New strategies to enhance photodynamic therapy for solid tumors

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Chapter 8

Inhibition of hypoxia inducible factor 1 with acriflavine sensitizes tumor cells to photodynamic therapy with zinc phthalocyanine-encapsulating cationic liposomes

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Abstract

Photodynamic therapy (PDT) is a tumor treatment in which a tumor-localized photosensitizer is excited with light, resulting in the local production of reactive oxygen species, destruction of tumor vasculature, tumor hypoxia, tumor cell death, and an anti-tumor immune response. However, pre-existent tumor hypoxia may desensitize tumors to PDT by activating the hypoxia-inducible factor 1 (HIF-1) survival pathway. Accordingly, it was hypothesized that inhibition of HIF-1 with acriflavine (ACF) exacerbates cell death in A431 tumor cells. PDT of human epidermoid carcinoma (A431) cells was performed with newly developed PEGylated cationic liposomes containing the photosensitizer zinc phthalocyanine. Molecular docking revealed that ACF binds to the dimerization domain of HIF-1α and confocal microscopy experiments confirmed the translocation of ACF from the cytosol to the nucleus under hypoxia. Hypoxic but not normoxic A431 cells stabilized HIF-1 following PDT. Inhibition of HIF-1 with ACF increased the extent of PDT-induced cell death under hypoxic conditions and reduced the expression of HIF-1 target genes VEGF, PTGS2, and EDN1. Neither hypoxia, PDT, nor ACF affected the expression of genes related to glycolysis. In conclusion, HIF-1 contributes to A431 tumor cell survival following PDT with liposomal zinc phthalocyanine. Inhibition of HIF-1 with ACF leads to improved PDT efficacy.

1. Introduction

Photodynamic therapy (PDT) is a non-to-minimally invasive treatment modality approved for the treatment of various types of solid tumors. The therapy encompasses the administration of a photosensitizer that accumulates in the tumor tissue and the subsequent irradiation of the photosensitizer-replete tumor with laser light that excites the photosensitizer. The excited photosensitizer interacts with molecular oxygen (O2) and produces singlet oxygen (¹O2) and/or superoxide (O2•−) through type II and type I photochemical reactions, respectively 1. These reactive oxygen species (ROS) induce oxidative damage that leads to the death of tumor cells and cells that comprise the...
tumor vasculature. These events culminate in tumor vascular shutdown, tumor tissue hypoxia and hyponutrition, and an anti-tumor immune response. PDT is successfully used in the treatment of a variety of tumors, although bladder cancers and nasopharyngeal carcinomas have been reported to respond relatively poorly to the therapy.

An improvement in PDT efficacy may be achieved by selecting a photosensitizer with better physicochemical properties in combination with tumor targeting. Metallated phthalocyanines such as zinc phthalocyanine (ZnPC) hold several important advantages over currently approved photosensitizers. For instance, ZnPC absorbs light at a longer wavelength (674nm) and has a substantially higher molar extinction coefficient (2.74 × 10^5 M^-1 cm^-1) than conventional photosensitizers. However, ZnPC is highly hydrophobic (logP of ~8), and must therefore be employed in conjunction with a biologically compatible photosensitizer delivery system. ZnPC retains its photophysical and photochemical properties in liposomes and its liposomal encapsulation enables the delivery of high payloads of ZnPC to tumor cells. Another advantage of the utilization of liposomes is that the lipid bilayer can be compositionally modified to accommodate a specific pharmacokinetic purpose, including targeting. For these reasons, we have previously developed a neutrally charged liposomal formulation containing ZnPC intended for passive targeting towards the tumor stroma via the enhanced permeability and retention effect. Although this modality was effective in vitro, the induction of damage to tumor stroma and perivascular tumor cells may not account for complete tumor eradication in vivo. Targeting of photosensitizers to the tumor vascular endothelium therefore poses an interesting alternative or addition, which can be achieved with cationic liposomes. The development and application of such liposomes for PDT may result in more effective shutdown of the tumor vasculature, severe tumor hypoxia, and an enhanced therapeutic outcome.

However, a predisposition of tumor cells to survive hypoxic conditions due to preexisting tumor hypoxia may reduce the tumoricidal efficacy of PDT. Preexisting tumor hypoxia and the constitutive activation of the hypoxia inducible factor 1 (HIF-1) transcription factor is the result of the tumor growth rate exceeding the rate of neoangiogenesis (Fig. 1). Moreover, HIF-1 has been related to chemotherapeutic resistance. With respect to PDT, HIF-1 activity was found to be increased following irradiation in a variety of in vitro and in vivo models. Additionally, HIF-1 has been associated with resistance to PDT in vitro and in esophageal cancer patients. Inhibition of HIF-1 activity and downstream survival signaling may therefore improve the therapeutic efficacy of PDT.

Since hypoxia is a preexisting condition in most tumors, and the induction of HIF-1 by hypoxia triggers a plethora of survival mechanisms, HIF-1 has been targeted for pharmacological intervention in cancer therapy. The HIF-1 activation pathway is summarized in Fig. 1 in relation to normophysiological conditions and PDT. Under normoxic conditions, HIF-1α is constantly degraded via O2-dependent hydroxylation of Pro402 and Pro564 by prolyl-hydroxylases (PHD) and/or Asn803 by factor inhibiting HIF (FIH). Hydroxylated HIF-1α is bound by Von Hippel-Lindau tumor suppressor protein (VHL), which promotes complexation to E3 ubiquitin ligase and subsequent polyubiquitination and proteasomal degradation. Hypoxia deters HIF-1α hydroxylation by PHDs and FIH, resulting in its stabilization and nuclear translocation. An alternative route to HIF-1 activation is through ROS, which deter the activity of PHDs and FIH via oxidation of the redox-sensitive Fe(II) in the catalytic center, thereby inhibiting the enzymatic activity of these HIF-1 hydroxylases. When translocated to the nucleus, HIF-1α dimerizes with HIF-1β to bind DNA at hypoxia-responsive elements in the promoter regions of a plethora of genes involved in glycolysis, angiogenesis, survival, and apoptosis.

Given its prominent role in the survival of (tumor) cells in hypoxic microclimates, this study aimed to determine the feasibility to inhibit HIF-1 during PDT in an attempt to exacerbate tumor cell death in vitro. Acriflavine (ACF) has been reported to be a specific inhibitor of HIF-1 that prevents the HIF-1α/HIF-1β dimerization by binding the dimerization domain of HIF-1α. The effects of ACF in combination with PDT with a recently developed cationic liposomal formulation containing the photosensitizer ZnPC was therefore assessed in A431 human epidermal squamous cell carcinoma cells, representing a tumor type that is treated by PDT. The main findings of the study are that...
ACF exerts an adjuvant effect on PDT efficacy in hypoxic cells and reduces the expression of HIF-1 target genes.

2. Material and methods

2.1 Chemicals, lipids, reagents, and antibodies

1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzozadiazol-4-yl)amino]hexanoyl}-sn-glycero-3-phosphocholine (NBD-PC) and 3β-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol (DC-chol) were purchased from Avanti Polar Lipids (Alabaster, AL). L-α-phosphatidylethanolamine, distearoyl methoxypolyethylene glycol conjugate (DSPE-PEG, average PEG molecular mass of 2,000 amu), ZnPC (97% purity), HEPES, fibronectin, sodium chloride (NaCl), β-mercaptoethanol, cholesterol, chloroform, Nile Red, paraformaldehyde, sucrose, bovine serum albumin (BSA), Tween 20, CoCl$_2$, ACF, and pyridine were obtained from Sigma-Aldrich (St. Louis, MO). Tris-HCl and DMSO were acquired from Merck (Darmstadt, Germany). Ethanol was obtained from Biosolve (Valkenswaard, the Netherlands). Water-soluble tetrazolium-1 (WST) and RNase A were purchased from Roche Applied Science (Basel, Switzerland). The mouse anti-human HIF-1α antibody (clone 54/HIF-1α) was from BD Transduction Laboratories (Franklin Lakes, NJ), mouse anti-human β-actin (AC-74) was from Sigma-Aldrich, and mouse anti-human phospho-H2AX-AlexaFluor647 was from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase-labeled polyclonal goat-anti-mouse IgG1 secondary antibodies were from Dako Cytomation (Glostrup, Denmark). Sep-
hadex G50 fine was from GE Healthcare (Piscataway, NJ).

All (derivatized) lipids were dissolved in chloroform, purged with nitrogen gas, and stored at -20 °C. Phospholipid stock concentrations were determined by the inorganic phosphate assay modified from 41. ZnPC was dissolved in pyridine at a 178-μM concentration and stored at room temperature (RT) in the dark. CoCl2 was dissolved in MilliQ at a concentration of 50 mM and filter-sterilized (0.2 μm, Corning, Corning, NY). Physiological buffer (10 mM HEPES, 0.88% (w/v) NaCl, pH = 7.4, 0.292 osmol/kg) was prepared in MilliQ. Nile Red was dissolved in DMSO at a 5-mM concentration.

2.2 Preparation and characterization of liposomes

Liposomes composed of DPPC:DC-chol:cholesterol:DSPE-PEG (66:25:5:4 molar ratio, unless indicated otherwise) and ZnPC (ZnPC:lipid molar ratio of 0.003) were prepared by the lipid film hydration technique as described in 7, 42. The hydrated lipid film was bath-sonicated (60 °C) and the resulting cationic liposomes (referred to as endothelium-targeting liposomes, ETLs) were stored under nitrogen gas at 4 °C in the dark. The ZnPC-ETLs were characterized for size and polydispersity by photon correlation spectroscopy (Zetasizer 3000, Malvern Instruments, Malvern, Worcestershire, UK) using settings reported previously 7. The lipid concentrations of the liposomal preparations were determined as described in section 2.1 and corrected for the (DC-)chol content based on predefined molar ratios. Typically, the ZnPC-ETLs in this study had a diameter of 185.9 ± 8.3 nm, a polydispersity index (PDI) of 0.214 ± 0.05 and a ζ-potential of 3.9 ± 1.2 mV.

2.3 Absorption and fluorescence spectroscopy

Absorption spectroscopy was performed using a Lambda Bio spectrophotometer (Perkin Elmer, Waltham, MA). For the liposomal stability assays, liposomes were prepared and purged with N2 or O2 as indicated, after which they were stored in the dark at 4 °C. Spectra were recorded in triplicate every 7 days.

Fluorescence excitation and emission spectra of ACF were recorded on a Cary Eclipse fluorescence spectrometer (Varian, Palo Alto, CA).

2.4 Cell culture

A431 cells were cultured under standard conditions (humidified atmosphere containing 95% air and 5% CO2, 37 °C) in phenol red-containing Dulbecco’s modified Eagle’s medium (DMEM, Gibco / Life Technologies, Gaithersburg, MD) supplemented with 4 mM L-glutamine, 10% fetal bovine serum (FBS, Gibco) and penicillin/streptomycin (Lonza, Basel, Switzerland, 50 U/mL and 50 U/mL, respectively). The cells were subcultured once a week at a 1:25 ratio and seeded in 24-wells or 6-wells plates (Corning) at a density of 2×10^5 cells/mL and 500 μL medium/well (24-wells plates) or 1.5 mL/well (6-wells plates).

Cells were maintained at standard normoxic culture conditions (95% air, 5% CO2, 37 °C) or at hypoxic culture conditions (<1% O2, 5% CO2, 37 °C using a gas mixture of 95% nitrogen, 5% CO2 (Linde Gas, Schiedam, the Netherlands)). Hypoxic culture conditions were achieved in a custom-built air-tight plastic incubator (11.6 × 9.1 × 5.4 inches) comprised of a gas inlet, a gas outlet connected to a bubble trap, a temperature regulation system (silicone tubing), closed loop system connected to a dual temperature circulator (model TLC 3, Tamson Instruments, Bleiswijk, the Netherlands), a metal grid for the placement of the wells plate, wetted gauze in a petri dish to obtain 99% humidity, and a 2-inch computer fan secured to the metal grid for homogenous gas distribution. The O2 percentage in the chamber was measured with an OdaLog gas monitor (App-Tek International, Brendale, Australia). The temperature inside the incubator was continuously monitored using a wireless thermometer (Oregon Scientific, Tualatin, Oregon).
2.5 Liposome uptake assays

Fluorescently labeled liposomal formulations were prepared for the uptake assays, in which 4% NBD-PC was added at the expense of DPPC. A431 cells were seeded as described in section 2.4 and grown to subconfluence overnight. Cells received fresh, serum-free medium in which the 100 μM (final lipid concentration) NBD-ETLs were suspended. Cells were incubated for 24 h and washed thrice with 1 mL of PBS, after which NBD fluorescence was measured using a BioTek multiplate reader (BioTek, Winooski, VT) at λex = 460 ± 40 nm, and λem = 520 ± 20 nm. Data were corrected for background fluorescence (control cells).

2.6 Photodynamic therapy

Prior to PDT, the culture medium was removed and cells were washed once with 1 mL of PBS (room temperature, RT). Cells received fresh, serum-free medium supplemented with Zn-PC-ETLs at concentrations indicated separately in the Results section. The cells were incubated for 1 h at standard culture conditions, washed twice with 1 mL of PBS (RT), and supplemented with fresh serum- and phenol red-free medium. Subsequently, PDT was performed at RT with a 671-nm solid state diode laser (CNI Laser, Changchun, China) at a power of 500 mW. The spot size and duration of irradiation was adjusted to the surface of a single well (1.9 cm², 57 s) or a 6-wells plate (9.5 cm², 285 s) to achieve a cumulative radiant exposure of 15 J/cm².

Cells were treated by PDT as follows. On day 0, cells were seeded as described in section 2.3 and allocated to the control group (CTRL), ACF group (only ACF preconditioning), PDT group (only PDT), or ACF + PDT group (ACF preconditioning followed by PDT). On day 1, cells received serum-free medium (CTRL and PDT groups) or serum-free medium containing 3 μM ACF. On day 2, the medium was removed and cells received fresh, serum-free culture medium (CTRL and ACF groups) or serum-free medium containing 10 μM ZnPC-ETLs (final lipid concentration, PDT and PDT + ACF groups). Cells were incubated with ZnPC-ETLs for 1 h at standard culture conditions and irradiated as described above. Subsequently, cells were kept at standard culture conditions for 4 h, i.e., normoxia. Alternatively, cells were photosensitized with 5 μM ZnPC-ETLs for 1 h, irradiated, and subsequently maintained for 4 h under hypoxic culture conditions to mimic vascular shutdown conditions post-PDT.

2.7 Cell viability assays

Cell viability was determined using the water-soluble tetrazolium-1 (WST-1) method as described in 7.

2.8 Immunoblotting

A431 cells were seeded in 6-wells plates (section 2.4). After 24 h, cells were incubated with 10 μM ZnPC-ETLs (final lipid concentration) and treated with PDT (section 2.6). At predefined time points after PDT, cells were placed on ice and immediately lysed in ice-cold Laemmli buffer (for composition, see Cold Spring Harbor recipes for 2 × Laemmli buffer) supplemented with protease inhibitor cocktail (1 tablet per 5 mL buffer, Roche Applied Science). As a positive control for HIF-1α stabilization, cells were incubated with 500 μM CoCl₂ for 20 h prior to lysis 24. The lysates were passed 10 × through a 25-gauge needle (BD Biosciences, San Jose, CA) to mechanically shear DNA. Next, samples were incubated at 95 °C for 10 min and centrifuged at 13,000 × g for 15 min at 4 °C. Proteins (30 μg) were separated on 10% SDS-PAGE precast gels (50 μL slot volume, Bio-Rad Laboratories, Hercules, CA) for 90 min and centrifuged at 13,000 × g for 15 min at 4 °C. Proteins (30 μg) were separated on 10% SDS-PAGE precast gels (50 μL slot volume, Bio-Rad Laboratories, Hercules, CA) for 90 min at 125 V. Subsequently, the gels were blotted onto PVDF membranes (Millipore, Billerica, MA) that had been primed in methanol for 10 min. Blotting was performed for 1 h at 330 V at 4 °C. Protein membranes were blocked for 1 h in Tris-buffered saline (TBS, 20 mM Tris-HCl, 150 mM NaCl, pH = 7.5) with 0.2% Tween 20 (TBST) supplemented with 5% dried milk powder (Protifar,
Nutricia, Cuijk, the Netherlands). Next, the membranes were incubated with antibodies (anti-HIF-1α 1:500, anti-β-actin 1:4,000) for 16 h at 4 °C on a rocker, washed 4 × in TBST, and incubated with secondary antibodies (goat-anti-mouse, 1:1,000) for 1 h at RT. Membranes were washed 3 × in TBST and 2 × in TBS. Detection of β-actin was performed with the enhanced chemiluminescence (ECL) kit (Thermo Scientific), whereas detection of HIF-1α was performed with ECL plus (Thermo Scientific) on an ImageQuant LAS 3000 luminometer (GE Healthcare).

2.9 Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

RNA extraction from cells seeded in 6-wells plates was performed by lysing cells in 0.5 mL TRizol according to the manufacturer’s protocol (Life Technologies). RNA was quantified with a Nanodrop 2000 UV-VIS spectrophotometer (Thermo Scientific) and checked for genomic DNA contamination (A260/A280 ratio ≥ 1.80). Reverse transcription was performed on 1 µg of total RNA using oligo-dT primer and the forward primer of S18 rRNA (Table 1). cDNA was synthesized using the Superscript reverse transcriptase kit according to the manufacturer’s protocol (Roche). Primers (Biologio, Nijmegen, the Netherlands) for each target gene were designed using the NCBI primer design tool (Table 1). qRT-PCR was performed with Sensifast SYBR green (Bioline, London, UK) on 25 ng of cDNA using final primer concentrations of 500 nM in a reaction volume of 10 µL. The qRT-PCR run program comprised 3 min at 95 °C, 45 cycles of 1 s at 94 °C, 7 s at 65 °C, and 10 s at 72 °C, followed by melting curve analysis (65-97 °C in 60 s, 4 °C ∞) (LightCycler 480, Roche). Each primer pair in Table 1 was designed to allow transcript variant amplification and passed the quality checks (PCR-efficiency (typically >80%), single-amplicon melting curve, and correct amplicon size). Data analysis was performed using LinReg as described in 43. A log2 transformation was performed in order to obtain absolute fold-differences in expression levels of the genes of interest.

2.10 Molecular docking

The X-ray structure of the ligand-bound PAS domain was retrieved from the Protein Data Bank (http://www.rcsb.org), which yielded the following structure/ligand combinations: 3F1O/2XY, 3H7W/018, 3H82/020, and 4GHI/0X3. Molecular docking calculations were carried out using AutoDock Vina software 44. Ligand structures were optimized using Dreiding force field 45 in Molconvert software (Chemaxon, Budapest, Hungary). Gasteiger partial charges 46 were calculated on ligand atoms. Polar hydrogen atoms were added to the protein and Gasteiger partial charges were calculated using AutoDock Tools. Water molecules and heteroatoms were removed from the structures. Simulation boxes were centered on the originally crystallized ligands. A 20 × 20 × 20-Å simulation box was used in each docking calculation with an exhaustiveness option of 8 (average accuracy). A new term was introduced into the AutoDock Vina scoring function that enables the addition of a distance-dependent restrain for a given interaction between a ligand atom type and a specific protein atom. The AutoDock Vina source code was modified accordingly and an executable file was generated using gcc. The restrain was defined as a hydrogen bond donor atom type of the ligand and the NH backbone atom of the critical residue, being minimized at -5 kcal/mol. The restrained bonding distance had a cut-off value of 4 Å.

Redocking experiments on the retrieved PAS domain/ligand structures were performed to assess the predictive power of the general scoring function implemented in AutoDock Vina. The experimental complex geometry could fully be reproduced with the docking calculations using the general scoring function. Thus, no further refinement of the scoring function was implemented.

2.11 Extracellular lactate determination

Extracellular lactate levels were determined with The Edge blood lactate analyzer (Apex Biotechnology, Hsinchu, Taiwan). Lactate concentrations were determined using a standard curve of lactate in phenol red free DMEM, and corrected for the average protein content/group to correct for
the toxicity of the treatment

2.12 Confocal laser scanning microscopy

The fluorescence excitation and emission spectra of ACF in MilliQ water were determined using a Cary Eclipse fluorescence spectrophotometer (Varian, Palo Alto, CA). The intracellular localization of ACF and the occurrence of DNA damage were investigated prior and after PDT using confocal laser scanning microscopy. Microscope cover slips (24 × 40 mm, VWR, Lutterworth, UK) were placed in 6-wells plates and coated with 5×10⁻⁴% (w/v) fibronectin in 1 mL of sterile 0.9% NaCl solution (Fresenius Kabi, Bad Homburg, Germany) for 2 h at 37 °C prior to cell seeding. The fibronectin-containing solution was removed and the cells were seeded onto the cover slips (densities specified in section 2.3) and incubated overnight.

For the ZnPC uptake experiment, cells were incubated for 4h with 100 μM ETLs in which ZnPC was encapsulated at a 0.024 ZnPC:lipid ratio. Cells were subsequently incubated with 50 nM Mitotracker Red (MTR, Life technologies, Carlsbad, CA) for 30 min at standard culture conditions. After incubation, cells were washed with 1 mL of PBS (RT) and fixed in 4% paraformaldehyde, 0.2% sucrose for 5 min, after which the coverslips were mounted on microscope slides using Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

For the ACF uptake experiment, cells were subjected to PDT (section 2.6) and subsequently incubated with 3 μM ACF for 4 h at normoxic or hypoxic conditions as indicated. After incubation, cells were washed with 1 mL of PBS (RT) and fixed in 4% paraformaldehyde, 0.2% sucrose for 5 min. After fixation, cells were washed with 1 mL of PBS (RT). Nile Red staining was performed with 1 μM Nile Red in PBS for 60 s. Cells were washed thrice with 1 mL PBS and mounted on microscope slides using Vectashield mounting medium.

For DNA damage assessment, cells were permeabilized after fixation by 5-min incubation in 1 mL PBS containing 0.1% TX-100 (RT). Cells were washed with 1 mL of PBS (RT), after which they were incubated with mouse-anti-human phospho-H2AX-AlexaFluor647 at a 1:100 dilution in 0.5% BSA and 0.15% glycine in PBS (staining buffer) for 16 h at 4 °C. Cells were washed thrice with staining buffer and subsequently mounted on microscope slides using Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Slides were dried for 1 h and sealed with nail polish. Cells were imaged on a Leica SP8 laser scanning confocal microscopy system (Leica Microsystems, Wetzlar, Germany). Fluorescence intensities were measured at λex = 405 nm, λem = 415-480 nm for DAPI, λex = 470 nm, λem = 480-550 nm for ACF, λex = 540 nm, λem = 550-650 nm for Nile Red, and λex = 660 nm, λem = 670-750 nm for phospho-H2AX. All experiments were performed using the same laser and microscope hardware settings.

### Table 1. Primer information of primer pairs used for qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
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<tr>
<td>HIF1α</td>
<td>GCCGGAACGACGAGAAGAAAAAGA</td>
<td>CCAAGAAGTTTCCATGACGCG</td>
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<td>BCL2</td>
<td>TTGTGGACACTCAGGCGCCC</td>
<td>CAGGCTGCACGTTGGTTTTCA</td>
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<tr>
<td>BCLN1</td>
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<td>TGTTCCAATCCGTTGTCT</td>
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<td>AGGACACCGCTCGCTCTAGCA</td>
<td>GTGTTCCAATCCGTTGTCT</td>
</tr>
<tr>
<td>EDN1</td>
<td>GGGCTGAAAGAGCCTTCTTTGA</td>
<td>TGCCAAGTTGTTCCCTTTCA</td>
</tr>
<tr>
<td>HNK1</td>
<td>GCGAAGTCCTGAGCTTAC</td>
<td>CATGTTACCTGCTGACCCGC</td>
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<tr>
<td>HMOX1</td>
<td>AGGAAATCTTCTCTGCTGCC</td>
<td>GCTGACACATTTGCTTCT</td>
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<tr>
<td>HSPA5</td>
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<td>TCATCCACGCTGCACGGAAT</td>
</tr>
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<td>LDHA</td>
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<td>PTGS2</td>
<td>GGGCATGGGTTGAGCTTAAA</td>
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<td>SERPINE1</td>
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<td>VEGF</td>
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<td>S18rRNA</td>
<td>TTCCGAGACGTGAGCAGTAG</td>
<td>CGAAACCTCGACTTCTTTG</td>
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</table>
2.13 Statistical analysis

Data were analyzed in GraphPad Prism software (GraphPad Software, San Diego, CA). Data was analyzed for normality using a Kolmogorov-Smirnov test. Normally distributed data sets were analyzed with either a student’s t-test or a one-way ANOVA and a Tukey’s post-hoc test. All data are reported as mean ± standard deviation. In the figures, relevant intergroup differences are indicated with (*), differences between treated groups versus the untreated (CTRL) group are indicated with (#), and differences between normoxic and hypoxic data are indicated with ($). The level of significance is reflected by a single (p < 0.05), double (p < 0.01), triple (p < 0.005), or quadruple sign (p < 0.001).

3. Results and discussion

3.1 HIF-1 activation is exacerbated by PDT with ZnPC-ETLs

Several studies have shown the potential of cationic liposomes to target the tumor vasculature in cancer therapy 13, 47, 48. PEGylation is essential to prevent uptake of these liposomes by non-target cells, bestow long-circulating capacity, and minimize adverse events in vivo related to the toxicity of cationic lipids (reviewed in 11). Although liposomes that contain ZnPC have been developed by us 7 and others 49, 50, the application of photosensitizer-containing cationic liposomes in the field of PDT of cancer is limited 51. Consequently, a cationic liposomal formulation for the delivery of ZnPC to tumor cells in vitro and the tumor vasculature in vivo has been previously developed 7 and was further investigated here.

Prior to assessing the effects of PDT on HIF-1 activation, the cytotoxicity of PDT with ZnPC-ETLs was determined. There was no ZnPC-ETL cytotoxicity in the absence of light up to a final lipid concentration of 50 μM, measured after a 24-h incubation period (Fig. 2A). PDT (15 J/cm²) resulted in a ZnPC concentration-dependent decrease in relative cell viability after 24 h of normoxic incubation (Fig. 2B). The photochemical production of ROS and the short period of hypoxia induced by the conversion of O₂ to ROS are therefore not sufficient to stabilize HIF-1α. Consequently, the experiments were also performed under hypoxic culture conditions to mimic vascular shutdown following PDT.

Under hypoxic conditions, A431 cells were more amenable to PDT-induced cell death, as evidenced by the almost similar extent of cell death achieved at ≤ 50% of the photosensitizer concentration used in the normoxia groups (Fig. 2B). Approximately 50% cell death was achieved with 10 and 5 μM ZnPC-ETLs under normoxic and hypoxic conditions, respectively. Subsequent experiments regarding the involvement of HIF-1 in the PDT response were therefore conducted using these final lipid concentrations.

3.2 Persistent HIF-1α stabilization desensitizes A431 cells to PDT

Ji et al. showed that CoCl₂ treatment could desensitize Het1A esophageal tumor cells to PDT via upregulation of HIF-1 24. To investigate whether this phenomenon also occurs in A431 cells, the susceptibility of A431 to PDT was determined after 24-h preconditioning with 500 μM CoCl₂. Cells were subjected to PDT with 10 μM ZnPC-ETLs (final lipid concentration), after which the viability was measured at 4 h post-PDT (N = 12). CoCl₂ preconditioning significantly (p < 0.001) reduced PDT efficacy in A431 cells (Fig. 2C). Cells preconditioned with CoCl₂ exhibited a post-PDT viability of 75.4 ± 14.9% compared to a post-PDT viability of 36.6 ± 6.0% in the group not preconditioned with ACF, most likely as a result of HIF-1 and HO-1 overexpression 24, 52, 53.

3.3 PDT exacerbates HIF-1 signaling

HIF-1α stabilization did not occur under normoxic conditions (results not shown), which
may be explained by the short half-life of HIF-1α under normoxic conditions (~5-8 min\(^54\)). Under hypoxic conditions and in the absence of PDT, A431 cells stabilized HIF-1α from 30 min onwards. HIF-1α stabilization was more pronounced following PDT under hypoxic conditions, whereby the extent of stabilization was proportional to the duration of hypoxia (Fig. 2C). The delay in the onset of hypoxic incubation and stabilization of HIF-1α was most likely caused by the gradual depletion of residual O\(_2\) in the culture medium\(^55\).

Several studies have investigated HIF-1 in the context of PDT. In vitro, chemically induced HIF-1 activation correlated positively with increased tumor cell survival post-PDT\(^24\). However, HIF-1 stabilization can occur in an oxygen-independent manner\(^19\) and the degree of HIF-1 activation differs between cell lines\(^20\). In vivo, PDT has shown to induce HIF-1α stabilization and increased VEGF protein levels in a murine model of Kaposi’s sarcoma\(^22\). In order to confirm whether PDT has an auxiliary effect on HIF-1 activation in comparison to hypoxia alone in A431 cells, pertinent and frequently used reporter genes for HIF-1 activity\(^17, 36, 56-58\) were screened. As shown in Fig. 2D, the most prominent effects of PDT on HIF-1 target genes entailed the increased expression of prostaglandin synthase 2 (PTGS2, COX-2), heme oxygenase 1 (HMOX1, HO-1), and vascular endothelial growth factor receptor (VEGF), the reduced downregulation of endothelin 1 (EDN1), and the downregulation of HIF1A and b-cell lymphoma 2 (BCL2). The fold change in expression levels of survivin (BIRC5) and pyruvate kinase muscle 2 (PKM2) was < 1.

The HIF-1-related findings in A431 cells are in agreement with the previously referenced in vitro\(^19, 20, 24\) and in vivo studies\(^21-23, 59\). The in vivo studies demonstrated that HIF-1 was stabilized in hypoxic tumor tissue as a result of vascular shutdown following PDT, leading to an increase in mRNA levels of HIF1A, VEGF, and PTGS2\(^59\).

3.4 ACF binds the dimerization domain of HIF-1α

Inhibition of the function of proteins that are produced downstream of HIF-1 signaling is as-
sociated with improved PDT efficacy. For example, inhibition of VEGF with bevacizumab was shown to increase PDT outcome. These results have been confirmed in BA mouse mammary carcinomas, which exhibited HIF-1α stabilization and increased protein levels of VEGF and survivin following PDT. Moreover, tumor growth rate and overall survival could be increased by blocking tyrosine kinase receptors in PDT-treated CNE2 xenografts in mice, i.e., a class of receptors that includes the receptor for VEGF. These data attest that the HIF-1 signaling axis is important in the context of PDT. However, rather than inhibiting the downstream products of HIF-1, the inhibition of HIF-1 itself is pharmacologically more sensible in terms of an optimal adjuvant effect on PDT efficacy insofar as all downstream signaling pathways are blocked at once.

The specific inhibition of HIF-1 by ACF has been previously attributed to its binding to the Per Arnt Sim (PAS) domain of HIF-1α, thereby preventing HIF-1α/HIF-1β dimerization. To corroborate those findings and to determine which residues are involved in ACF/HIF-1α complex formation, molecular docking of ACF to the HIF-1α PAS-domain was performed. It should be noted that docking of ACF to the PAS-domain of HIF-1 resulted in favorable binding only in case of the 3H7W crystal structure, which can be explained by minute differences in side chain conformations in the crystal structures. ACF and the binding site possessed excellent shape complementarity as revealed in the docking simulation (Fig. 3). The ligand of 3H7W in the original study is an aniline derivative with no specific hydrogen bonding capacity, indicating that shape complementarity is essential to accommodate planar molecules in case of this narrow, closed binding site. As illustrated in Fig. 3, the main driving force for complex formation is a π-π interaction with the aromatic pocket formed by Phe244, His248, His293, and Tyr307 of the binding site. In addition, the amino group of ACF is in a hydrogen bond with Tyr306, potentially indicating the presence of a strong interaction.

3.5 ACF exacerbates tumor cell death in vitro

To determine the most suitable concentration of ACF for the combinatorial treatment mo-
dality, the concentration-dependent uptake and toxicity of ACF were assessed. Uptake was deter-
mined by utilizing the intrinsic fluorescent properties of ACF as depicted in Fig. 4A. ACF uptake fol-
lowed a linear pattern up to a concentration of 4 μM (Fig. 4B). The toxicity of ACF was determined
during a 24-h incubation period under either normoxic or hypoxic conditions. ACF became slightly
toxic at concentrations > 3 μM under normoxic conditions, with a 29% decrease in cell viability at 5
μM (Fig. 4C). Under hypoxic conditions, ACF exhibited a decrease of ~40% in viability at concentra-
tions > 1 μM that did not decrease further at higher concentrations (Fig. 4C). Based on these data,
the ACF concentration that was used for further experimentation was 3 μM.

Next, the neoadjuvant potential of ACF was investigated in terms of PDT efficacy. First, cells
were preconditioned for 24 h with 3 μM ACF, treated by PDT with 10 μM ZnPC-ETLs, and kept under
normoxic conditions. It was found that ACF alone did not induce any toxicity in A431 cells and, when
used in conjunction with PDT, also did not impart an additional effect (Fig. 4D). However, under hy-
poxic conditions, the extent of A431 cell death in the ACF + PDT group was more extensive than in
the ACF group, indicating that ACF exerted an adjuvant effect on PDT efficacy (Fig. 4D).

To determine whether the toxicity imparted by the different treatments was effectuated
through apoptosis, the activity of caspases 3 and 7 were assayed 4 h after treatment. Under nor-
moxic conditions, A431 cells exhibited no significant increase in apoptotic signaling following ACF
treatment and displayed a 2-fold increase in caspase 3/7 activity after PDT compared to control cells
(Fig. 4E). However, ACF + PDT resulted in 4-fold higher caspase 3/7 activity. Surprisingly, the adjuvant
effect of ACF on PDT-induced apoptosis was abrogated in hypoxic A431 cells, indicating that the
mechanisms of post-PDT cell death are reliant on the prevailing oxygen tension.

These results are in favor of the hypothesis that the beneficial effect of ACF on PDT outcome
most likely stemmed from the downstream effects of HIF-1α antagonism. One of the major functions
of HIF-1 is the stimulation of anaerobic glycolysis over oxidative phosphorylation. In order to assess
whether anaerobic glycolysis was stimulated in A431 cells by hypoxia, exacerbated by PDT, and in-
hibited by ACF, the excretion of lactate was investigated. Lactate excretion by hypoxic cells was sig-
nificantly higher than that of normoxic cells in all treatment groups (Fig. 4F). ACF + PDT significantly
lowered the extent of lactate production under normoxic conditions (grey bars). Under hypoxic con-
ditions (white bars), the extent of lactate excretion was only affected by ACF but not PDT alone or
in combination with ACF. These results warrant further investigation regarding the involvement of
glycolysis in response to PDT.

Taken altogether, there was significant increase in PDT efficacy when cells were pretreated
with ACF and subjected to post-therapeutic hypoxia. Although PDT induced cell death primarily
through apoptosis as shown by the increased caspase 3/7 activity, the adjuvant effect of ACF on
PDT efficacy did not correlate with increased caspase 3/7 activity, indicating that cells perish via an
alternate mechanism. In a previous study by Tennant et al. it was demonstrated that inhibition of
HIF-1 activation by the reactivation of PHDs with α-ketoglutarate under hypoxic conditions resulted
in metabolic catastrophe in HCT116 human colon carcinoma cells that was characterized by reduced
glucose uptake, lowered lactate production, and loss of plasma membrane functionality (i.e., (pro-
grammed) necrosis) 61. Since ACF + PDT-treated A431 cells exhibited a similar pattern of reduced
lactate production and increased caspase 3/7-independent cell death, our data corroborates the
previous findings and suggest that A431 cells subjected to ACF + PDT perish as a result of metabolic
catastrophe with a necrotic phenotype. However, it should be noted that the hypoxia-induced in-
crease in lactate production was hardly affected by PDT, implying that the HIF-1-induced metabolic
switch from oxidative phosphorylation to anaerobic glycolysis may not be an acute mechanism of
cell survival following PDT.

3.6 ACF uptake and intracellular localization

Since ACF was avidly taken up by A431 cells, the uptake and intracellular localization of
ACF was investigated with confocal laser scanning microscopy to determine its intracellular fate
before and after PDT. ACF was imaged utilizing its intrinsic fluorescent properties (Fig. 4A). Nile Red,
a lipophilic fluorogenic dye, was used to stain the membrane of paraformaldehyde-fixed cells and organelles. To observe an effect of intracellular ACF translocation as a result of PDT and/or oxygen tension, ACF was added to cells during hypoxic incubation and/or after PDT. Confocal images (Fig. 5) show that ACF did not abundantly localize to the endoplasmic reticulum (ER) and/or Golgi apparatus given that the ACF fluorescence intensity was low in the perinuclear areas with intense Nile Red fluorescence. ACF fluorescence was not observed as concrete intracellular foci, suggesting that ACF did not preferentially accumulate in mitochondria or lysosomes.

In the absence of PDT, cells displayed a healthy morphology and ACF localized in the cytoplasm and nucleus (Fig. 5A and B). PDT-treated cells cultured under normoxic conditions post-treatment exhibited shrinkage and blebbing (Fig. 5C). ACF fluorescence was substantially increased, particularly in the nuclei. Under hypoxic conditions, there were no profound effects of ACF on the intracellular localization and morphology of the cells. PDT-treated cells that were kept under hypoxic conditions displayed a similar degree of shrinkage and blebbing, but a reduced ACF fluorescence intensity (Fig. 5D).

3.7 ACF reduces the expression of angiogenesis and survival-associated genes

To investigate whether the increase in PDT efficacy with adjuvant ACF was indeed attributable to HIF-1 inhibition, the expression levels of a variety of HIF-1 target genes were determined after hypoxic incubation only (CTRL) or in combination with ACF, PDT, or ACF + PDT treatment using qRT-PCR. Genes were clustered according to angiogenesis-, glycolysis-, or survival-related genes (Fig. 6). PDT strongly induced the expression of PTGS2, VEGF, and HMOX1 (also shown in Fig. 3D). ACF alone and in combination with PDT reduced the extent of PTGS2 and VEGF expression. This observation provides important clues to the enhanced cytotoxicity of ACF + PDT, since the protein products of both genes have been identified to stimulate tumor cell survival post-PDT 59, 62-64. HMOX1 was also induced by hypoxia and PDT, but its expression was unaltered following ACF pretreatment,
suggesting that its expression was modulated by an unknown HIF-1-independent mechanism (e.g., by nuclear factor E2-related factor 2 (NRF2) 65). BIRC5, the gene that encodes survivin - a protein that regulates survival in cancer cells 66 - was found to be downregulated after hypoxia and PDT, but was upregulated when cells were pretreated with ACF. Accordingly, the ACF-induced upregulation of survivin may counter the neoadjuvant efficacy of this HIF-1α inhibitor. In the angiogenesis gene cluster, it is interesting to note that EDN1 mRNA-levels were reduced after hypoxia and PDT yet strongly upregulated following ACF and ACF + PDT treatment. EDN1 is a putative gene target of activated HIF-1 that induces proliferation by binding to EDN-1-associated receptor (ETAR) that in turn activates β-catenin and induces the expression of e.g., CCND1 (cyclin D1). EDN1 also stimulates cell survival via the promotion of nuclear factor κB activity and subsequent upregulation of BCL2 and BIRC5, as well as by stimulating the activity of COX-2. Consequently, EDN1 excreted from tumor cells stimulates angiogenesis, proliferation, and survival (for an elaborate review on the downstream signaling events of EDN1, see 67). In contrast with the previous, an increase in EDN1 expression after ACF treatment, especially in combination with PDT, may indicate that HIF-1 has an inhibitory function on the expression of this gene rather than a stimulatory effect. The expression of EDN1 has been

Figure 5. The intracellular localization of ACF was determined in A431 cells using confocal microscopy. Cells were treated as indicated and placed under either normoxic or hypoxic culture conditions in the presence of 3 μM ACF for 4 h. All images were taken with a 63× oil immersion lens and digital zoom.
proposed to be potentially triggered by PDT via alternative signaling, namely through JUN and FOS transcription factors \(^{67}\), and was found to be strongly upregulated upon ER stress \(^{68}\). PDT-induced \textit{EDN1} upregulation was apparently offset in A431 cells, but the ACF-mediated ER stress may have materialized as alluded to previously.

Most other genes were either slightly up or down regulated in a treatment-independent fashion. In addition to hypoxia and PDT (Fig. 2E), ACF and ACF + PDT did not influence the expres-

Figure 6. Heat map of gene expression patterns in A431 cells analyzed 0 h, 2 h, or 4 h post-treatment under hypoxic conditions. The plotted data represents the log2-transformed fold change of each data point in relation to the 0-h normoxic CTRL. Upregulated genes are depicted in red, downregulated genes in green.

Figure 7. (A-H) Analysis of DNA damage after CTRL (A and E), ACF (B and F), PDT (C and G), and ACF + PDT (D and H) treatment in A431 cells. Cells were kept for 4 h under normoxic (A-D) or hypoxic conditions (E-H) post-treatment. Cells were stained with DAPI (nuclei, blue) and phospho-H2AX (DNA double strand breaks, red).
Table 2. Mean ± SD log2-transformed fold-change in mRNA levels in control cells or ACF/PDT/ACF + PDT-treated cells as a function of time of established HIF-1 target genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>CTRL</th>
<th>ACF</th>
<th>PDT</th>
<th>ACF + PDT</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>2 h</td>
<td>4 h</td>
<td>2 h</td>
<td>4 h</td>
</tr>
<tr>
<td>HIF1</td>
<td>0.02 ± 0.09</td>
<td>0.50 ± 0.45</td>
<td>0.12 ± 0.31</td>
<td>-0.47 ± 0.42</td>
</tr>
<tr>
<td>ESM1</td>
<td>0.00 ± 0.01</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>1.11 ± 0.11</td>
</tr>
<tr>
<td>SFRP1</td>
<td>0.00 ± 1.18</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>1.65 ± 0.09</td>
</tr>
<tr>
<td>GCN5</td>
<td>0.00 ± 0.39</td>
<td>-0.27 ± 0.34</td>
<td>0.06 ± 0.36</td>
<td>0.06 ± 0.36</td>
</tr>
<tr>
<td>HK1</td>
<td>0.00 ± 1.04</td>
<td>0.27 ± 0.40</td>
<td>0.00 ± 0.00</td>
<td>-0.15 ± 0.37</td>
</tr>
<tr>
<td>LAMA4</td>
<td>0.00 ± 0.30</td>
<td>-0.26 ± 0.68</td>
<td>0.10 ± 0.96</td>
<td>-0.16 ± 0.53</td>
</tr>
<tr>
<td>PDKM</td>
<td>0.00 ± 0.42</td>
<td>0.22 ± 0.40</td>
<td>0.40 ± 0.96</td>
<td>0.74 ± 0.47</td>
</tr>
<tr>
<td>PIK3C1</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.10 ± 0.53</td>
<td>0.20 ± 0.21</td>
</tr>
<tr>
<td>PIK3C2</td>
<td>0.00 ± 0.20</td>
<td>-0.21 ± 0.40</td>
<td>0.00 ± 0.62</td>
<td>-0.24 ± 0.37</td>
</tr>
<tr>
<td>ROC1</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.10 ± 0.53</td>
<td>0.20 ± 0.21</td>
</tr>
<tr>
<td>BECH1</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.10 ± 0.53</td>
<td>0.20 ± 0.21</td>
</tr>
<tr>
<td>BCR3</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.10 ± 0.53</td>
<td>0.20 ± 0.21</td>
</tr>
<tr>
<td>NMDQY1</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.10 ± 0.53</td>
<td>0.20 ± 0.21</td>
</tr>
<tr>
<td>HSPM5</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.10 ± 0.53</td>
<td>0.20 ± 0.21</td>
</tr>
<tr>
<td>PSST</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.10 ± 0.53</td>
<td>0.20 ± 0.21</td>
</tr>
</tbody>
</table>

**3.8 Neither ACF, PDT, nor hypoxia induce DNA damage**

Many anticancer agents exert chemotherapeutic effects by inducing DNA damage, which signals the p53 tumor suppressor protein to induce cell cycle arrest and apoptosis. Given the knowledge that p53 can affect HIF-1 activation and that ACF was prominently present in the nucleus (Fig. 5), we investigated whether ACF induced DNA damage under any of the experimental conditions. Cells were stained for the presence of DNA double strand breaks using the epigenetic marker phospho-H2AX. The results illustrate that there was preexisting DNA damage in A431 cells, which was not exacerbated by any of the experimental conditions. In conclusion, ACF does not induce DNA damage despite its nuclear localization.

**4. Conclusion**

Given that the activation of HIF-1 by PDT has been readily established and its overexpression has been associated with decreased susceptibility of tumors and tumor cells to PDT, this study aimed to determine the feasibility of HIF-1 inhibition with ACF in combination with PDT in vitro. The results of this study are clearly in favor of such a combination therapy since it was shown that the inhibition of HIF-1 with ACF in cultured A431 cells significantly increased the efficacy of PDT. Further in vivo investigations on the (neo)adjuvant potential of ACF in PDT are warranted to extrapolate these findings in the context of vascular shutdown, hypoxia, hyponutrition, angiogenic signaling, and the tumor microenvironment. Since HIF-1 is constitutively active in most – if not all – tumors and has been associated with therapy resistance, this study further underscores the potential of ACF in cancer therapy.
neuronal cell lines. 


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