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5,10,15,20-Tetrakis(4-sulfonatophenyl)porphyrin Zinc and Chloro-aluminum Phthalocyanine Disulfonate in Photodynamic Therapy of Colorectal Adenocarcinoma *In Vitro*

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Abstract. *Background/Aim: Colorectal cancer (CRC) is one of the most widespread malignancies. One of the alternative therapeutic methods appears to be photodynamic therapy (PDT). Materials and Methods: This study investigated the efficiency of 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrin zinc ($ZnTPPS_4$) and chloro-aluminum phthalocyanine disulfonate ($ClAlPcS_2$) with two commercial photosensitive compounds 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin (TMPyP) and tetramethylthionine chloride (methylene blue, MB) in PDT for CRC in vitro. In addition to the study of the photodynamic effect on the viability of the colorectal carcinoma cell line HT29, cellular uptake, ROS production, and DNA damage were investigated. Results: All photosensitizers showed good accumulation within HT29 cells, high efficiency in killing the cells, and a concentration-dependent increase in the production of ROS. Conclusion: PDT using $ZnTPPS_4$ and $ClAlPcS_2$ may be effective in the treatment of CRC, achieving a similar photocytotoxic effect at much lower concentrations compared to MB.*

The increasing prevalence of cancer has prompted scientists and clinicians from various fields to explore new, safer, and more potent treatment options. Colorectal cancer (CRC) is one of the most widespread malignancies and ranks as the second leading cause of tumor-related deaths in women and the third most common in men worldwide (1). Moreover, it has been predicted that the incidence and mortality rate of CRC will increase by 60% by 2030 (2). Despite progressions in science and medicine, a third of patients diagnosed with CRC have metastases, and cancer recurrence occurs in every second patient, even after radical treatment. The most common treatments for CRC include surgery, chemotherapy, radiotherapy, and immunotherapy, with their effectiveness highly dependent on tumor size, stage, and advancement of cancer. Surgery is the most widely used treatment modality. It is based on excising the lesion and is mostly effective during the early stages of colorectal carcinoma. However, during the procedure, tumor cells may spread into the circulatory system, or some tumor parts may remain unresected.

One of the alternative therapeutic methods appears to be photodynamic therapy (PDT). PDT is a non-invasive method that uses light to destroy tumors. After applying a photoactive compound (photosensitizer) and exposing it to visible light, a photodynamic reaction occurs that produces reactive oxygen species (ROS) (3). ROS harm biomolecules, such as lipids, proteins, and nucleic acids, leading to cell death and tumor eradication. In addition, the photosensitizer tends to concentrate in tumor cells, resulting in fewer side effects. Although this minimally invasive approach can have many of the above-mentioned benefits, it is still not as widely used.

One of the major research interests in PDT is the development of selective photosensitizers that accumulate preferentially in malignant tissue compared to normal tissue. Photosensitizers used in PDT represent diverse groups of chemical compounds including porphyrins, chlorophylls, bacteriochlorines, phthalocyanines, pheoforbides, purpurines, 5-aminolevulic acid (ALA), texaphirines, *etc*. (4). Their efficacy in photosensitization reflects their physicochemical properties, light-absorption characteristics and yield of the photodynamic reaction *via* electron transitions and energy transfer to oxygen molecule. In addition, pharmacokinetic and pharmacodynamic properties as well as the microenvironment must be included in a supposed photoanticancer profile. Over the last three decades, a large number of porphyrin- and phthalocyanine-based photosensitizers have been synthesized

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and evaluated for their photodynamic activity. Porphyrins are cyclic organic compounds, composed of four pyrrole units linked by four methine bridges (5). Their characteristics include a high absorption of light in the Soret region and an increased production of singlet oxygen and free radicals (6). However, their major drawback, apart from their lipophilicity, is poor tumor specificity and long-term sensitivity of the skin to light (7). Phthalocyanines are larger aromatic compounds consisting of four isoindole units connected by a ring of nitrogen atoms. Their absorption spectrum is in the red region (630-700 nm) thereby enabling a better penetration into the tissue. They cause minimal skin phototoxicity and exhibit significant cytotoxic activity against most tumor cells (8). To improve the phototherapeutic potential of the porphyrin- and phthalocyanine-based photosensitizers, researchers have developed diverse methods to chemically modify and functionalize these compounds, *e.g.* by triazines, polyethylene glycol, coordination with metal atoms (9) or their incorporation in nanostructures (4).

The goal of our presented study was to investigate the difference in the efficiency of four different water-soluble photosensitizers (Figure 1), 5,10,15,20-tetrakis(1-methyl pyridinium-4-yl)porphyrin (TMPyP), 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrin zinc ($ZnTPPS_4$), tetramethylthio nine chloride (methylene blue, MB), and chloro-aluminum phthalocyanine disulfonate ($ClAlPcS_2$), in the PDT of the human colorectal adenocarcinoma cells HT29.

Materials and Methods

Cell line, photosensitizers, cultivation, and irradiation conditions. Colorectal carcinoma cell line HT29 (ATCC, Manassas, VA, USA) was grown in Dulbecco's modified Eagle´s medium (DMEM) (Merck, Darmstadt, Germany) supplemented with fetal bovine serum (10% v/v, Biowest, Nuaillé, France), penicillin and streptomycin solution (100 U/ml and 100 μg/ml, respectively, Merck), and photosensitizers in 96-well microplates (10,000 cells/well), in darkness and under a humidified atmosphere with 5% CO_2 at 37˚C. TMPyP and MB were purchased from Merck (Merck), whereas $ZnTPPS_4$ and $ClAlPcS_2$ were synthesized and donated by Jiří Mosinger (Department of Inorganic Chemistry, Charles University in Prague, Czech Republic) and Jan Rakušan (Centre for Organic Chemistry Ltd, Rybitvi, Czech Republic), respectively. Their synthesis was described previously (10, 11). The chemical structures of these photosensitizers are shown in Figure 1.

Two LED light sources providing emission maxima at 415 nm (full width at half maximum (FWHM) 20 nm) and 634 nm (FWHM 20 nm) were used for cell irradiation. The light sources were specifically designed for the experimental microplate irradiation and are protected by the National Patent CZ302829B6. Cells treated with TMPyP and $ZnTPPS_4$ were exposed to the 415 nm source at 38 mW/cm^2 whereas those treated with MB and $ClAlPcS_2$ to the 634 nm source at 45 mW/cm^2 at total irradiation doses of 2.5, 5, and 10 J/cm^2.

Fluorescence microscopy. The intracellular distribution of photosensitizers at the initial concentration of 10 μM after 24 h of incubation was visualized using the inverted fluorescence microscope Olympus IX 70 equipped with a CCD camera (Olympus, Tokyo, Japan) and the Olympus imaging software CellM and CellR (Olympus Soft Imaging Solutions, Planegg, Germany) were used for analysis. Before fluorescence microscopy, the extracellular medium was properly replaced with fresh PBS buffer.

Viability measurement. The cytotoxic and phototoxic effect of photosensitizers was evaluated using the MTT viability test (MERCK). After incubation of HT29 cells with photosensitizer, irradiation in PBS, and another 24 h of incubation in the fresh DMEM, an aliquot of MTT solution was added, so that the final concentration of MTT was 0.5 mg/ml. After 3 h, the medium was removed, and the resulting formazan product was dissolved in dimethyl sulfoxide and quantified by measurement of absorbance at 570 nm.

Estimation of ROS. After 24 h of cell incubation with photosensitizer, DMEM was replaced with PBS, fluorescence probe 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H2DCFDA, Thermo Fisher Scientific, Invitrogen, Carlsbad, CA, USA) was added at the final concentration of 10 μM and left incubated for 20 min in the dark. After PDT, the fluorescence of CM-DCF within sensitized cells (excitation and emission wavelength was 480 nm and 530 nm, respectively) was recorded using the multi-reader Tecan Infinite 200pro (Tecan Group, Männedorf, Switzerland).

Comet assay – DNA fragmentation analysis. After irradiation with a dose of 5 J/cm^2, PBS was replaced with the DMEM, and the cell line HT29 was incubated for 6 or 24 h. Microscope slides were precoated with 1% high melting point (HMP) agarose (SERVA Electrophoresis, Heidelberg, Germany) dissolved in distilled water and placed in a drying oven for 30 min at a temperature of 60˚C. Then an aliquot of 1% HMP agarose was also applied on the precoated slides, covered with a coverslip, and placed in a refrigerator for agarose gelling enhancement. The cells were detached from the wells using TrypLe solution (TrypLe, Thermo Fisher Scientific, Gibco, Grand Island, NY, USA). The cell suspensions were centrifuged for 4 min at 300 × *g*. Pellets were then dispersed in 20 μl of PBS and vortexed. After the addition of 80 μl of 1% low melting point (LMP) agarose (Merck), the suspensions were placed on the solidified agarose on the precoated microscope slide, covered with the original coverslips and transferred to a refrigerator for 15 min. After solidifying, the samples were immersed in a lysis buffer (2.5 M NaCl, 100 mM EDTA (ethylenediaminetetraacetic acid), 10 mM Tris [tris(hydroxy-methyl)aminomethane], 1% Triton X-100, pH 10) at 4˚C for 60 min. After the lysis, the slides were placed in an electrophoretic tank and dipped in a cool electrophoretic solution (300 mM NaOH, 1 mM EDTA) for 40 min. The electrophoresis was run at 350 mA and 0.8 V/cm for 20 min. After the electrophoretic separation, the slides were carefully rinsed twice for 10 min with a neutralization buffer (0.4 M Tris, pH 7.5) at 4˚C, stained using SYBR Green (Merck), and visualized using a fluorescence microscope with CCD camera. Finally, the cell DNA was manually scored using CometScore 1.5 software (TriTek, Corp., Sumerduck, VA, USA). For evaluation, we randomly chose 100 cells from each given sample. Median values of the amount of the DNA in the tail, which is directly proportional to the DNA damage, were evaluated.

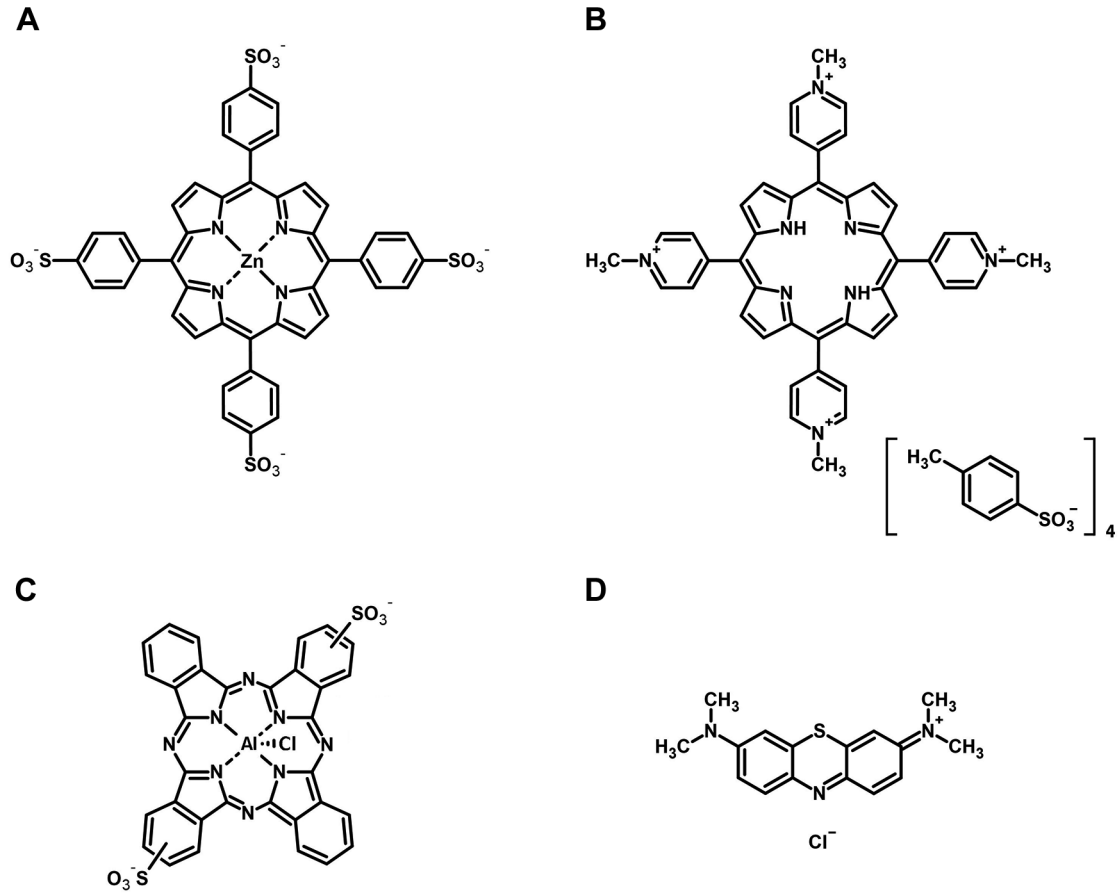

Figure 1. *Chemical structure of photosensitizers: (A) 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrin zinc ($ZnTPPS_4$); (B) 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin (TMPyP); (C) chloro-aluminum phthalocyanine disulfonate ($ClAlPcS_2$); (D) tetramethylthionine chloride (methylene blue, MB).*

Statistical analysis. The data are shown as means±standard errors for all measurements except the Comet assay, where the evaluating parameters correspond to the medians±standard errors. IC_{50} values and Hill coefficients (h) were determined from three independent experiments by non-linear regression fits to sigmoidal curves using ORIGIN 6.0 software (OriginLab, Corp., Northampton, MA, USA). One-way analysis of variance (ANOVA) was used for comparisons between experimental groups. A p-value of <0.05 was considered statistically significant.

Results

Fluorescence microscopy. Cellular localization of all studied photosensitizers in HT29 cells after 24 h of incubation shows inhomogeneous distribution creating several small clusters within the cells (red fluorescence, Figure 2). The sites with significantly less accumulation were the nuclear regions.

Determination of the cell viability. The phototoxic effect of photosensitizers was investigated in HT29 cells 24 h after the application of PDT. Results showed (Figure 3) that the cell viability was significantly influenced in concentration-dependent manners. The lowest IC_{50} value was found for TMPyP-mediated PDT. MB turned out to be the least potent phototoxic compound. Here, the potency was more than 57 times lower compared to TMPyP–PDT. All the analyzed photosensitizers did not show a significant viability reduction when no light was applied (Figure 4), thus they all meet the basic criterion of ideal photosensitizer.

ROS production measurement. The increase in ROS production in HT29 cells was observed immediately after PDT using the fluorescent probe CM-H2DCFDA. All photosensitizers tested caused the increase in ROS production (Figure 5). Higher values in fluorescence intensities were achieved when using light with a wavelength of 415 nm, which the photooxidation of the fluorescent probe itself can cause.

DNA fragmentation analysis using Comet assay. The effect of the photosensitizers at the concentration of IC_{50} and

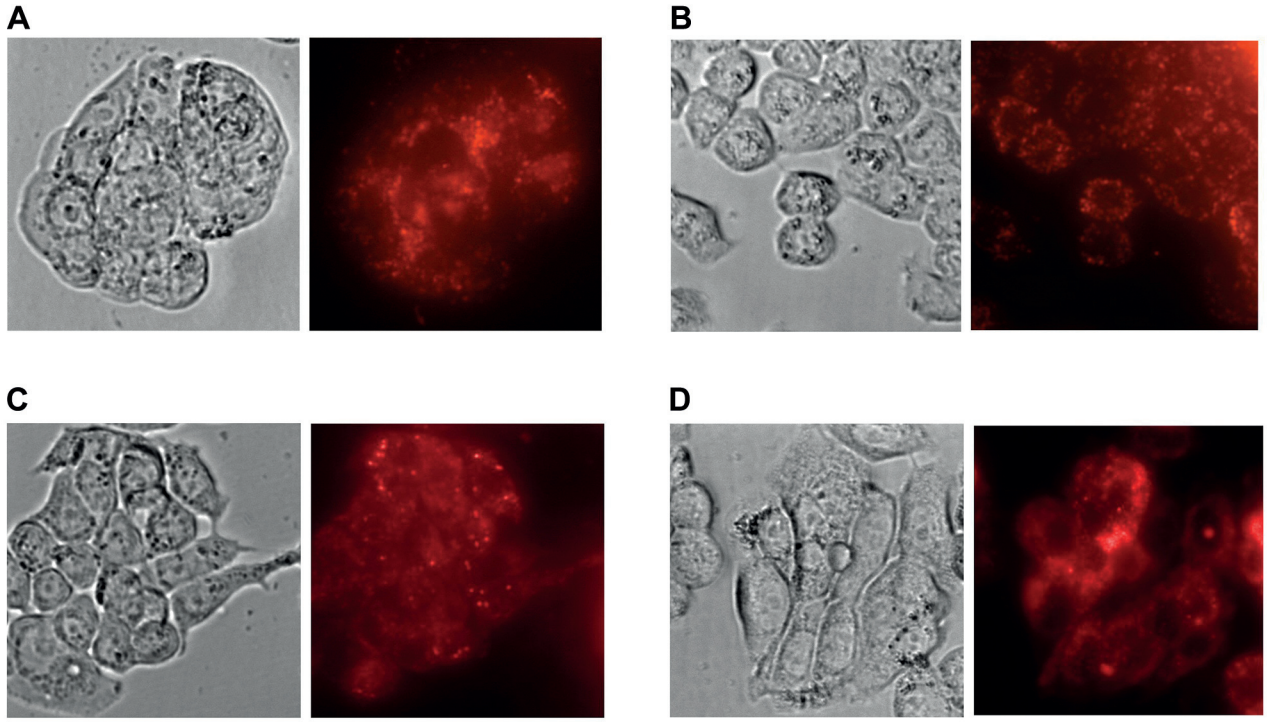

Figure 2. *Microscope images of live HT29 cells in transmission and fluorescence mode at an original image magnification of 400 times. The cells were incubated with: (A) $ZnTPPS_4$; (B) TMPyP; (C) $ClAlPcS_2$; (D) MB.*

irradiation dose of 5 J/cm^2 on DNA double-strand break formation was evaluated using the Comet assay. Significant DNA fragmentation was observed after 24 h from PDT and only when MB was applied. Other photosensitizers caused minimal DNA damage similar to non-treated cells. The results are stated in Table I.

Discussion

Our study aimed at comparing the effectiveness four different photosensitizers, $ZnTPPS_4$, TMPyP, $ClAlPcS_2$, and MB, against CRC *in vitro*. Although all photosensitizers showed good efficacy in the PDT of HT29 cells, significantly lower IC_{50} value was found for tetracationic TmPyP (0.47 μM). The highest IC_{50} value at the same irradiation dose was obtained for MB (27 μM).

Nyman and Hynninen reported that the formation of a complex with a diamagnetic cation, for example with Al^{3+}, Zn^{2+} and Ga^{3+}, increases the yield and lifetime of the triplet state (12) and therefore the production of singlet oxygen and the subsequent oxidation of neighboring molecules can be supported. The comparison of $ZnTPPS_4$ with the commercially available TMPyP showed that the production of singlet oxygen was higher in the case of the non-zinc complex. This can be explained by the fact that we used a source with an emission at 415/20 (maximum/FWHM) nm to induce the PDT, but the excitation peaks for TMPyP and $ZnTPPS_4$ are at 420/20 nm and 418/55 nm, respectively. The formation of ROS by $ClAlPcS_2$ and MB, both activated by red light (634/20 nm), and the dependence on concentration and irradiation dose is similar except for the highest used concentration (6 μM) and irradiation dose (10 J/cm^2). Because the IC_{50} value was more than 10 times lower for $ClAlPcS_2$, a difference in their cell target structures can be considered.

It is known that the uptake and localization of the photosensitizer in the target cells are influenced by the hydrophilic-lipophilic balance (13, 14). After incubation of HT29 cells with $ZnTPPS_4$, TMPyP, $ClAlPcS_2$, and MB, fluorescence microscopy revealed a high intracellular fluorescence, indicating that there was a substantial uptake of the photosensitizers into the cells. Although this method does not show a significant accumulation of the studied hydrophilic photosensitizers in the cell nucleus, the comet analysis showed that MB significantly damages DNA 24 h after PDT. MB is a positively charged molecule that binds to numerous cancer cells probably due to their hyperpolarized resting membrane potential in comparison to normal cells (15). In addition, the mitochondrial membrane in tumor cells also exhibits hyperpolarization, and therefore cationic photosensitizers may target this organelle (16). Thus, cell

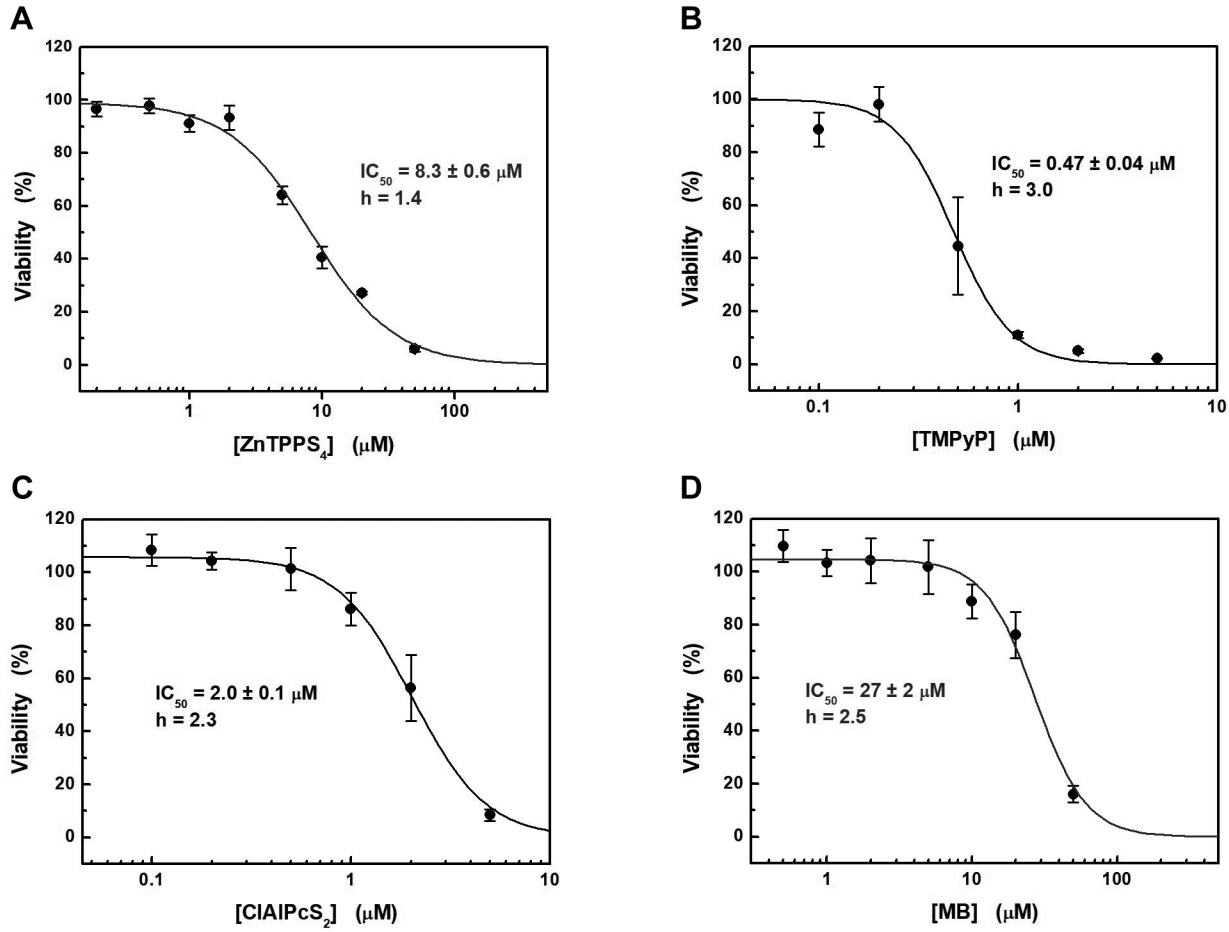

Figure 3. *The dependence of HT29 cell viability on photosensitizer concentration: (A) $ZnTPPS_4$; (B) TMPyP; (C) $ClAlPcS_2$; (D) MB. The dependence was determined by measuring the enzyme activity of living cells using the methylthiazol tetrazolium bromide test. The controls are represented by irradiated cells in the absence of photosensitizers and these values were set as 100%. Each data point is expressed as the mean±SD of three independent measurements.*

death by MB-PDT can be caused *via* apoptosis induced through mitochondria damage and subsequent activation of caspase 3 (17, 18) followed by late DNA fragmentation. However, MB also accumulates in lysosomes (17), induces high production of ROS, and is effective in the breast adenocarcinoma cell line MCF-7 (17, 19, 20) lacking expression of caspase 3. Therefore, the phototoxic effects of MB-PDT appear to be nonspecific, resulting in apoptosis, necrosis, necroptosis, and autophagy. It should also be mentioned that the type of cell death is significantly affected not only the type and concentration of the photosensitizer, but also other factors, including the cell genotype, oxygen level, and irradiation conditions (wavelength, fluence rate and exposure time) (21). In the case of the second cationic sensitizer, TMPyP, it was found that in addition to mitochondria and Golgi complex (22), it can accumulate in the cell nucleus of tumor cells, especially at a higher concentration or prolonged incubation time (23). However,

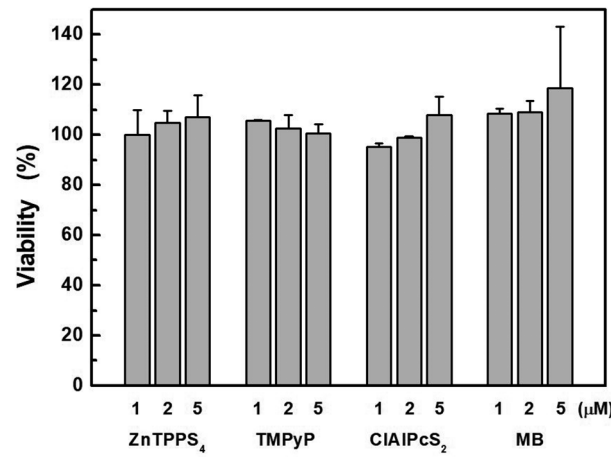

Figure 4. *Dark cytotoxicity measurement of photosensitizers on HT29 cells. The cells were incubated with $ZnTPPS_4$, TMPyP, $ClAlPcS_2$, or MB. The controls are represented by the non-irradiated cells in the absence of photosensitizers and these values were set as 100%.*

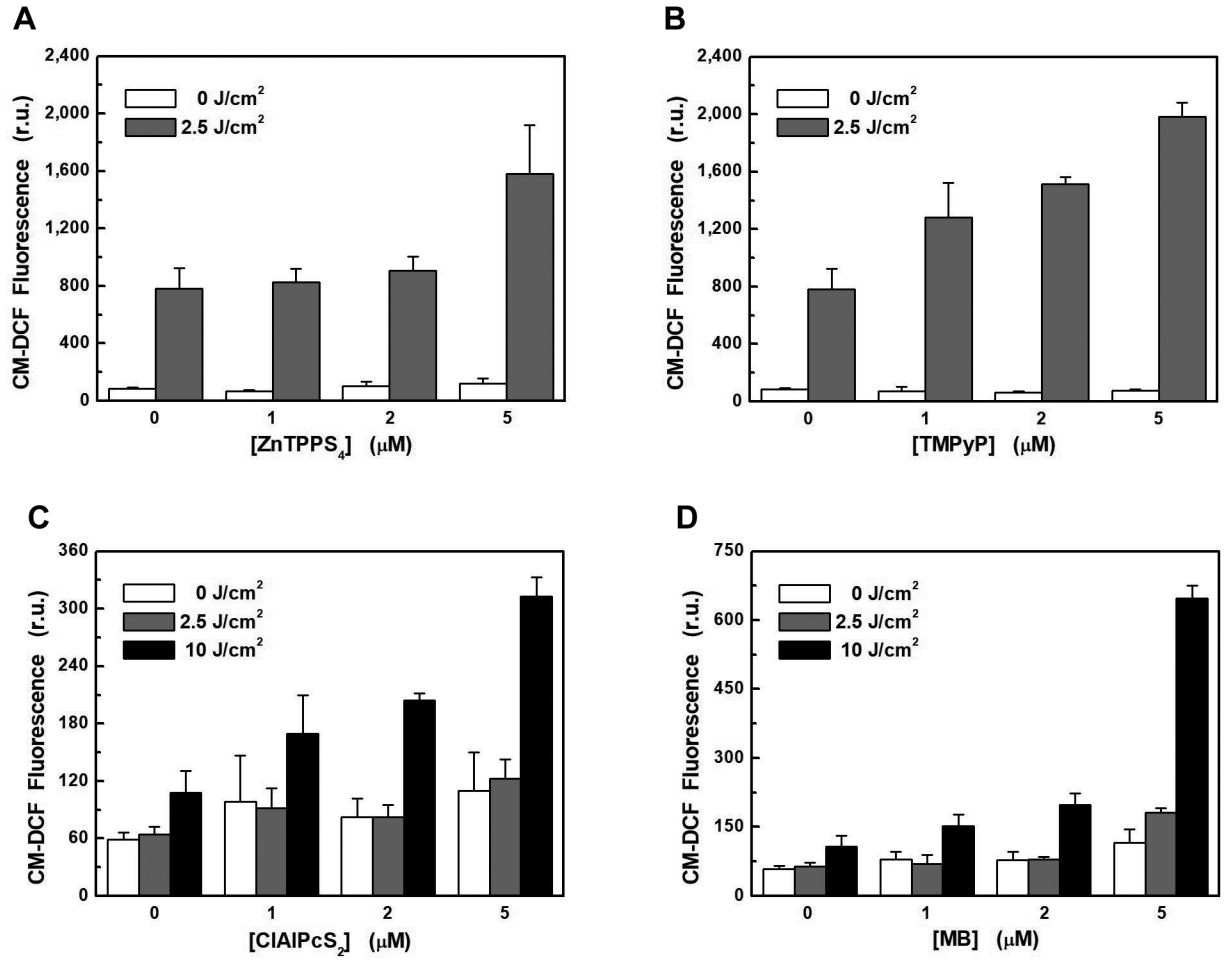

Figure 5. *The dependence of ROS production in HT29 cells on the concentration of: (A) $ZnTPPS_4$; (B) TMPyP; (C) $ClAlPcS_2$; (D) MB and irradiation dose. Data are expressed as the mean±SD of three independent measurements.*

our results showed that TMPyP-PDT did not lead to DNA fragmentation in HT29 cells. A similar finding in HeLa cells (cervical carcinoma) has been reported previously by Acedo *et al*. (21) who showed DNA fragmentation by TMPyP only in combination with zinc phthalocyanine. Surprisingly, the anionic photosensitizer $ZnTPPS_4$ also shows a certain affinity to mitochondria, because it induces changes in the mitochondrial membrane potential in HeLa and G361 (melanoma) cells in a concentration-dependent manner as was shown in one of our previous articles (24). Furthermore, it seems that TMPyP-PDT produces significant DNA fragmentation only at high doses (26 J/cm^2) (25), which also confirms our observation in HT29 cells exposed to the relatively low dose of 5 J/cm^2. The second generation of photosensitizers, such as phthalocyanines, chlorins, and purpurins, absorb light in the red region and therefore allow better tissue penetration. However, their disadvantage is poor solubility in water. Sulfonation can be a solution to increase their tissue distribution as the formation of a complex with metal elements increases the yield and lifetime of the photosensitizer's triplet state as well as their ability to

Table I. *DNA fragmentation after PDT using different photosensitizers at the IC_{50} concentration and irradiation dose of 5 J/cm^2. Control represents non-irradiated cells in the absence of photosensitizer.*

	DNA fragmentation [%]	
	6 h after PDT	24 h after PDT
ZnTPPS4	0.01±0.00	0.01±0.01
TMPyP	0.07±0.12	0.36±0.52
ClAlPcS2	0.81±1.41	2.16±3.73
MB	0.03±0.04	40.42±6.52
Control	0.86±0.64	0.01±0.00

PDT: Photodynamic therapy.

produce ROS (12). We found that to induce the same lethality in HT29 cells, about 10 times less concentration of $ClAlPcS_2$ is needed compared to MB. Similar to TMPyP and $ZnTPPS_4$, $ClAlPcS_2$ did not lead to DNA cleavage. $ClAlPcS_2$ was also effective in HeLa cells, where its mechanism of action was related to the mitochondria causing damage to the respiratory chain, as we showed earlier (26).

Conclusion

Conventional diagnosis and treatment methods for CRC have limitations, including severe side effects, invasiveness, and the inability to treat late-stage CRC effectively. Current screening tests, both invasive and noninvasive, lack sensitivity to detect early-stage CRC. Therefore, researchers have been investigating diagnostic and therapeutic alternatives, such as PDT. The advantage of PDT in the treatment of CRC over conventional methods is its ability to specifically target and treat cancer cells without damaging surrounding tissues. This approach using light colonoscopy allows selective multiple dosage administration with negligible side effects. As our *in vitro* study shows, PDT using sulfonated porphyrin- and phthalocyanine-based photosensitizers coordinated with metal atoms such as $ZnTPPS_4$ and $ClAlPcS_2$ can be effective in the treatment of CRC.

Conflicts of Interest

All Authors declare that they have no competing interests in relation to this study.

Authors' Contributions

Jakub Hosik, Barbora Hosikova, and Robert Bajgar performed the experiments, analyzed the data, interpreted the results, and wrote the manuscript. Hana Kolarova provided assistance in editing the final version of the manuscript. All Authors reviewed the manuscript.

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