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Chapter 11

Photodynamic therapy with liposomal zinc phthalocyanine and tirapazamine increases tumor cell death via DNA damage

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Abstract

The efficacy of photodynamic therapy (PDT) in some solid tumors is limited by the poor biodistributive properties of conventional photosensitizers and a natural predisposition of tumor cells to survive hypoxia and oxidative stress. This study investigated the therapeutic potential of a third-generation photosensitizer, liposomal zinc phthalocyanine (ZnPC), in combination with the hypoxic cytotoxin tirapazamine (TPZ). TPZ induces DNA double strand breaks (DSBs) under hypoxic conditions and subsequent apoptosis via p53 signaling. Experiments were performed in tumor cells with functional p53 (Sk-Cha1) and dysfunctional p53 (A431). The combination therapy of TPZ and PDT induced DNA-DSBs and cell cycle stalling and enhanced the cytotoxicity of PDT by exacerbating apoptotic and non-apoptotic tumor cell death. These phenomena occurred regardless of oxygen tension and the mechanism of cell death differed per cell line. Liposomes containing both ZnPC and TPZ exhibited no dark toxicity but were more lethal to both cell types after PDT compared with ZnPC-liposomes lacking TPZ – an effect that was more pronounced under hypoxic conditions. In conclusion, TPZ is a suitable pharmaceutical compound to increase PDT efficacy by exploiting the post-PDT tumor hypoxia. The inclusion of TPZ and ZnPC into a single liposomal delivery system was feasible. The PDT strategy described in this study may be valuable for the treatment of PDT-recalcitrant tumors.

1. Introduction

Extrahepatic cholangiocarcinomas (EHCCs) are tumors that most frequently arise at the bifurcation of the common bile duct. The prevalence of these tumors with unknown etiology is merely ~0.0015% but is increasing in the Western population and is associated with a very high mortality rate ¹. The only curative treatment is radical resection of the malignancy, which is not possible in 50-90% of the cases, depending on the treatment center ². Patients with non-resectable EHCCs have a median survival time of 6-9 months post-diagnosis ³. Current palliative radiotherapy and/or che-
motherapy regimens can only marginally improve life expectancy (~12 months post-diagnosis) 4, contributing to the dismal prognosis associated with the malignancy. A relatively promising last-line intervention for non-resectable EHCCs is photodynamic therapy (PDT), a treatment modality that was shown to extend patients’ average life expectancy to 16 months post-diagnosis 5. Despite the high cure rates of PDT in many types of cancer 6, PDT of non-resectable EHCCs is not curative but only palliative. Accordingly, there is a clear medical need to develop a more effective last-line and preferably curative intervention for this group of patients.

PDT for solid tumors consists of the oral or systemic administration of a photosensitizer, which preferentially accumulates in endothelial cells of intratumoral vasculature and perivascular tumor mass 7-12, followed by irradiation of the tumor with laser light. Irradiation causes excitation of the photosensitizer to a singlet and subsequent triplet state and the transfer of energy or the triplet state electron to molecular oxygen (O2), yielding singlet oxygen (‘O2) or superoxide anion (O2•–), respectively 13. These reactive oxygen species (ROS) oxidize various intracellular biomolecules and cause necrotic, apoptotic, and/or autophagic death of the photosensitized tumor cells and tumor-associated cells as a result of oxidative stress 14. Additionally, PDT inflicts shutdown of the tumor microvasculature, resulting in tumor tissue hypoxia/anoxia and hyponutrition 7. Finally, the destruction of tumor(-associated) cells attracts phagocytic and antigen-presenting cells to the tumor site that fuel a prolonged anti-tumor immune response 15.

With respect to PDT, one experimental approach that may lead to improved therapeutic efficacy is nanoparticle-mediated delivery of the photosensitizer to pharmacologically relevant locations in solid tumors; namely the tumor cells, tumor vasculature, and tumor interstitium 16. For these purposes our group has developed tumor-targeted liposomes, tumor endothelium-targeted liposomes (ETLs), and interstitium-targeted liposomes 17 that encapsulate the second-generation photosensitizer zinc phthalocyanine (ZnPC), which can be combined in a single PDT modality. Additionally, the liposomes may co-encapsulate pharmacological agents that detrimentally interfere with vital biological and biochemical processes in cancer cells before and/or after PDT so as to further improve the therapeutic efficacy. Suitable pharmacological targets include the survival pathways that are activated in cancer cells after sublethal PDT, as addressed in 18, 19.

Another important biological process that can be exploited therapeutically is intratumoral hypoxia insofar as a constitutive state of hypoxia is propitious to tumor survival and hence common in many types of cancer 20. This constitutive and PDT-induced state of hypoxia may be targeted by employing a cytotoxic agent that is activated at low oxygen tension, which can further reduce tumor viability after PDT. A candidate drug for this purpose is tirapazamine (TPZ), a hypoxic cytotoxin capable of inducing oxidative DNA damage at low intracellular oxygen tensions 21. Several clinical trials in which TPZ was combined with chemotherapy or chemoradiotherapy yielded promising results in non-small cell lung cancer- 22, 23, head-and-neck cancer- 24, 25, and cervical cancer patients 26, 27. However, clinical trials in which TPZ did not exhibit beneficial effects on the efficacy of chemotherapy have been published 28. TPZ has been combined with PDT in a R1F1 murine cancer model using Photofrin (a first-generation photosensitizer) on one occasion, in which the combined therapy yielded a small adjuvant effect 29.

This study therefore explored the potential of TPZ pretreatment to increase efficacy of PDT with liposomal ZnPC and also explored the feasibility of using a single liposomal formulation that contains both TPZ and ZnPC. Since DNA damage and apoptosis are mainly linked via the tumor suppressor protein p53, experiments were performed with p53-functional Sk-Cha1 extrahepatic cholangiocarcinoma cells (derived from a tumor type that is recalcitrant to PDT) and with p53-dysfunctional A431 human epidermoid carcinoma cell line (PDT is generally highly effective in skin cancers) 30. It is demonstrated that, despite obvious differences in the cellular responses to TPZ, PDT efficacy was significantly improved by TPZ in both cell lines. Furthermore, both TPZ and ZnPC can be combined in a singular drug delivery system to enhance PDT efficacy.
2. Materials and Methods

2.1 Chemicals and reagents

1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), cholesterol, and 3β-[N-(N',N'-dime-thylaminoethane)-carbimoyl]cholesterol (DC-cholesterol) were obtained 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), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), bovine serum albumin (BSA, fraction V), L-tryptophan, paraformaldehyde, sucrose, glycine, β-mercaptoethanol, fibronectin, cholesterol, TPZ, ferrous sulfate (Fluka), DMSO, Triton X-100, and pyridine were from Sigma-Aldrich (St. Louis, MO). MitoTracker Red CMX-ROS (MTR), 2',7'-dichlorodihydrofluorescein-diacetate (DCFH2-DA), 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), and propidium iodide (PI) were acquired from Life Technologies/Molecular Probes (Eugene, OR). Ethanol and methanol were from Biosolve (Valkenswaard, the Netherlands). Water-soluble tetrazolium-1 (WST-1) and RNase A were purchased from Roche Diagnostics (Basel, Switzerland). Anti-phospho-H2AX (Ser139)-AlexaFluor647 (clone 20E3) rabbit monoclonal antibody was from Cell Signaling Technology (Danvers, MA).

For the oxidation and antioxidant assays, DCFH2 (40 mM stock in methanol) was prepared from DCFH2-DA as described in 31. All (derivatized) lipids were dissolved in chloroform, purged with nitrogen gas, and stored at -20 ºC. ZnPC was dissolved in pyridine at a 178-μM concentration, purged with nitrogen gas, and stored at room temperature (RT). The following compounds (stock concentration) were dissolved in DMSO and stored at -20 ºC: TPZ (10 mM), JC-1 (5 mg/mL), MTR (10 mM), DCFH2-DA (5 mM), PI (1 mg/mL).

2.2 Absorption and fluorescence spectroscopy

Absorption and fluorescence emission and excitation spectroscopy were performed on a Lambda 18 spectrophotometer (Perkin Elmer, Wellesley, MA) and a Cary Eclipse luminescence spectrometer (Varian, Palo Alto, CA), respectively. Spectra were normalized to the maximum absorption or fluorescence emission intensity.

2.3 Oxidation assays in cell-free environment

The oxidative and antioxidant properties of TPZ were assayed using an in vitro test system as described in 17,31. In a first test arm, TPZ-mediated oxidation of the redox-sensitive fluorogenic molecular probe, DCFH2, was assayed 31. DCFH2 is nonfluorescent at λex = 500 nm but is converted to the highly fluorescent dichlorofluorescein (DCF) upon oxidation. Accordingly, 1,483 µL of ‘HEPES buffer’ (10 mM HEPES in MilliQ, pH = 7.4) was transferred to a cuvette containing a magnetic stirrer that was placed in a temperature-controlled (20 ºC) cuvette holder of the spectrophotometer (operated in kinetics mode). At t = 60 s, 1.5 µL of 40 mM DCFH2 in methanol was added to the buffer. At t = 120 s, 15 µL of 10 mM TPZ, 5 mM of TPZ, 1.25 mM of TPZ, or DMSO; t = 180 s, 15 µL of Fe(II)SO4 (30 mM in MilliQ, positive control for TPZ), or MilliQ (negative control for Fe(II)SO4) was added. Spectral acquisition was performed at λex = 500 ± 5 nm and λem = 523 ± 5 nm (DCF fluorescence). The antioxidant properties of TPZ were assessed in two separate runs using DCFH2 as a reducing agent and a similar approach as in the previous experiments. In the first run, Fe(II)SO4 was employed as an oxidizing agent, whereas in the second laser-irradiated ZnPC-ETLs (section 2.4) were used as ROS generator. For run 1, the settings were as follows: t = 0 s, 1,468 µL of HEPES buffer; t = 60 s, 1.5 µL of DCFH2; t = 120 s, 15 µL of 10 mM TPZ, 5 mM of TPZ, 1.25 mM of TPZ, or DMSO; t = 180 s, 15 µL of Fe(II)SO4. Run 2: t = 0 s, 1,468 µL of HEPES buffer; t = 60 s, 1.5 µL of DCFH2; t = 120 s, 15 µL of 10 mM TPZ or DMSO; t = 180 s, 15 µL of ZnPC-ETLs; t = 240-360 s, laser irradiation (300 mW, applied as described in 17). Data in all experiments were corrected for the average fluorescence intensity of the last 10s of acquisition before addition of the last compound to the cuvette.
2.4 Liposome preparation and characterization

ZnPC-containing cationic liposomes (referred to as ‘ZnPC-ETLs’) were prepared according to the lipid film hydration technique. DPPC (66 mol%), DC-cholesterol (25 mol%), cholesterol (5 mol%), DSPE-PEG (4 mol%), and ZnPC (at a 0.003 molar ratio relative to the final lipid concentration) were mixed at the indicated ratios. Liposomes were prepared as described in. ZnPC, with a logP value of ~8, was incorporated into the lipid bilayer of the liposomes. The ZnPC-ETLs had a diameter of 111.6 ± 3.3 nm, a PDI of 0.151 ± 0.038, and a ζ-potential of +4.3 ± 1.1 mV.

TPZ liposomes (‘TPZ-ETLs’) were prepared in a similar manner as the ZnPC-ETLs, with the exception that TPZ was added to the lipid mixture at predefined molar ratios. TPZ was dissolved in methanol:DMSO:water at a 20:0.2:0.2 volume ratio at a stock concentration of 1 mM. The solution was heated at 60-70 °C until complete dissolution was achieved. Unless indicated otherwise, unencapsulated TPZ was removed by size exclusion chromatography in a 2.5-mL syringe (Sephadex G50 fine, 2.5-mL column volume, centrifugation at 2,000 ×g for 3 min at 4 °C for column drying, 250 µL loading volume, elution at 800 × g for 8 min at 4 °C). The TPZ-ETLs were stored under nitrogen gas at 4 °C in the dark. Size exclusion chromatography was performed within 24 h prior to use. TPZ has an estimated logP of between -0.061 and 1.31 (http://www.drugbank.ca/drugs/DB04858), is therefore mainly water-soluble and thus localizes to the aqueous core of the liposome.

2.5 Cell culture

Human epidermoid (A431) carcinoma cells were cultured in phenol red-containing Dulbecco’s modified Eagle’s medium (DMEM, Gibco/Life Technologies) supplemented with 10% fetal bovine serum (Gibco), 2 mM L-glutamine, penicillin/streptomycin (100 U/mL and 100 µg/mL, respectively, both from Lonza, Basel, Switzerland). A431 were typically subcultured once a week at a 1:25 ratio and seeded at 1.5×10^5 cells/mL. Seeding was performed in 24-wells plates (500 µL of medium per well) or 6-wells plates (2 mL of medium per well) (Costar, Corning, Corning, NY). For all procedures not involving standard cell culture (i.e., the experimental steps), cells were incubated in serum- and phenol red-free, fully supplemented DMEM equilibrated at 37 ºC. Human EHCC (Sk-Cha1) cells were cultured in Roswell Park Memorial Medium (RPMI) 1640 as described in 17.

Cells were maintained at standard normoxic culture conditions (20% O₂, 5% CO₂, 37 ºC) or at hypoxic culture conditions (<1% O₂, 5% CO₂, 37 ºC 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).

2.6 Photodynamic therapy and neoadjuvant therapy with TPZ

For PDT, the culture medium was replaced with fresh medium containing ZnPC-ETLs. The ZnPC-ETL final lipid concentration was 10 µM for cells in the normoxia group and 5 µM for cells in the hypoxia group. After 1-h incubation, cells were washed twice with PBS (RT) and resuspended in fresh medium. Next, the cells were irradiated with a 671-nm laser (CNI, Changchun, China) at 500 mW, 60-s pulse duration, and a spot size of 1.9 cm², corresponding to an irradiance of 263 mW/cm² and a cumulative radiant exposure of 15.8 J/cm². Following PDT, cells were incubated at either standard culture conditions or at hypoxic culture conditions, the duration of which is indicated separately per experiment.
Cells were subjected to PDT and normoxic incubation conditions post-PDT as follows. On day 0, all cells were seeded as described in section 2.5 and allocated to the control group (CTRL), TPZ group (only TPZ pretreatment), PDT group (only PDT), or TPZ + PDT group (TPZ pretreatment followed by PDT). On day 1, cells received serum-free medium (CTRL and PDT groups) or serum-free medium containing either 50 μM (A431 cells) or 100 μM TPZ (Sk-Cha1 cells). On day 2, the medium was removed and cells received fresh, serum-free culture medium (CTRL and TPZ groups) or serum-free medium containing 10 μM ZnPC-ETLs (final lipid concentration, PDT and PDT + TPZ groups). Cells were incubated with ZnPC-ETLs for 1 h at standard culture conditions and irradiated as described in section 2.5. Subsequently, cells were kept at standard culture conditions for 4 or 24 h.

Alternatively, cells were subjected to PDT and hypoxic incubation conditions post-PDT as follows. Cells were seeded on day 0 and received TPZ on day 1 (50 μM for A431 cells, 100 μM for Sk-Cha1 cells) for 24 h. Following replacement of medium on day 2, cells were incubated with 5 μM ZnPC-ETLs for 1 h, irradiated, and subsequently maintained under hypoxic culture conditions for 4 or 24 h to mimic vascular shutdown conditions post-PDT. All PDT experiments with TPZ-ETLs were performed with liposomes from which the non-encapsulated TPS was removed by size exclusion chromatography (section 2.4).

2.7 Determination of cell viability

Cell viability was determined using the WST-1 assay as described in 17.

2.8 Measurement of intracellular reactive oxygen species formation

Intracellular ROS formation was detected using DCFH\(_2\)-DA. The cells were incubated with TPZ (50 μM final concentration) for 24 h, resuspended in fresh medium, and incubated for 4 h at either normoxic or hypoxic conditions (section 2.6). The medium was then replaced by fresh medium containing 25 μM DCFH\(_2\)-DA (final concentration) and the cells were incubated for 30 min at standard culture conditions. Next, the cells were washed twice with 0.5 mL PBS (RT) and resuspended in 0.5 mL PBS (RT). Intracellular DCF formation was measured at \(\lambda_{ex} = 460 \pm 40\) nm and \(\lambda_{em} = 540 \pm 15\) nm in a fluorescence plate reader (BioTek Synergy HT). Immediately thereafter, the PBS was removed and the cells were lysed with lysis solution (20% methanol and 0.1% Triton X-100 in MilliQ). Total protein was determined with the bicinchoninic acid assay (Pierce, Rockford, IL). DCF fluorescence intensities were corrected for background fluorescence intensity (control cells not incubated with DCFH\(_2\)-DA) and divided by the total protein content.

2.9 Confocal laser scanning microscopy

For PDT/TPZ-induced DNA damage experiments, cells were seeded in 6-wells plates containing fibronectin-coated microscope coverslips (24 × 40 mm, VWR, Lutterworth, UK) at the densities described in section 2.5. Two lines of experiments were performed in which cells were seeded, treated by PDT, and kept under either normoxic or hypoxic conditions. In the normoxic test arm, the cells were untreated (CTRL; no TPZ or PDT) or treated with TPZ as described above, PDT (10 μM ZnPC-ETLs (final lipid concentration) for 1 h as described in section 2.6), or TPZ + PDT. Cells in all groups were kept under normoxic culture conditions for 4 h after treatment. In the hypoxic test arm, the cells were treated as described for the normoxic groups except that a 5-μM ZnPC-ETL final lipid concentration was used in the PDT and TPZ + PDT groups and that the cells were kept for 4 h under hypoxic culture conditions after treatment. When indicated, cells were stained with 50 nM MTR in fresh medium for 30 min at standard culture conditions. For the staining of damaged DNA, cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min and washed with 1 mL of PBS. Subsequently, cells were incubated with anti-phospho-H2AX-AlexaFluor-647 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. Subsequently, cells were washed with 1 mL of PBS (RT), fixed in 1 mL of 4% (w/v) paraformaldehyde
and 2% (w/v) sucrose for 5 min, and washed with 1 mL of PBS (RT). The coverslips were mounted onto microscope slides using 10 μL of 4',6-diamidino-2-phenylindole (DAPI)-containing Vectashield mounting medium (Vector Labs, Burlingame, CA). The slides were dried for 1 h and sealed with nail polish. Cells were imaged with a Leica TCS SP8 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany). Fluorescence was acquired at λex = 405 nm and λem = 415-480 nm for DAPI, λex = 470 nm and λem = 480-550 nm for TPZ, λex = 579 nm and λem = 589-650 nm for MTR, and λex = 647 nm and λem = 657-750 nm for phospho-H2AX-AlexaFluor647.

2.10 Cell cycle analysis

For cell cycle analysis, A431 cells were seeded at 7.5×10^4 cells/mL and Sk-Cha1 cells at 1.25×10^5 cells/mL in 6-wells plates. Cells were cultured for 24 h at standard culture conditions until approximately 80% confluence was reached. Afterwards, the cells were incubated for 24 h in medium containing either 50 μM TPZ (A431) or 100 μM TPZ (Sk-Cha1). The cells were washed with PBS (RT) and resuspended in fresh medium containing either 10 μM (normoxia group) or 5 μM (hypoxia group) ZnPC-ETLs (final lipid concentration) for 1 h. Next, the cells were washed twice with PBS (RT), resuspended in fresh medium, and subjected to PDT as described in section 2.6. After PDT the cells were incubated under either normoxic conditions or hypoxic conditions for 24 h. Next, cells were washed with PBS (RT, 1 mL/well) and harvested by Accutase treatment (250 μL/well, 10 min). The supernatant was removed and cells were resuspended in 0.3 mL PBS (RT) and kept on ice. Subsequently, cells were fixed by the dropwise addition of 0.7 mL of ice-cold 96% ethanol during gentle swerving. The cells were pelleted by centrifugation and the supernatant was aspirated, followed by resuspension of the pellet in 0.5 mL PBS containing 50 μg/mL PI and 20 μg/mL RNase A (both final concentrations) and incubation for 30 min at RT in the dark. Cell cycle profiles were obtained by flow cytometry (FACSCantoll, BD Biosciences, Franklin Lakes, NJ) at λex = 488 nm and λem = 585 ± 42 nm (PI fluorescence) and analyzed with FlowJo software (Treestar, Ashland, OR) based on 10,000 events. Cell cycle quantitative metrics (‘quantrics’) were determined based on the PI fluorescence intensity histograms. The G1 population (one copy of the genome per cell) was characterized by a PI fluorescence peak between 80 and 120 a.u., the G2 population (two copies of the genome per cell) typically exhibited a peak between 180 and 220 a.u. The S-phase population was distinguishable between the G1 and G2 peaks (i.e., PI fluorescence intensity between 120 and 180 a.u.).

2.11 Determination of mode of cell death.

Loss of mitochondrial membrane potential (MMP) as a result of mitochondrial permeability transition (MPT) was measured with JC-1 as an early marker for apoptosis. Cells were grown in 6-wells plates as described in section 2.5 and treated by TPZ (section 2.6), PDT (section 2.6), or TPZ + PDT. Following irradiation, the cells were washed with 1 mL of PBS (RT) and resuspended in 500 μL of fresh medium containing JC-1 (10 μg/mL final concentration). The cells were incubated for 30 min at standard culture conditions, washed twice with 1 mL PBS (RT), and detached by Accutase treatment (250 μL/well, ~10 min) at standard culture conditions. Next, 1 mL of medium was added and the cells were centrifuged for 5 min at 400 ×g (4°C), followed by resuspension in 500 μL of ice-cold PBS and flow cytometric analysis (FACSCantoll). JC-1 aggregates in mitochondria (signifying intact MMP) were determined at λex = 488 and λem = 585 ± 42 nm, whereas JC-1 monomers in the cytosol (signifying induction of MPT and loss of MMP) were determined at λex = 488 and λem = 530 ± 30 nm. Viable cells were gated based on forward- and side-scattering properties and subsequently characterized based on their fluorescence properties; green fluorescence indicated cells with perturbed MMP, red fluorescence indicated cells with intact MMP. The percentage of green fluorescent cells was calculated from the total (red + green) events. The data were processed in FlowJo software and presented as average ± SD of 1×10^5 events for N = 3 measurements.
2.12 Intracellular TPZ determination

A431 and Sk-Cha1 cells were seeded into 6-wells plates as described section 2.5 and grown to confluence overnight. Cells were incubated for 1 h with 100 μM TPZ or 100 μM TPZ-ETLs that contained 1 mM intraliposomal TPZ. It should be noted that the liposomes were not subjected to size exclusion chromatography. The concentration of free TPZ in the medium was therefore 50 μM upon incubation of cells with 100 μM TPZ-ETLs. After incubation, cells were lysed by the addition of 200 μL of ice-cold MilliQ and 10-min incubation on ice. The lysates were harvested and the residual cells on the plate were dissolved in 750 μL lysis solution (section 2.8). Intracellular TPZ concentrations were measured by spectrophotometric analysis of the lysates (Lambda Bio, Perkin Elmer). The absorption was measured at 470 nm, corrected for background (lysates obtained from cells not incubated with TPZ), and the concentrations were calculated by means of a standard curve (linear regression). The intracellular TPZ concentration was corrected for total protein content using the bicinchoninic acid assay (section 2.8).

2.13 Statistical analysis

Statistical analysis was performed in GraphPad Prism (GraphPad Software, La Jolla, CA). Normal distribution of data sets was evaluated with a Kolmogorov-Smirnov test (N ≥ 5). Differences between normally distributed ordinal variables were tested with one-way ANOVA and a Bonferroni post-hoc test (N ≥ 5). Non-Gaussian data was analyzed using a Mann-Whitney U-test or a Kruskall-Wallis analysis and Dunn’s post-hoc test. In the figures, intergroup differences are indicated with an asterisk and differences between treated groups versus the untreated (CTRL) group are indicated with a pound sign. The level of significance is depicted as a single (p < 0.05), double (p < 0.01), triple (p < 0.005), or quadruple sign (p < 0.001). Data are presented as mean ± standard deviation throughout the manuscript.

3. Results

3.1 Oxidant and antioxidant properties of tirapazamine

Measuring the optical properties of TPZ yielded a strong absorption peak at 470nm (Fig. 1A). As shown in Fig. 1B, TPZ was unable to oxidize DCFH₂⁻, whereas the addition of Fe(II)SO₄ caused gradual oxidation of DCFH₂ by various ROS intermediates (e.g., HO₂⁻, H₂O₂, •OH) formed as a result of reactions between Fe(II) and O₂.³³ For a compound to be used as adjuvant in PDT, it is also essential that the compound possesses minimal-to-no antioxidant properties so that the photoproduced ROS are not scavenged. In a test system where DCFH₂ was used as ROS substrate for Fe(II)SO₄-produced ROS, the addition of TPZ decreased the oxidation of DCFH₂ in a concentration-independent manner by 32.2% on average (Fig. 1C). Similarly, the ZnPC-ETL-mediated oxidation of DCFH₂ by photoproduced ¹⁰O₂ ³⁴ was reduced by 21.9% in the presence of TPZ, altogether indicating that TPZ is an antioxidant capable of quenching radical- and non-radical ROS (Fig. 1D). This may potentially interfere with PDT efficacy if the intracellular TPZ and ZnPC localization are similar, given the short diffusion distance of photoproduced ROS in a biological milieu.³⁵,³⁶

3.2 Tirapazamine is toxic under hypoxic conditions in a cell type-dependent manner

Before determining the adjuvant efficacy of TPZ, the inherent toxicity of increasing concentrations TPZ was assessed under normoxic and hypoxic conditions in A431 and Sk-Cha1 cells in the absence of PDT. Under normoxic conditions, TPZ was moderately toxic to A431 (Fig. 2A) and Sk-Cha1 cells (Fig. 2E) as evidenced by a relative viability of 66% and 60%, respectively, at a 100-μM TPZ concentration. Under hypoxic culture conditions, A431 cells exhibited profound toxicity at a TPZ
concentration of >10 μM. A similar pattern was observed for Sk-Cha1 cells, although hypoxic A431 cells were more susceptible to TPZ than hypoxic Sk-Cha1 cells. However, hypoxia sensitized both cell types to TPZ, which is potentially beneficial for an acute hypoxia-inducing treatment such as PDT.

3.3 Tirapazamine-induced cell death is associated with increased oxidative stress

Since intracellular TPZ is known to form radical species under hypoxic conditions, such as TPZ, O$_2^-$, and ‘OH $^{37}$, we tested whether cancer cells exhibit increased oxidative stress in the presence of TPZ. Accordingly, cells were incubated with 50 μM (A431 cells) or 100 μM TPZ (Sk-Cha1 cells) for 24 h under normoxic or hypoxic conditions and assayed for oxidative stress using the cell-permeant and redox-sensitive probe DCFH$_2$-DA. In both cell lines, hypoxia resulted in a moderate increase in oxidative stress (Fig. 2B and F); a phenomenon that has been reported before $^{38}$. TPZ did not result in increased oxidative stress in Sk-Cha1 cells and induced very mild oxidative stress in A431 cells (Fig. 2B and F). On the other hand, TPZ + hypoxia was associated with a 316% and 162% increase in the degree of oxidative stress in A431 and Sk-Cha1 cells, respectively. These results suggest that the cytotoxicity of TPZ is caused by ROS generation, which is significantly exacerbated under hypoxic conditions.

3.4 Tirapazamine exacerbates PDT-induced cell death in a cell type-dependent manner

To test whether normoxic pre-incubation of cells with minimally toxic concentrations of TPZ could exacerbate PDT-induced cell death, cells were incubated for 24 h with either 50 μM TPZ (A431 cells) or 100 μM TPZ (Sk-Cha1 cells) as these concentrations resulted in a ~40% reduction in viability in the cell lines (Fig. 2A and E). Subsequently, cells were treated with PDT and cultured for 4 h under either normoxic or hypoxic conditions. It should be underscored that, when normoxic incubation was performed post-PDT, cells were photosensitized with 10 μM ZnPC-ETLs. When incubation was performed under hypoxic conditions post-PDT, cells were photosensitized with 5 μM ZnPC-ETLs. This regimen induced a ~50% reduction in viability under the given experimental conditions, and was employed in all experiments unless noted otherwise. The hypoxic conditions were tested as a model for the post-PDT tumor microenvironment (vascular shutdown-mediated hypoxia).

TPZ exhibited no normoxic toxicity and mild hypoxic toxicity in A431 cells (Fig. 2C and D). PDT alone decreased the relative viability of A431 cells to 52-51% under both normoxia and hypoxia. The combination of PDT with TPZ pre-incubation (TPZ + PDT) did not further reduce cell viability in normoxic A431 cells (relative viability of 60%) compared to PDT alone, but significantly decreased the viability to 39% when A431 cells were incubated under hypoxic conditions after PDT.

For Sk-Cha1 cells (Fig. 2G and H), TPZ pre-incubation was mildly toxic under both normoxic and hypoxic conditions (relative viability of 70-75%). PDT alone reduced the relative cell viability to 42% under normoxic conditions (10 μM ZnPC-ETLs) and to 70% under hypoxic conditions (5 μM 224
ZnPC-ETLs). In contrast to A431 cells, Sk-Cha1 cells were more sensitive to TPZ + PDT compared to PDT alone, yielding an additional reduction in relative cell viability of 22% and 28% under normoxic and hypoxic conditions, respectively. As such, these data represent a 54% and 41% increase in efficacy when PDT was combined with TPZ versus PDT alone under normoxic and hypoxic conditions, respectively.

In sum, the findings indicate that TPZ preconditioning can be used to considerably increase the therapeutic efficacy of PDT in Sk-Cha1 cells and to a much lesser extent in A431 cells.

3.5 Tirapazamine induces DNA damage in a PDT-independent manner

To investigate the manifestation of TPZ-induced DNA damage after PDT in accordance with literature, cells were either left untreated or treated by TPZ for 24 h, PDT (5 μM ZnPC-ETLs), or TPZ + PDT, incubated for 4 or 24 h under hypoxic conditions to mimic the in vivo conditions, and stained with anti-phospho-H2AX, an epigenetic marker for DNA DSBs. The DNA DSB data obtained at 4 h post-PDT are presented in Fig. 3. Representative cell cycle profiles of similarly treated cells were assayed after 24 h of hypoxic incubation post-PDT/CTRL treatment and are also presented in Fig. 3. The cell cycle quantrics are provided in Fig. 4.

A431 cells in the control (Fig. 3A and B) and PDT group (Fig. 3E and F) exhibited only minor levels of phospho-H2AX foci, indicating that neither hypoxia nor PDT induced DNA DSBs. Constitutive DNA damage was more prevalent in A431 cells compared to Sk-Cha1 cells (Fig. 3E versus F and Fig 5A/E versus B/F), which is consistent with the fact that A431 cells do not contain a functional p53 protein and thus better tolerate DNA damage, which is not the case for Sk-Cha1 cells. PS3 is
Figure 3. Confocal laser scanning microscopy of DNA damage and cell cycle profiles of differently treated cells following hypoxic incubation. A431 (panels A,C,E,G) and Sk-Cha1 cells (panels B,D,F,H) were pretreated with non-supplemented medium (Ctrl and PDT) or medium supplemented with TPZ (A431, 50 µM; Sk-Cha1, 100 µM) and incubated under normoxic conditions for 24 h. Afterwards, cells were either not irradiated (Ctrl, TPZ) or treated by PDT (PDT and TPZ + PDT, 5 µM ZnPC-ETLs). All cells were incubated under hypoxic conditions for 4 h and assayed for DNA DSBs by confocal microscopy following phospho-H2AX (red) and DAPI (blue) staining. Additionally, the cell cycle profiles were determined in each group by flow cytometry after 24 h of hypoxic incubation. The G1, S, and G2/M populations are indicated in the histograms (representative histograms are provided from N = 3 experiments/group). The groups are labeled in green at the top of each panel set.

responsible for DNA repair and initiation of DNA damage-induced apoptosis and regulates cell cycle progression based on the extent of DNA damage. The cell cycle profiles confirm that the distribution of cells in G1, S, or G2/M phase was only minimally affected by PDT, although A431 cells did exhibit a mild proliferative tendency in response to PDT as evidenced by the slightly elevated S- and G2/M phase cell populations (Fig. 3A and Fig. 4A).

TPZ pretreatment and subsequent hypoxic incubation induced visibly higher levels of phospho-H2AX foci in both A431 and Sk-Cha1 cells (Fig. 3C and D, respectively). The DNA DSBs led to prominent changes in cell cycle profiles after 24 h. The distribution of cells in the S-phase reveals that S-phase inflow was increased in A431 cells (Fig. 3C and Fig. 4A) but unaltered in Sk-Cha1 cells (Fig. 3D and Fig. 4B) compared with control cells. Accumulation of cells in late S-phase occurred in
both cell lines, supporting the hypothesis that the stalling of DNA replication forks during S-phase by inhibition of topoisomerase (necessary for DNA unwinding to allow replication) leads to an intra-S-phase arrest in the presence of TPZ. The increase in DNA DSBs in TPZ-treated hypoxic cells may also explain the increase in G2/M populations, since DNA damage typically stimulates G2-arrest prior to M-phase entry.

Similar results regarding DNA DSBs and effects on cell cycle profiles were observed in cells pre-incubated with TPZ and subsequently treated with PDT (Fig. 3G and H and Fig. 4A and B). Taken together, the reduction in cell viability following TPZ + PDT seems to stem from TPZ-induced DNA-damage and cell cycle stalling.

3.6 Tirapazamine induces DNA damage under normoxia in a PDT-independent manner

As was shown in Fig. 2C and G, TPZ also affected cell viability under normoxic conditions. In order to determine whether TPZ exerts its DNA-damaging effects under normoxic conditions, A431 and Sk-Cha1 cells underwent similar treatments as in the previous section, with the exception that the cells were kept under normoxic conditions under all circumstances and were photosensitized with 10 μM ZnPC-ETLs.

The phospho-H2AX staining in untreated cells revealed that the nuclei of A431 cells contained multiple phospho-H2AX-positive foci, whereas these were largely absent in Sk-Cha1 cells (Fig. 5A and B, respectively). The DNA damage translated to similar cell cycle characteristics as reported for hypoxic cells in both cell lines (Fig. 5A and B and Fig. 4C and D). The degree of DNA DSBs did not increase in Sk-Cha1 cells after PDT (Fig. 5F), whereas the extent of DNA damage was slightly exacerbated after PDT in A431 cells (Fig. 5E), which was accompanied by a mild increase in cell populations in the S-phase and G2/M-phase (Fig. 5E and Fig. 4C). TPZ treatment increased the number of phospho-H2AX foci under normoxic conditions (Fig. 5C and D), although the effect was not as extensive as under hypoxic conditions (Fig. 3C and D). As reported for the hypoxic conditions, the cell cycle profiles under normoxic conditions revealed an increased proliferation rate in TPZ-subjected A431 cells (Fig. 5C and Fig. 4C) and an increase in the number of late-S-phase Sk-Cha1 cells (Fig. 5D and Fig. 4D), which suggests that the implicated topoisomerase inhibition is hypoxia-independent. TPZ + PDT (Fig. 5G-H) visibly increased the phospho-H2AX foci to a comparable level as observed under hypoxic conditions (Fig. 3G and H). Accordingly, the transient depletion of oxygen by PDT (O2 -> ROS) may be sufficient to produce TPZ capable of inducing DNA oxidation and subsequent DNA DSBs insofar as prolonged hypoxia did not greatly exacerbate the occurrence of these lesions (Fig. 3G and H). Cell cycle analysis of cells treated with TPZ + PDT and subsequent normoxic incubation further confirms that the resulting DNA damage culminates in S-phase stalling and G2-arrested cells (Fig. 5G/H and Fig. 4C/D). Thus, the adjuvant effect of TPZ to the efficacy of PDT is not limited by oxygen tension, as TPZ induces DNA damage and increases the PDT effect even under normoxic culture conditions.
3.7 Tirapazamine is a potent inducer of apoptosis in combination with PDT

During imaging of DNA DSBs (Fig. 3 and 5) we frequently observed blebbing nuclei in the treated Sk-Cha1 cells, which is indicative of apoptosis. Additional confocal microscopy was therefore performed with Sk-Cha1 and A431 cells that had been treated with TPZ and TPZ + PDT and labeled with MTR, DAPI, and phospho-H2AX. Both treatments yielded a substantial amount of apoptotic Sk-Cha1 cells (exposed to 100 μM TPZ) with distinct blebbing morphology (Fig. 6A-D, arrows) regardless of oxygen tension. In addition, the apoptotic nuclei were intensely positive for phospho-H2AX, which may be a consequence of nuclease activity inherent to apoptosis \(^{48}\) or the result of extensive TPZ/TPZ\(^{•}\)-mediated DNA DSB formation. The apoptosis-associated blebbing was not observed in

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**Figure 5.** Confocal laser scanning microscopy of DNA damage and cell cycle profiles of differently treated cells following normoxic incubation. A431 (panels A,C,E,G) and Sk-Cha1 cells (panels B,D,F,H) were pretreated with non-supplemented medium (Ctrl and PDT) or medium supplemented with TPZ (A431, 50 μM; Sk-Cha1, 100 μM) and incubated under normoxic conditions for 24 h. Afterwards, cells were either not irradiated (Ctrl, TPZ) or treated by PDT (PDT and TPZ + PDT, 10 μM ZnPC-ETLs). All cells were incubated under normoxic conditions for 24 h and assayed for DNA DSBs by confocal microscopy following phospho-H2AX (red) and DAPI (blue) staining. Additionally, the cell cycle profiles were determined in each group by flow cytometry after 24 h of normoxic incubation. The G1, S, and G2/M populations are indicated in the histograms (representative histograms are provided from \(N = 3\) experiments/group). The groups are labeled in green at the top of each panel set.
A431 cells incubated with 50 μM TPZ (not shown).

The intrinsic pathway of apoptosis is characterized by mitochondrial permeability transition (MPT) and loss of MMP, which ensues e.g., p53-mediated pro-apoptotic BCL-2 protein activation (including BAX, PUMA, and NOXA). Loss of mitochondrial membrane integrity as a result of MPT can be measured with the fluorescent probe JC-1 (section 2.11).

Under normoxic conditions, the majority of control and 50 μM TPZ-treated A431 cells did not exhibit mitochondrial membrane perturbations (Fig. 6E), which is in line with the viability data (Fig. 2C). PDT with ZnPC-ETLs (10 μM) caused a 31% increase in MPT, whereas the TPZ + PDT regime had no additional effect on mitochondrial integrity (Fig. 6E). These findings are in agreement with the viability profiles of comparably treated normoxic A431 cells (Fig. 2C). Similar trends were observed in hypoxic A431 cells in the control, TPZ (50 μM), and PDT (5 μM ZnPC-ETLs) groups (Fig. 6F), although the effect of PDT on MPT was dampened under hypoxic conditions relative to normoxic cells while TPZ exacerbated the loss of mitochondrial integrity in PDT-treated A431 cells. These findings were mirrored by significantly increased oxidative stress by TPZ (Fig. 2B) and relative viability data (Fig. 2D) under hypoxia.

With respect to Sk-Cha1 cells, normoxic cells exhibited similar levels of MMP loss (4% of cells) as normoxic A431 cells, whereas hypoxia considerably increased MMP loss to 21% of cells (Fig. 6G). TPZ (100 μM) had no significant effect on MMP loss under normoxic conditions (Fig. 6G), despite the fact that it was associated with reduced cell viability (Fig. 2G) in a ROS-independent manner (Fig. 2F). Furthermore, 100 μM TPZ exhibited a tendency towards cytoprotection (albeit not significant) under hypoxia (Fig. 6G) despite the considerable TPZ-induced increase in oxidative stress (Fig. 2F) and reduction in cell viability (Fig. 2H). PDT (10 μM ZnPC-ETLs) caused MMP loss in a significantly higher percentage of normoxic cells (49%), which was further aggravated in cells treated with 100 μM TPZ + 5 μM ZnPC-ETL PDT (60%). The effect of PDT on MMP loss was slightly reduced under hypoxic conditions, where TPZ exhibited no additional effect on MMP loss in PDT-subjected Sk-Cha1 cells (Fig. 6H), suggesting that other cell death pathways may cause the reduction in cell viability (Fig. 3H).
3.8 Encapsulation of tirapazamine in ZnPC-ETLs increases the efficacy of PDT

TPZ is very poorly taken up by cells yet exacerbates the extent of PDT-induced cell death in hypoxic A431 cells (Fig. 2D) and in Sk-Cha1 cells regardless of oxygen tension (Fig. 2G and H). The inclusion of TPZ into ZnPC-ETLs may therefore increase the intracellular availability of TPZ and further increase PDT efficacy using a single drug delivery system. In the last set of experiments, ZnPC- and TPZ-ETLs were prepared with fixed ZnPC content and increasing TPZ concentrations. To investigate whether these liposomes remain stable in the presence of ZnPC, the size and PDI of TPZ-ETLs was followed for 7 days post preparation, at which the liposomes were either stored at 4 °C or 37 °C. The results, shown in Table 1, show that the sizes and PDI's remain highly stable throughout the experiment, and were neither affected by the storage temperature nor the presence or absence of ZnPC.

Fig. 7A and E illustrate that incubation of cells with TPZ-ETLs (1 mM intraliposomal TPZ concentration) versus cells incubated with free TPZ (100 μM) resulted in an approximately 10-fold increase in intracellular TPZ in case of the TPZ-ETLs. This proved that liposomal encapsulation of TPZ results in increased intracellular availability of TPZ. All further experiments were performed with TPZ-ETL solutions from which the non-encapsulated TPZ had been removed. Fig. 7B and F show that there is no dark toxicity of TPZ-ETLs in A431 and Sk-Cha1 cells at a final lipid concentration of 10 μM. More importantly, the therapeutic efficacy of PDT with TPZ-ETLs was substantially higher compared to PDT with free TPZ preconditioning. Inclusion of 0.1 and 1 mM of TPZ into ZnPC-ETLs decreased A431 cell viability after PDT to 62 and 67% under normoxic conditions (Fig. 7C), respectively, and to 32 and 34% under hypoxic conditions (Fig. 7D), respectively, compared to PDT with TPZ-lacking ZnPC-ETLs (representing 100% relative viability). A similar dose effect was observed in Sk-Cha1 cells; incorporation of 0.1 and 1 mM of TPZ into ZnPC-ETLs decreased the relative viability after PDT to 73 and 63% under normoxic conditions (Fig. 7G), respectively, and to 54 and 55% under hypoxic conditions (Fig. 7H), respectively, compared to ZnPC-ETLs not containing TPZ. The data provide compelling in vitro proof-of-concept for the use of fourth-generation photosensitizers (i.e., a second-generation photosensitizer (ZnPC) encapsulated in a targeted photosensitizer delivery system (liposomes) with co-encapsulated adjuvant chemotherapeutics (TPZ)) for PDT.

4. Discussion

This study investigated the adjuvant effect of TPZ on PDT efficacy in cells derived from a therapy-resistant tumor (EHCC, Sk-Cha1) and in cells (A431) derived from a tumor that is equally relevant for PDT but that is distinct from Sk-Cha1 cells with respect to p53 functionality. Although Sk-Cha1 cells carry a missense mutation in the TP53 gene that encodes the DNA binding domain, there...
is ample evidence that p53 is functional in these cells. In contrast, A431 cells have lost a TP53 allele and carry a missense mutation in the region that encodes the DNA binding domain of the remaining protein, substantially lowering their p53 signaling capability.

The most important findings of this study were that, firstly, TPZ exacerbated PDT-induced cell death under hypoxic conditions in both cell lines. This appeared to be the result of a chain of events that included TPZ formation by nuclear reductases, the consequent induction of DNA DSBs, stalling of cell division, loss of MMP, and cell death via apoptosis. However, there were several important differences observed between the cell lines. Under hypoxic conditions, TPZ was more toxic to A431 cells compared to Sk-Cha1 cells, yet TPZ exhibited an adjuvant effect on PDT efficacy under normoxic conditions in Sk-Cha1 cells but not in A431 cells (albeit at a two-fold higher TPZ concentration than was used for A431 cells). Secondly, the extent of TPZ's adjuvant efficacy was not reliant on the p53 protein insofar as a decrease in post-PDT cell viability was observed in both cell lines under hypoxic conditions. Thirdly, these positive results prompted the development of liposomes that included both TPZ and ZnPC in a single liposomal formulation so as to enable the optimal delivery of both compounds to tumor cells. The TPZ-ETLs exhibited no dark toxicity but were more lethal to either cell type after PDT compared with ZnPC-ETLs lacking TPZ — an effect that was more pronounced in A431 cells than in Sk-Cha1 cells, especially under hypoxic conditions.

Before discussing the neoadjuvant effect of TPZ on PDT efficacy, it is imperative to address TPZ as a stand-alone pharmaceutical compound in the absence of PDT. The putative mechanism of action entails the TPZ -> TPZ conversion in the nucleus under hypoxic conditions, resulting in oxidative damage to DNA bases through the formation of formamidopyrimidine and 5-hydroxy-6-hydroxypyrimidine, potentially leading to mismatches and single strand breaks (SSBs). This type of base damage is either repaired via base excision repair or results in DNA DSBs when cells with DNA lesions progress through S phase. Accordingly, TPZ-induced DNA base damage, SSBs, and DSBs have been reported previously. The different types of DNA damage may emanate from TPZ's...
hypoxia-independent inhibition of topoisomerases and the hypoxia-dependent formation of TPZ• and subsequent base oxidation.

Although the hypoxia-driven mechanism of action was confirmed by our experiments, TPZ/TPZ• also exerted a pharmacological effect under normoxic conditions when combined with PDT. Since PDT rapidly depletes intracellular O₂ levels by the conversion into ROS, the transient post-PDT hypoxia appeared to be sufficient to form TPZ• and induce DNA DSBs and subsequent cell death, even under normoxic culture conditions. The reason behind TPZ being more toxic to p53-dysfunctional A431 cells compared to p53-functional Sk-Cha1 cells is currently elusive. In addition, non-irradiated, TPZ-treated hypoxic Sk-Cha1 and A431 cells exhibited a considerable increase in intracellular oxidative stress as well as an elevated degree of DNA DSBs. This TPZ-mediated increase in oxidative stress translated to augmented MPT in PDT-subjected A431 cells but not Sk-Cha1 cells, suggesting that the mechanism behind the cell death exacerbation in the TPZ + PDT groups differed between Sk-Cha1 and A431 cells. These phenomena may be a consequence of the higher proliferation rate of A431 cells versus Sk-Cha1 cells, causing the DNA damage to be propagated more quickly in A431 cells, resulting in mitotic catastrophe and cell death.

In contrast to the above, the other results were in agreement with the p53-status of the two cell types. TPZ induced oxidative stress under hypoxic conditions that led to DNA DSBs in both cell lines. The cell cycle of p53-dysfunctional A431 cells was minimally affected by adjuvant TPZ, whereas the cell cycle was affected in Sk-Cha1 cells. In addition, while increased cell death occurred in both cell lines, the increased cell death was associated with more extensive MPT in Sk-Cha1 cells but not A431 cells. Thus, the augmented cell death signaling in TPZ + PDT-treated A431 cells appeared to circumvent the p53-MPT route, whereas cell death and growth arrest in Sk-Cha1 cells may have proceeded through p53 via DNA DSB-induced MPT and activation of the p21 cell cycle inhibitor, respectively. Based on the above it appears that TPZ has prominent adjuvant potential in combination with PDT, although the mechanism of cell death appears to be dependent on the p53-status and proliferation rate of the tumor cells.

Given TPZ’s confirmed hypoxic cytotoxin properties, we tested whether PDT-induced hypoxia, which was emulated by the incubation of PDT-subjected cells under hypoxic conditions, would be a potent activator of TPZ that would exacerbate cancer cell death via auxiliary pathways not induced by PDT, such as DNA damage. Most photosensitizers localize predominantly to the endoplasmic reticulum, Golgi apparatus, mitochondria, and/or lysosomes. Their intracellular localization in combination with the fact that ROS have a short action radius in a biological milieu causes PDT-induced damage to be typically confined to the aforementioned organelles, whereby the nucleus is left unscathed. Nuclear ‘protection’ from PDT was corroborated by the absence of increased phospho-H2AX staining of DNA DSBs in both cell lines. Although TPZ exhibited mild antioxidative properties in a cell-free environment (Fig. 1), TPZ did not reduce the therapeutic efficacy of PDT. This may be due to the induction of DNA damage by TPZ in the nucleus and not the cytoplasm, although the intracellular distribution of TPZ was not investigated. The damage inflicted by PDT is mainly cytoplasmic as a result of the non-nuclear localization of ZnPc (unpublished results). The TPZ-induced DNA damage and corollary induction of apoptosis were hence expected to have an additive impact on the extent of cell death following PDT. Furthermore, increased cell cycle arrest and cell death following PDT + TPZ treatment were hypothesized to mainly occur in hypoxic Sk-Cha1 cells, i.e., cells that are able to regulate these processes via p53 following DNA damage.

There is a large range of applications for liposomal drug delivery in biomedicine that includes delivery of cytostatic or diagnostic drugs for cancer, leishmaniasis, heart disease, port wine stains, and thrombosis, just to name a few. Liposomes are versatile drug carriers since the liposome surface can be modified to gain long circulating capacity using e.g. glucoronide or PEG, cross the blood-brain barrier using p-aminophenyl-α-d-mannopyranoside, and target specifically to tumor cells using antibody-conjugation. Moreover, the lipid component of liposomes can be modified to bestow specific biodistributive properties upon the liposomes. For instance, cationic liposomes have the ability to accumulate specifically at tumor vascular endothelial cells in vivo. A distorted blood flow in angiogenic tumor vessels and the consequential shedding...
of the barrier-forming glyocalyx of endothelial cells is believed to be the underlying mechanism for an increased interaction of the cationic liposomes and the anionic endothelial cell membranes. Cationic liposomes are suitable carriers for \textit{in vitro} drug delivery since they are effectively taken up by many different cell types in culture. For instance, cationic liposomes composed of DPPC, cholesterol, and stearylamine (45:45:10 mol%, 120nm) were previously used to assess the delivery of the radiosensitizer 5-bromo-2-deoxy-uridine (BrdU) to human HMV-II melanoma cells \textit{in vitro}. The cationic liposomes containing BrdU were substantially more effective in radiosensitizing HMV-II cells in comparison to anionic or nonionic liposomes. Taken together, the available literature suggests that liposomes constitute a promising carrier for target-specific delivery of hydrophilic and hydrophobic pharmacological agents, such as ZnPC and TPZ.

Clinically, TPZ has been employed as a stand-alone treatment or as a radio- and chemosensitizer in the treatment of non-squamous cell lung cancer, head and neck cancer, cervical cancer, and melanoma, which are hypoxic tumor types. TPZ has shown limited clinical efficacy in non-hypoxic tumors, and the functionality of p53 has been implicated in the responsiveness of tumor cells to TPZ inasmuch as neuroblastoma cell lines without a functional p53 were significantly less sensitive to TPZ-induced apoptosis than their wild type counterparts. Contrarily, p53-deficient cell lines have also been shown to be hypersensitive to TPZ. The exact mechanism notwithstanding, the results presented in Fig. 2 are in favor of a combined treatment approach of TPZ + PDT for EHCCs, especially since merely 14-26% of EHCC patients carry a mutation in the TP53 gene. However, there are three major drawbacks of TPZ in regard to clinical use, which include (1) poor cellular uptake, (2) suboptimal pharmacokinetics, and (3) the moderate adverse events reported in completed and ongoing clinical trials. Poor uptake enforces the use of higher TPZ dosing to achieve a desired clinical effect. Since TPZ has been associated with muscle cramping, anemia, diarrhea, skin rash, nausea, vomiting, and (reversible) blindness, the use of higher TPZ dosages is contra-indicated. Moreover, the plasma half-life of TPZ is extremely short, namely 36 min in mice and 47 min in humans at LD10 doses, resulting in poor drug delivery efficiency.

In order to resolve these drawbacks for clinical use, TPZ can be co-encapsulated with ZnPC in ETLs to specifically deliver these compounds to pharmacologically important locations in the tumor and achieve an adjuvant therapeutic effect after PDT. Liposomal encapsulation and tumor targeting of TPZ may prevent some of the pharmacokinetic and toxicity hurdles mentioned above and improve pharmacodynamic efficacy. Indeed, it was shown that the encapsulation of TPZ into ZnPC-ETLs resulted in a substantial increase in intracellular TPZ concentrations and PDT efficacy, yet did not exert the toxic effects induced by free TPZ. This increase in efficacy was observed in both A431 and Sk-Cha1 cells, indicating that the adjuvant effect of liposome-delivered TPZ on PDT efficacy occurs in a p53-independent manner. Taken altogether, it was demonstrated that TPZ and ZnPC can be combined in a singular drug delivery system for the treatment of tumor cells. Moreover, the study provides compelling evidence for the use of a fourth-generation liposomal formulation (i.e., a liposome-encapsulated second-generation photosensitizer with co-encapsulated TPZ or pharmacological agents that for example inhibit survival pathways) for PDT of recalcitrant solid cancers.

5. Conclusions

TPZ is a hypoxic cytotoxic that confers an adjuvant effect on PDT outcome, despite the varying responses of cells to TPZ treatment. The adjuvant effect stems from TPZ-mediated radical formation, DNA damage, loss of MMP, and consequent induction of cell death. These phenomena occurred under both normoxic and hypoxic conditions and independently of p53 functionality. The poor uptake of TPZ by tumor cells as well as unfavorable pharmacokinetics was circumvented by liposomal co-encapsulation, which nearly doubled the PDT efficacy. These \textit{in vitro} results provide proof-of-concept for the use of fourth-generation photosensitizers and furnish mechanistic insight into the adjuvant potential of TPZ in PDT.
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