the Norwegian Radium Hospital Research Foundation for financial support.

Abbreviations Used

EGFR, epidermal growth factor receptor; IT, immunotoxin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCI, photochemical internalization; PDT, photodynamic therapy; PS, photosensitizer; RIP, ribosome-inactivating protein; ROS, reactive oxygen species; TKI, tyrosine kinase inhibitor; $TPPS_{2a}$, meso-tetraphenylporphine with two sulfonate groups on adjacent phenyl rings.

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