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DISSERTATION

New photosensitizers and nanomolecules for photodynamic therapy of cancer

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Abbreviations

$_1O^2$	singlet oxygen
CAM	chick chorioallantoic membrane (assay)
CdSe	cadmium selenide
DMF	Dimethylformamide
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures)
GSH	$_L$-glutathione
GSH-CdSe/ZnS	cadmium selenide zinc sulfide QD with $_L$-glutathione capping
IC_{50}	half maximal inhibitory concentration
ISC	intersystem crossing
NaCl	natrium chloride
ns	nanoseconds
Pc	phthalocyanine
PDR	photodynamic reaction
PDT	photodynamic therapy
PS	photosensitizer
QD	quantum dot
ROS	reactive oxygen species
S_0	ground state
S_1	excited state
T_1	excited triple state
ZnPc	zinc phthalocyanine
ZnPc-PDT	photodynamic therapy with zinc phthalocyanine
ZnS	zinc sulphide
WHO	World Health Organisation

Abstract

1.1. Abstract in German language

Laut WHO leiden jährlich über 14 Millionen Menschen an Krebs. Die Photodynamische Therapie (PDT) ist eine sich in intensiver Entwicklung befindende onkologische Behandlungsalternative zu den bisherigen Standardbehandlungen. So konnte sie beispielsweise neben der gängigen Chemotherapie weltweit erfolgreich etabliert werden. Die auf Lichtaktivierung einer photoaktiven Verbindung (Photosensibilator, PS) basierende PDT ist eine non-invasive Methode, welche über Freie-Radikal-Entstehung (ROS) den Tod der Zielzelle induziert. In der letzten Dekade konnten Phthalocyanine (Pc) als vielversprechende photoaktive Substanzen ein hohes Maß an Interesse erzeugen. Sie besitzen verschiedene günstige Eigenschaften, wie etwa eine hohe Effizienz in der Erzeugung freier Radikale. Andere interessante Entwicklungen auf dem Feld der PDT ergeben sich zum einen in der Möglichkeit des Energietransfers auf den PS über Energiedonoren, und zum anderen in der Möglichkeit der diagnostischen Bildgebung des Tumorgewebes. Diesbezüglich könnten Nanopartikel wie Quantum Dots (QD) einerseits als Energiedonoren, sowie andererseits als Fluorophore für das Tumormonitoring dienen. Das Ziel dieser Dissertation war die Untersuchung des photoaktiven Potentials von neuen Phthalocyaninen als PS für die PDT, sowie der Eignung von neuentwickelten QDs für die biomedizinischen Anwendungen.

Verschiedene Zelllinien gastrointestinaler Karzinome wurden mit den neu synthetisierten Phthalocyaninen inkubiert und im Anschluss photoaktiviert (10-60 J/cm^2). Die in-vitro-Studien konzentrierten sich auf Zellproliferationsinhibition, Zellzyklusarrest, induktion von Apoptose und Freie-Radikal-Entstehung nach PDT. Gleichfalls waren die Aufnahme und Sicherheit der nicht-photoaktivierten Pc‘s von Interesse. Die in-vivo-Studien fokussierten sich auf die antiangiogene und antitumorale Aktivität der Pc‘s, sowie ihre Sicherheit im nicht photoaktivierten Zustand. Eine Gruppe von QDs wurde im Hinblick auf Biotolerabilität in-vivo und in-vitro getestet. Tetra-triethyleneoxysulfonyl substituiertes Zink Phthalozyanin (ZnPc) zeigte unter allen getesteten Pc‘s das größte photodynamische Potential. ZnPc wurde von Krebszellen in einem Zeit/Dosis-abhängigen Verhältnis mit einer homogenen zytoplasmatischen Verteilung aufgenommen. Photoaktiviertes ZnPc verursachte eine starke Proliferationsinhibition der Krebszellen (>95%), einen G_1-Phase-Zellzyklusarrest und eine Induktion der Cytochrom C-vermittelten Apoptose. Der antiproliferative Effekt basierte auf einer ROS-vermittelten Zytotoxizität. Die In-Vivo-Untersuchungen zeigten einen starken, langanhaltenden antiangiogenen, sowie antineoplastischen Effekt nach ZnPc-PDT. Nicht-photoaktiviertes- ZnPc induzierte sowohl in-vivo, als auch in vitro keine Toxizität. Unter allen getesteten QDs scheint CdSe mit ZnS Schale und Glutathione (GSH)

Capping für den biomedizinischen Gebrauch sicher zu sein. Dabei zeigte es neben einer guten intrazellulären Aufnahme eine gute in-vivo und in-vitro Biotolerabilität.

Diese Studie konnte das außergewöhnliche photoaktive Potenzial von ZnPc als zukünftigen Photosensibilisator für die PDT aufzeigen. Das neu synthetisierte QD GSH-CdSe/ZnS erscheint hinsichtlich der guten Biotolerabilität als vielversprechnender Kandidat innerhalb der biomedizinischen Anwendungen.

1.2. Abstract in English language

According to the WHO, over 14 million people suffer from cancer each year. One treatment option under intensive development is photodynamic therapy (PDT), which alongside standard treatments such as chemotherapy is being successfully applied in the oncology worldwide. PDT is a non-invasive method based on the light activation of a photoactive compound (photosensitizer, PS) that causes free radicals formation (ROS) and death of the target cells. In the past decade, phthalocyanines (Pc) have attracted much interest as promising PS-candidates, because they possess favourable properties e.g. high efficiency in free radical formation. Another interesting development in the field of PDT concerns the improvement of the energy transfer to PS and diagnostic imaging of the tumor tissue. In this respect, nanoparticles such as quantum dots (QDs) may qualify as energy donors and fluorophores for tumor monitoring. The goal of this thesis was to evaluate the photoactive potential of novel phthalocyanines as PS for PDT and to explore whether newly developed QDs are suitable for biomedical application.

Newly synthesized phthalocyanines were incubated with a panel of gastrointestinal cancer cell lines and photoactivated (10 - 60 J/cm^2). The *in vitro* studies focused on the inhibition of cell proliferation, cell cycle arrest, induction of apoptosis and free radical formation after PDT, as well as the uptake and safety of non-photoactivated Pcs. The *in vivo* studies emphasised the antiangiogenic and antitumor activity of Pc and its safety in the non-photoactivated state. A set of QDs was tested *in vitro* and *in vivo* with regard to biotolerability. The tetra-triethyleneoxysulfonyl substituted zinc phthalocyanine (ZnPc) displayed outstanding photodynamic potential among all tested Pcs. ZnPc was taken up by cancer cells in a dose- and time-dependent manner with homogenous cytoplasmic distribution. Photoactivated ZnPc caused a dramatic decrease in the number of cancer cells (>95%), G_1-phase arrest in the cell cycle and induction of cytochrome c mediated apoptosis. The antiproliferative effect was based on ROS-induced cytotoxicity. *In vivo* investigations showed a strong, long-lasting antiangiogenic and antineoplastic effect of ZnPc-PDT. Non-photoactivated ZnPc did not induce any toxicity *in vitro* as well as *in vivo*. Among all

tested QDs, CdSe with ZnS shell and GSH capping appeared safe for biomedical use, showing high intracellular uptake and good bio-tolerability *in vitro* and *in vivo.*

This study provides strong evidence that ZnPc possesses extraordinary photoactive potential as a future photosensitizer for PDT. Newly synthesized QD GSH-CdSe/ZnS, with its good bio-tolerability, appears to be a promising candidate for biomedical application.

2. Introduction

2.1. Photodynamic therapy

The idea of using photodynamic therapy (PDT) for a treatment of neoplastic tissue was proposed over 100 years ago by Jesionek and von Tappeiner (1). Today, PDT is a clinically approved therapeutic procedure used for the treatment of several types of cancers and dysplasia/neoplasia such as Barret's dysplasia, choroidal neovascularization and esophageal cancer (1–5).

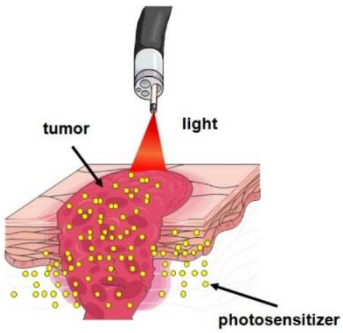

Figure 1: The principle of PDT. The PS applied systemically or topically accumulates in the tumor tissue which is subsequently illuminated. Photoactivation of PS causes a photochemical reaction that results in death of the target cells (6).

Photodynamic therapy involves systemic application of a photosensitizer (PS) that preferably accumulates in the tumor tissue. Local light exposure activates the photosensitizer, which absorbs a photon and causes an energy transfer cascade. That results in production of reactive oxygen species (ROS) that damage and eventually kill the illuminated tumor cells (6) (Fig.1). The effectiveness of the treatment largely depends on the photophysical features of the photosensitizer (7,8).

In detail, the photosensitizer when activated by light is excited from the ground state (S_0) to the first excited state (S_1), and then, via intersystem crossing, to the excited triplet state (T_1) (Fig. 2). The PS can relax back to its ground state (S_0) by emitting fluorescence (from S_1) or

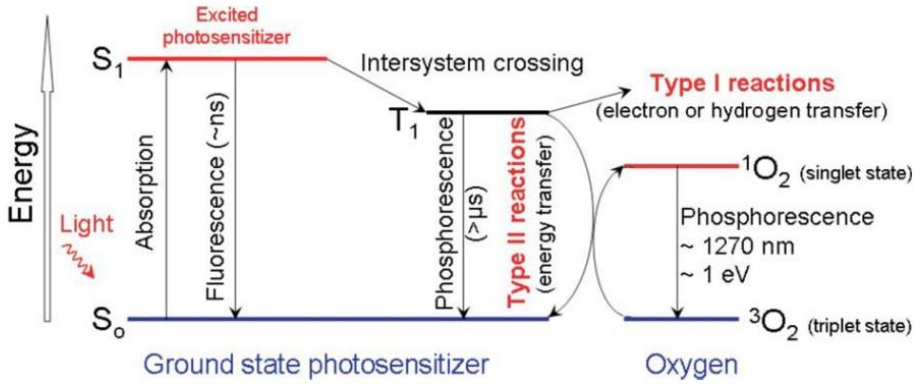

Figure 2: Jablonski diagram illustrating the energy transfer between the photosensitizer and oxygen molecule in PDT. S0 – ground state, S1 – first excited state, T1 – excited triplet state, ns – nanoseconds, µs – microseconds, nm – nanometers, eV – electron volts (2).

phosphorescence (from T_1). The excited photosensitizer can also transfer energy directly to the surrounding molecules (3). The detailed mechanism of the photodynamic action inside the cell is still uncharted; however, it is known that the excited photosensitizer can proceed in two ways, depending on the oxygen level. A type I reaction occurs in an environment with a reduced oxygen level. PS in its excited state can react directly with neighbouring molecules, leading to a transfer of a proton or an electron, thereby forming free radicals. These radicals may react with oxygen and produce reactive oxygen species. In the type II reaction, the triplet-state photosensitizer transfers its energy directly to the oxygen molecule, forming singlet oxygen ($^1O_2$). Highly reactive singlet oxygen may react and cause damage to many biological molecules, such as proteins or nucleic acids. It is impossible to measure directly but it is assumed that type II is the dominant mechanism of PDT (8–10).

Despite the great potential of PDT as a cancer treatment, a tumor's location and thickness may limit PDT application. First of all, photosensitizers have to be activated with light, so the cancer site has to offer access to light directly (skin cancer) or via endoscope/colonoscope (esophageal/ colorectal cancer). Moreover, the depth of light penetration is a maximum 10 mm (depending on the tissue's structural features and the PS used). For that reason, in the beginning, clinical applications of PDT were limited to superficial neoplastic tissues such as skin cancer. Recent technological development, especially of light devices (e.g. optical fibers), make it possible to treat many more types of cancer, including varieties previously deemed as not amenable to PDT (e.g. brain, prostate) (11–13). Nonetheless, PDT is applied today mostly in gastroenterology and dermatology (14,15) (Tab.1). Finally, and perhaps most importantly, it has been shown that PDT can be successfully applied in combination with other treatment modalities such as chemotherapy or surgery without risk of inducing cross-resistance (15–17).

Type of cancer	Photosensitizer	Country
Actinic keratosis	ALA (Levulan®, Metvix®)	U.S., EU
Basal cell carcinoma	ALA (Metvix®)	EU
Barrett's HGD	Porfimer sodium	U.S., Canada, EU, UK
Cervical cancer	Porfimer sodium	Japan
Endobronchial cancer	Porfimer sodium	Canada, Denmark, Finland, France, Germany, Ireland, Japan, The Netherlands, UK, U.S.
Esophageal cancer	Porfimer sodium	Canada, Denmark, Finland, France, Ireland, Japan, The Netherlands, UK, U.S.
Gastric cancer	Porfimer sodium	Japan
Head and neck cancer	Foscan	EU, Norway, Iceland
Papillary bladder cancer	Porfimer sodium	Canada

Abbreviations: ALA, 5-aminolevulinic acid; EU, European Union; HGD, high-grade dysplasia; UK, United Kingdom.

Table. 1: Examples of cancer types treated with PDT, drugs used and countries of approval (1).

2.2. Photosensitizers

Since photosensitizers are the key players in PDT, several new compounds are currently being investigated for their photoactive potential. Years of clinical experience with PDT have revealed the advantages and disadvantages of approved PS. Referring to multiple studies, a high quality PS should: (a) have a known chemical composition, (b) have high light absorption in the infrared spectrum (600-800nm) for deep tissue penetration with high quantum yield of $^1O_2$,(c) exhibit no cytotoxicity in absence of light and be cytotoxic in the photoactivated state, and (d) have high uptake in the neoplastic tissue and rapid clearance from normal tissue (16,18).

The structure of almost all photosensitizers is based on a heterocyclic ring structure similar to those of chlorophyll or heme of hemoglobin. Photosensitizers can be divided into three groups: porphyrin-based (e.g. Photofrin, 5-ALA), chlorophyll-based (e.g. Foscan, Verteporfin) and dyes (e.g. Photosens) (19). Photofrin (porfimer sodium) was the first clinically approved PS and belongs to the group of so-called first generation photosensitizers. Although Photofrin was a great success and is still widely used, it has several drawbacks, including "impure" chemical composition, long-lasting skin photosensitization and low tissue penetration (9,20). The second generation of PSs were developed to overcome these problems. Among the most intensively investigated 2nd generation PSs are phthalocyanines (21–24). Phthalocyanines (Pc) are dyes, structurally related to porphyrins, with a central atom (usually zinc, silicon or aluminum) to increase singlet oxygen production (Fig.3).

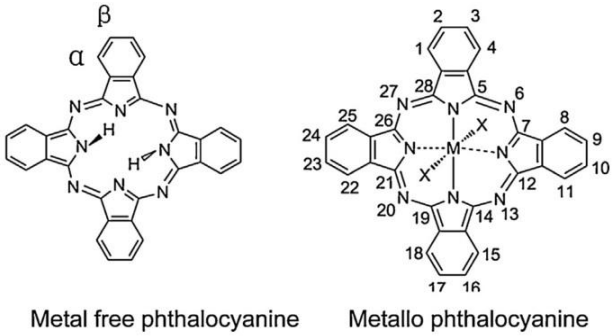

Figure. 3: Chemical structures of representative phthalocyanines (23).

Pcs are very potent as photosensitizers and exhibit many optimal properties of desirable PS, such as an easy-to-synthetize chemical structure of high purity, light absorption in the near-IR spectrum, high efficiency of ROS production and low toxicity in a non-photoactivated state. Moreover, the possibility to incorporate more than 70 different metal atoms into a Pcs ring and conjugation with different molecules allows improvement of the physical and biological properties of PSs (25). For example, slight changes in the chemical structure can improve solubility and conjugation, e.g. with antibodies or liposomes, enabling better uptake in the tumor tissue or targeted therapy. Second generation PSs modified in this way belong to the group of so-called 3rd generation photosensitizers (26,27).

Recent development of nanotechnology has had a strong impact on PDT and the creation of new PSs. One of the most interesting modifications is phthalocyanine-quantum dots conjugate (28,29).

Quantum dots are semiconductor nanocrystals (1-20 nm) with unique optical properties such as high photostability, broad absorption spectra, narrow sharply-defined symmetrical emission spectra and a large biochemically-accessible surface that enables the aforementioned Pc-QD conjugation. The photoactive interface between QDs and PS allows broadband light absorption and effective conversion of excitation energy into free radicals directly in the tumor cells. Moreover, by increasing the molecular weight of such a QD-PS nanodrug, better accumulation in the tumor tissue is expected (30). Additionally, it has been already shown that QDs, due to their bright fluorescence and high photostability, can be used successfully to monitor cancer cells *in vivo* (31).

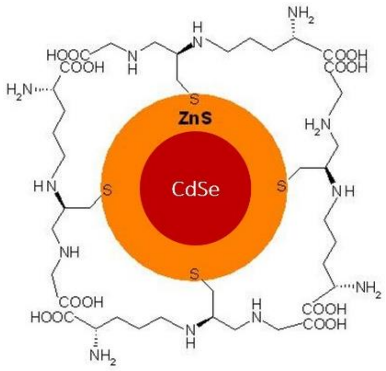

Figure 4: Schematic structure of representative QD. Cadmium-selenide with zinc sulfide shell (CdSe/ZnS) and glutathione (GSH) capping.

3. Objectives

The goal of this work was to evaluate the suitability of several newly synthesized zinc phthalocyanines as novel photosensitizers for the photodynamic therapy of cancer. Detailed *in vitro* studies focused on characterizing the underlying mode of the photodynamic action in terms of cytotoxicity and antiproliferative potency, as well as apoptosis induction, cell cycle arrest and ROS formation. The safety of Pc and its antineoplastic and antiangiogenic potential were examined *in vivo* by animal model and CAM assay. Additionally, seven different types of QDs were tested *in vitro* and *in vivo* in order to determine which QD may be promising candidate for biomedical application, such as photodynamic therapy.

4. Materials and Methods

4.1. Compounds

Photosenitizers and quantum dots used in this work were provided by our cooperation partners from Turkey (Prof. Ayşe Gül Gürek) and South Africa (Prof. Tebello Nyokong). Synthesis and photophysical examination of the compounds are described in the respective literature: (32–35).

4.2. Cell lines

PS-induced phototoxicity and cytotoxic effects of QDs were studied in human gastrointestinal cancer cell lines of different origins:

- <u>Esophageal cancer</u>

- Kyse-140 - esophageal squamous carcinoma; DSMZ no.: ACC 348
- Kyse-70 - esophageal squamous carcinoma; DSMZ no.: ACC 363

- OE-33 - esophageal adenocarcinoma; DSMZ no.: ACC 706

- Neuroendocrine gastrointestinal and colorectal cancer

- BON cells - pancreatic carcinoid; (36)
- HCT-116 - colorectal carcinoma; DSMZ no.: ACC 581

Kyse-140, Kyse-70 and OE-33 cells were cultured in RPMI 1640 medium (Biochrom AG). BON cells were cultured in DMEM/Ham's F-12 (1:1) medium (Biochrom AG). HCT-116 cells were cultured in DMEM medium (Biochrom AG). Each medium was supplemented with 10% fetal bovine serum (FCS, Biochrom), 100 U/ml penicillin and 100 µg/ml streptomycin (Biochrom AG). OE-33' medium was additionally supplemented with 2 mM L-glutamine (Biochrom AG).
All cell lines were kept at 37˚C in a humidified atmosphere (5% CO_2). The culture medium was changed every second day and once a week the cells were passaged using 1% Trypsin/EDTA.

4.3. Light source and PDT treatment

PDT treatment (power density: 10-60 J/cm²) was carried out by illumination with a broad band white light source equipped with a 100 W halogen lamp (EFR 12 V/100 W GZ-6.35 Lampe, OMNILUX, Germany). The spectral output of the lamp ranged from 400 to 800 nm. To prevent infrared irradiation, a heat-reflecting filter (Präzisions Glas & Optik, Iserlohn, Germany) that cuts off transmission at 700 nm and above was inserted into the optical path. During irradiation, the temperature of the samples never exceeded 37°C. The temperature was measured with a digital thermometer placed inside the irradiation system, and a fan connected to the thermometer was used to cool down the illumination unit once the internal temperature reached 37°C.

4.4. *In vitro* experiments

- Microscopy - intracellular distribution of PS was analyzed with a confocal laser microscope (Leica, DMI 6000, Germany) [excitation: HeNe laser (633nm), detection: PMT (400-800nm)]. Cellular uptake of QDs was analyzed with a fluorescence microscope (Zeiss Axioskop 40, Germany) at ex/em 546±12/575–640 nm (37).
- Measurement of growth inhibition - changes in the cell number were analyzed by crystal violet staining and by real-time imaging of cell growth using the iCelligence system (ACEA, Bioscences, USA) (38).
- Determination of cell viability - cell viability was investigated by using a cell viability/cytotoxicity assay kit (live/dead assay) from Life Technologies (CA, USA) (33).

- Determination of cytotoxicity - release of the cytoplasmic enzyme lactate dehydrogenase (LDH), indicating unspecific cytotoxicity, was measured in the supernatant of the samples by using a colorimetric kit (Roche Diagnostics, Germany) (39).
- Detection of apoptosis-specific caspase-3 activity - changes in caspase-3 activity were calculated from the cleavage of the fluorogenic substrate AC-DEVD-AMC (Calbiochem-Novabiochem, Germany). Substrate cleavage was measured fluorometrically using a VersaFluor fluorometer (Bio-Rad, Germany; filter sets: ex 360/40 nm, em 460/10 nm) (33).
- Cell cycle analysis - changes in the cell cycle were evaluated by using flow cytometry (FACSCanto II, BD Biosciences, Germany) and analyzed with FCS Express Software (De Novo, USA) (33).
- Measurement of reactive oxygen species (ROS) - formation and intracellular distribution of ROS were determined with CellROX Green (cytoplasmic) and CellROX Orange (nucleic) (ThermoFisher, USA) and fluorescence microscope (Axioskop 40, Zeiss; objective 40x, NA 1.30, Zeiss, Germany) equipped with a digital camera (Kappa, DX4-285FW, Germany) (37).
- Changes in the protein expression of proliferation-, apoptosis- and cell cycle -related proteins were evaluated by performing Western blot (33).

4.5. *In vivo* experiments

- Antiangiogenic effect of PDT - were analyzed by using chick chorioallantoic membrane (CAM) assay, which uses the chorioallantoic membrane of fertilized chicken eggs as a platform for observation of changes in the microvasculature caused by PDT (38).
- Antitumoral effect of PDT - was examined by performing CAM assay, in which tumor plaques were inoculated onto the CAM and PDT-treated (37).
- Biotolerability – the influence of the non-photoactivated PS on embryo survival and development was examined by performing CAM assay, in which a PS was injected into an embryo-feeding vein. Additionally, the safety of non-photoactivated PS was tested on male Wistar rats. Animals were injected intraperitoneally with a different dose of PS, in order to examine the influence of PS on the immune system and organs, e.g. liver and kidney (37).

  Determination of the potential toxicity of QDs was evaluated with the CAM assay. QDs were applied either intravenously into the CAM-vein or topically onto the CAM (35).

5. Results

5.1. Synthesis and characterization of novel zinc phthalocyanines as potential photosensitizers for photodynamic therapy of cancers. Moeno S, Krause RW, Ermilov EA, Kuzyniak W, Höpfner M. Photochem Photobiol Sci. 2014; 13(6):963-70.

This study reports on the synthesis of two novel phthalocyanines, namely: tetrakis-2,(3)-[(4-methyl-2-pyridyloxy)phthalo- cyaninato] zinc(II) (Pc 3) and its water soluble form tetramethyl tetrakis-2,(3)-[(4-methyl-2-pyridyloxy)phthalocyaninato] zinc(II) (Pc 4) and tetrakis-2,(3)-[(3-carboxylic-acid-6-sulfanylpyridine)phthalocyaninato] zinc(II) (Pc 5). Compounds were photophysically characterized and tested *in vitro* in terms of their photodynamic activity on human pancreatic carcinoid BON cells.

Phthalocyanines dissolved well in the dimethylformamide (DMF) with absorption/emission spectra between 675-695 nm. All Pcs showed appreciable intersystem crossing (ISC) quantum yield, which was calculated to be: 0.50 (Pc 3), 0.51 (Pc 4) and 0.62 (Pc 5). Since ISC quantum yield correlates with that of the singlet oxygen generation, it suggests strong photoactive potential of the examined compounds. Additionally, all Pcs showed fluorescence, with the fluorescence lifetime up to 2.93±0.18 ns. Unexpectedly, it was found that Pcs 3, 4 and 5 have a very high tendency to aggregate in the aqueous solution, which results in quenching of their fluorescence and ISC quantum yield.

Pc 4 and Pc 5 were tested *in vitro* with regard to their phototoxicity and safety in absence of light ("dark toxicity"). Both compounds were used in the concentration range of 5-20 µM and were incubated with cancer cells up to 12 h prior to PDT (30-60 J/cm^2). The maximal phototoxic effect of Pc 5 on BON cells was reached at the concentration of 10 µM and a light dose of 60 J/ cm^2, causing an 80% decrease in the cell number. Pc 4 showed lower phototoxic potential, even when used at high concentration (20 µM; 60 J/cm^2), causing only a 50% decrease in the number of cancer cells. Cells that survived PDT did not seem to regenerate (re-proliferate) in the following days, suggesting a long-lasting effect of PDT with either Pc 4 or Pc 5. Both Pcs in the non-photoactivated did not affect cell proliferation.

To compare the effectiveness of the novel Pcs with that of the clinically approved photosensitizer, Photofrin was tested *in vitro* under the same conditions (5-10 µM, 30-60 J/cm^2). Photoactivated Photofrin caused a dose-dependent decrease in the cell number of up to 85%, which is in the range induced by photoactivated Pc 5.

To summarize, results of this research showed Pc 4 and Pc 5 as interesting candidates for photodynamic therapy. Both Pc 4 and 5 possess favourable photochemical properties, such as appreciable ISC quantum yield and the ability to cause the long-term elimination of cancer cells.

5.2. Tetra-triethyleneoxysulfonyl substituted zinc phthalocyanine for photodynamic cancer therapy. Kuzyniak W, Ermilov EA, Atilla D, Gürek AG, Nitzsche B, Derkow K, Hoffmann B, Steinemann G, Ahsen V, Höpfner M. Photodiagnosis Photodyn Ther. 2016; 13:148-57.

In this study, the photodynamic potential of tetra-triethyleneoxysulfonyl substituted zinc phthalocyanine (ZnPc) was evaluated *in vitro* as well as *in vivo*. The photodynamic action of ZnPc was tested *in vitro* on a panel of human gastrointestinal cancer cell lines and *in vivo* by performing CAM assay.

The human pancreatic carcinoid cells (BON) and the human esophageal squamous carcinoma cells (Kyse-70) were incubated with ZnPc for 24 h and then illuminated with a light dose of 10 J/cm^2. Photoactivated ZnPc caused a dramatic decrease (>95%) in the number of BON and Kyse-70 cells in a time- and dose-dependent manner (Fig. 5). Moreover, ZnPc-PDT led to morphological changes in the cancer cells, which appeared flat and shrunken compared to untreated control cells. The observed effect was long-lasting only for Kyse-70 cells. Cells that survived PDT with a high

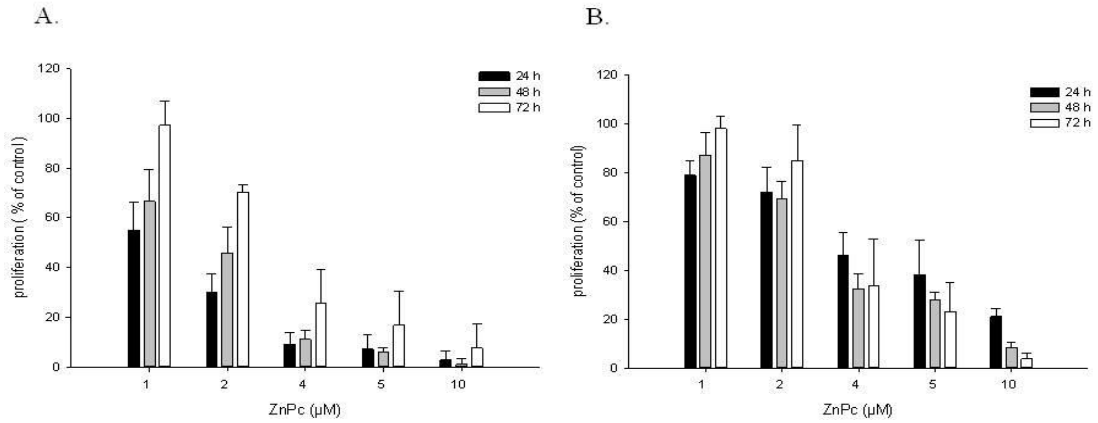

Figure. 5: Time- and dose-dependent growth inhibition of BON and Kyse-70 cells by ZnPc-PDT. BON (A) and Kyse-70 (B) were incubated with increasing concentrations of ZnPc for 24 h and illuminated (10 J/cm^2), which resulted in a dose- and time-dependent decrease in tumor cell proliferation of >95% (mean ± SD of 3 independent experiments).

dose of ZnPc (5-10 µM) did not seem to be able to regenerate in the following days (Fig. 5 B). Additionally, the expression of AKT and ERK ½, both being associated with cell proliferation and survival, was downregulated in both cell lines. Incubation with non-photoactivated ZnPc (1-10

μM) did not affect either cell proliferation or the integrity of the cell membrane, indicating the non-toxic character of ZnPc in the absence of light.

ZnPc-PDT induced apoptosis in both cell lines was reflected in a decrease of antiapoptotic protein Bcl-2 and a significant increase of caspase-3 activity. Flow cytometry revealed the induction of cell cycle arrest in a dose-dependent manner. 24 h after PDT, accumulation of BON and Kyse-70 cells in G_0/G_1 phase was observed with a corresponding decrease of cells in S- or G_2/M-phase, which was associated with a decrease of cyclin D1 expression.

In vivo evaluations showed an antiangiogenic potency of ZnPc-PDT. Photoactivated ZnPc caused degeneration of the blood vessels and capillary plexus of the vascular bed of the chicken chorioallantoic membrane (CAM-assay). Non-photoactivated ZnPc did not affect the vascularity of CAM.

Taken together, the results of this research show the extraordinary potential of ZnPc, which may become an interesting candidate for photodynamic cancer treatment. Future investigations will clarify the underlying modes of action of the novel ZnPc-PDT, including the uptake kinetics of the photosensitizer and its intracellular localization.

5.3. Novel zinc phthalocyanine as a promising photosensitizer for photodynamic treatment of esophageal cancer. Kuzyniak W, Schmidt J, Glac W, Berkholz J, Steinemann G, Hoffmann B, Ermilov EA, Gürek AG, Ahsen V, Nitzsche B, Höpfner M. Int J Oncol. 2017; 50(3):953-63

The results of previous work showed the extraordinary potential of tetra-triethyleneoxysulfonyl substituted zinc phthalocyanine (ZnPc) as a novel PS for photodynamic cancer treatment. This research continued with the characterization of ZnPc in terms of its photocytotoxic potential, with emphasis on the photodynamic treatment of esophageal cancer as well as on the safety of ZnPc in the non-photoactivated state. The *in vitro* experiments were carried on two cell lines: the human esophageal squamous carcinoma (Kyse-140) and the human esophageal adenocarcinoma (OE-33). The *in vivo* studies were performed by using the chlorioallantoic membrane assay (CAM assay) and on native Wistar rats.

Intracellular localization of ZnPc in the cancer cells was investigated by using confocal scanning microscopy, which revealed a dose-dependent uptake and homogenous cytoplasmic distribution of non-photoactivated ZnPc after 24 h of incubation in both cell lines. To establish the optimal loading time of ZnPc, cancer cells were incubated for 1-30 h with rising concentrations of ZnPc

(1-10 µM) and subsequently illuminated with a light dose of 10 J/cm². The longer the incubation time with ZnPc, the more evident was the ZnPc-PDT induced growth inhibitory effect, reaching its maximum after 24h of incubation. To evaluate the time course of growth inhibition of ZnPc-PDT treated cells (preincubated with ZnPc for 24 h), cell proliferation was examined for up to 72 h after PDT. Photoactivated ZnPc caused a dose- and time-dependent decrease in cell proliferation of up to >90 %. Squamous carcinoma cells responded better to the photodynamic treatment, which was reflected in the respective IC_{50}-values of 1.41±0.40 µM for Kyse-140 and 3.35±0.79 µM for OE-33. Kyse-140 cells that avoided being killed by ZnPc-PDT did not regenerate (re-proliferate) in the following days. Non-photoactivated ZnPc, even at concentrations as high as 10 µM, did not inhibit cell proliferation or cause changes in the morphology of both cell lines, showing the nontoxic nature of ZnPc itself.

The growth inhibitory effect of PDT was caused by the formation of highly reactive oxygen species (ROS), which were observed in the cytoplasm, nucleus and mitochondria of both esophageal cancer cell lines. To confirm this observation, Kyse-140 cells were incubated with the antioxidant vitamin C prior to PDT, causing a considerable decrease of ROS formation.

The dramatically elevated level of caspase-3 after ZnPc-PDT, with simultaneous downregulation of antiapoptotic Bcl-2 and an increase of proapoptotic Bax, indicated induction of apoptosis. Additionally, upregulation of cytochrome c expression indicates a breakdown of the mitochondria structure, denoting the intrinsic apoptotic pathway.

The *in vivo* evaluation revealed a long-lasting antiangiogenic effect and strong antineoplastic effect of ZnPc-PDT. Photoactivation of ZnPc (10 µM) caused a vasodegeneration of the vasculature and the capillary plexus with the respective connective vascular bed of CAM. The antiangiogenic changes were still evident 3 days after treatment, indicating the lasting antiangiogenic effect of ZnPc-PDT treatment. Tumor plaques grown from Kyse-140, treated with ZnPc (10 µM) for 24 h and illuminated (10 J/cm²), 72 h after treatment were significantly reduced in size (>70 %) compared to NaCl-treated control tumor plaques (Fig. 6). Non-photoactivated ZnPc, either injected into an embryo-feeding vein of the CAM or applied topically, did not influence either the survival of the chicken embryo or the development of the blood vessel network. Accompanying animal studies confirmed the good tolerability and systemic safety of non-photoactivated ZnPc. Young Wistar rats injected intraperitoneally with ZnPc (0.5-5 mg/kg) did not show signs of dark toxicity or incompatibility towards the non-photoactivated compound.

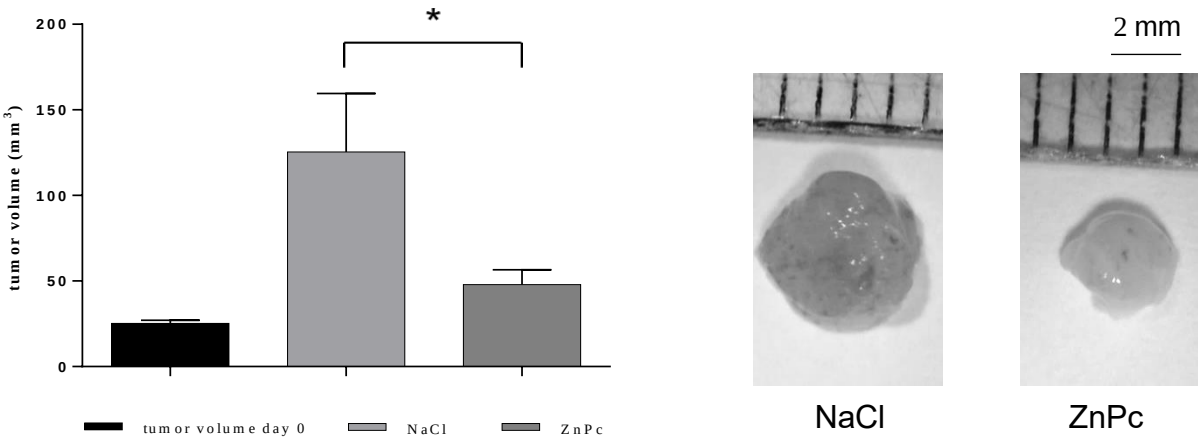

Figure 6. Anti-tumor activity of ZnPc-PDT. Esophageal tumor plagues (Kyse-140) were incubated with ZnPc (10µM/24h) and illuminated (10 J/cm²). 72 h post PDT the ZnPc-treated tumors were significantly smaller than the ZnPc-untreated but illuminated control tumors. (n=6; means ±S.E.M.; t-test, *p< 0,001).

In conclusion, this study confirms the outstanding photoactive potential of ZnPc as a photosensitizer for PDT, especially for treatment of esophageal cancer. Further *in vivo* investigations are necessary to estimate the anti-tumor activity of ZnPc-PDT in animal models.

5.4. Synthesis and characterization of quantum dots designed for biomedical use.

Kuzyniak W, Adegoke O, Sekhosana K, D'Souza S, Tshangana SC, Hoffmann B, Ermilov EA, Nyokong T, Höpfner M. Int J Pharm. 2014; 466(1-2):382-9.

The goal of this study was to evaluate the potential toxicity of seven different quantum dots (QDs) in order to determine which QD may be promising candidates for biomedical applications, such as energy donors in photodynamic therapy or fluorophores for tumor monitoring.

QDs were synthesized by the hydrothermal aqueous route. *In vitro* cytotoxicity studies were performed on the human pancreatic carcinoid cell line BON.

All QDs exhibited broad absorption with emission maxima laying between 560 and 630 nm (depending on the structure and size of the QDs), and well-resolved luminescence spectra. The QDs were in the size range of 2.0 – 3.5 nm.

Cadmium telluride QDs with or without a zinc sulfide shell and coated with 3-mercaptopropionic acid (MPA) were highly cytotoxic, even at nanomolar concentration. L-glutathione (GSH) or thioglycolic acid (TGA) capping reduced the cytotoxicity of cadmium telluride QDs and cadmium selenide QDs. Cadmium selenide with ZnS shell and GSH capping applied in concentrations below 0.5 µM exhibited no immediate cytotoxic effect and did not affect cell membrane integrity.

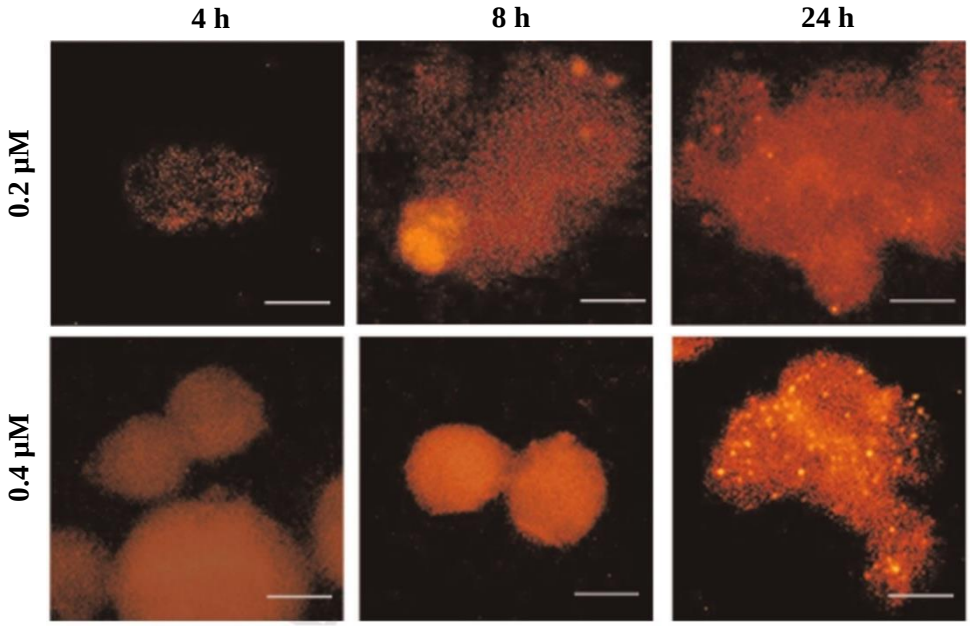

Figure 7. Time- and dose-dependent uptake of GSH-CdSe/ZnS QD in BON cells. QD uptake was determined by fluorescence microscopy after 4-24 h incubation. N=10-50 cells/concentration; representative findings of 3 independent experiments; scale bar, 20 µm.

Fluorescence microscopy analysis showed time- and dose-dependent uptake of GSH-CdSe/ZnS in the BON cells, with appreciable fluorescence (Fig. 7). However, it was impossible to determine the exact intracellular localization of QD.

Furthermore, the GSH-CdSe/ZnS QD when applied *in vivo*, either intravenously or topically to fertilized chicken eggs at the IC_{50} concentration (0.4 µM), did not affect the vascular network of the developing CAM or influence development of chicken embryos.

In summary, QDs with CdSe core, ZnS shell and GSH capping appear to be promising candidates for use in the biomedical field. High cellular uptake in the cancer cells with satisfying fluorescence and good tolerability both *in vitro* and *in vivo*, make GSH-CdSe/ZnS QD promising candidate for biological uses such as cancer monitoring. Further studies are necessary to estimate the potential of the GSH-CdSe/ZnS QD as an energy donor in photodynamic therapy.

5. Discussion

Cancer is still the most terrifying disease in existence, with 8.2 million cancer-related deaths each year (40). Current treatment options are far from satisfying. Oncological surgery or chemotherapy, which in the most cases are considered as the standard treatments, have severe side effects and may be very painful. Moreover, the number of new clinically approved drugs is inadequate. Therefore, there is an urgent need not only to develop new drugs but also to improve existing oncological approaches. Photodynamic therapy meets many current oncological needs, including

minimal invasiveness, high selectivity, almost painless and patient-friendly application, and the possibility of repeated use if needed (41,42). Nonetheless, the most important feature of PDT is that there is almost no resistance, which makes it especially attractive for cancer treatment (3,43). However, clinically approved photosensitizers have several drawbacks, such as long clearance from healthy tissue or inefficient production of singlet oxygen after photoactivation. The goal of this work was to evaluate the potential of a panel of newly synthesized phthalocyanines and quantum dots for use in photodynamic therapy and tumor monitoring.

Phthalocyanines, which belong to the 2nd generation of photosensitizers and which are known for their favourable physiochemical properties, are currently being intensively studied, as they usually exhibit many features of an ideal PS such as light absorption between 600-800 nm for deep tissue penetration, high quantum yield of singlet oxygen generation and non-toxic character in its non-photoactivated state (35, 36). Since it is possible to incorporate about 70 different atoms in the central ring of Pc, it is possible to synthesize almost infinite numbers of phthalocyanines. However, so far the only phthalocyanines that have received clinical approval or were at least tested in clinical trials are silicon-, aluminum- and zinc-based phthalocyanines (23). In this work, four kinds of zinc phthalocyanines have been examined for their potential use in PDT. All phthalocyanines showed favourable absorption spectra between 675-694 nm and exhibited no "dark" toxicity. However, the tetra-triethyleneoxysulfonyl substituted zinc phthalocyanine (ZnPc) revealed the greatest ability to eliminate cancer cells upon light activation. The maximal phototoxic effect of ZnPc caused >95% decrease in the number of cancer cells, while Pc 4 only <50 % and Pc 5 <80 %. Moreover, photoactivation of ZnPc required a lower light dose of 10 J/cm^2 as compared to 60 J/cm^2 for Pc 4 and Pc 5. Because it showed the highest efficiency of killing cancer cells after light activation with only 10 J/cm^2, ZnPc has been chosen for further studies.

While almost no resistance to PDT has been observed, low intracellular accumulation of PS may reduce the efficiency of the photodynamic reaction (PDR) (43,44). The cellular uptake and localization of the PS may influence PDR and play an important role in the induction of cell death via apoptosis or necrosis (9,45). Moreover, the effectiveness of the new photosensitizer correlates directly with the amount and localization of emerging free radicals (46).

In this work, by employing confocal laser scanning microscopy, notable dose-dependent intracellular uptake of ZnPc with homogenous cytoplasmic distribution was observed. It was impossible to recognize the exact localization of ZnPc, but ROS formation after photoactivation of ZnPc was observed not only in the cytoplasm, but also in nucleus and mitochondria, suggesting accumulation of PS near those structures. A prominent increase of ROS was observed especially

in the nucleus and mitochondria, which is attractive for productive PDT, because it may trigger the intrinsic apoptotic pathway by fracturing the structure of mitochondria.

Subsequent investigation confirmed the theory that ZnPc-PDT may induce apoptosis, in which the release of cytochrome c together with a significant increase of caspase-3 activity is observed. Moreover, photoactivation of ZnPc caused downregulation of antiapoptotic Bcl-2 and upregulation of proapoptotic Bax. This information may be important when PDT is applied for the treatment of tumors where inflammation and swelling are undesirable (e.g. brain tumors or esophagus cancers) (3), or when applied with new apoptosis-modulating drugs (currently being examined in human clinical trials) (47). In particular, the combination of apoptosis-modulating substances with PDT-inducing apoptosis may be an interesting clinical approach in the future.

Results of this study showed the ability of photoactivated ZnPc to induce cell cycle arrest. ZnPc-PDT caused a marked downregulation of the cell cycle promoter cyclin D1, leading to a G_1 cell cycle arrest. Additionally, *in vivo* investigation revealed a unique, long-lasting destructive influence on the blood vessels. Vascularity of the CAM examined 72 h after ZnPc-PDT did not regenerate after damage caused by photoactivation of ZnPc. Both the cell cycle arresting effect and the antiangiogenic potency of ZnPc-PDT may be of particular interest for combination therapy with clinically relevant chemotherapeutics. It has been already shown that PDT combined with different cancer modalities is beneficial (48). Zhang MD et al. have reported that PDT combined with chemotherapy (5-fluorouracil) increases the remission ratio by more than 20% and prolonged survival time by 6 months or more in treatment of advanced esophageal carcinoma (15). Another interesting approach for combination therapy was reported by Weiss et al. (49) who combined PDT with anti-angiogenic drugs (e.g. sorafenib) for treatment of the A2780 human ovarian carcinoma cells, achieving synergistic inhibition of the tumor growth.

A very important feature of an appropriate photosensitizer is its non-toxic character in the absence of light (so-called “dark toxicity”). PSs, when applied systemically, should exert minimum dark toxicity in healthy tissue (which is not illuminated) and maximum toxicity in the tumor tissue (upon light activation). This selective and local activation of PSs helps avoid systemic toxicity and saves healthy cells. Detailed *in vitro* and *in vivo* evaluations carried out in this work, validated ZnPc’s safety in the absence of light. Non-photoactivated ZnPc did not affect cell proliferation or cause changes in cell morphology. Cancer cells incubated with non-photoactivated PS showed no changes in the expression of proliferation, cell cycle or apoptosis-related proteins such as cyclin D1, Bcl-2 or AKT. Moreover, neither topical nor intravenous application of nonphotoactivated ZnPc influenced the vascularity of the CAM and chicken embryo development. Finally, in the first animal experiments on native Wistar rats, no signs of systemic toxicity was observed. These

findings support the notion that ZnPc does not exhibit unwanted dark toxicity, which further qualifies it as an interesting new compound for PDT.

Current trends in photodynamic therapy favour 3rd generation photosensitizers, a combination of well-known or promising new PSs with antibodies, liposomes or nanostructures such as QDs (29,50,51). For example, QD-PS conjugation may enable monitoring of tumor tissue and PDT using only one molecule. First, QDs known from their adjustable fluorescence spectra can be excited by a different wavelength than PS to visualize the neoplastic tissue precisely. Thereafter, PS can be excited at another wavelength or via FRET (fluorescence resonance energy transfer) from the QD (43). Moreover, by enhancing permeability and the retention effect, specialized PS should be selectively taken up by the tumor tissue (52). For that reason, a part of this work focused on analysing the safety of a panel of QDs that may be used for biomedical purposes.

The major drawback of the QDs is their potential toxicity, but this may be reduced by adjusting their structure. Shell and capping can stabilize a QD's structure and prevent an ion leakage from its core (35). Among several different types of QDs tested in this work, CdSe QD with ZnS shell and GSH capping showed particularly good biotolerability in the cell models as well as in living organisms. Applied in the micromolar range, this QD did not have immediate cytotoxic effects on cancer cells and did not influence chicken embryo vitality and development. GSH-CdSe/ZnS was taken up by cancer cells in a time- and dose-dependent manner, with detectable fluorescence. The nontoxic character of GSH-CdSe/ZnS, along with its sufficient cellular uptake and appreciable fluorescence, suggests that this QD may be a promising candidate for biomedical application, including future conjugation with Pc for 3rd generation PS.

In conclusion, this work presents the outstanding photoactive potential of a new zinc-phthalocyanine photosensitizer. The safety of ZnPc in its non-photoactivated state and strong antiproliferative and antiangiogenic efficiency after photoactivation makes ZnPc a promising candidate for PDT, applied on its own or together with established chemotherapeutics. The conjugation with GSH-CdSe/ZnS may be an interesting approach towards 3rd generation PS for innovative PDT. Further investigation is needed to explore the effectiveness of ZnPc-PDT in animal models and the efficacy of the QD-Pc conjugate *in vitro* and *in vivo*.

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7. Affidavit

I, Weronika Kuzyniak certify under penalty of perjury by my own signature that I have submitted the thesis on the topic "New photosensitizers and nanomolecules for photodynamic therapy of cancer". I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My contributions in the selected publications for this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author of correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

_____

Date Signature

Declaration of any eventual publications

Weronika Kuzyniak had the following share in the following publications:

Publication 1:

Moeno S, Krause RW, Ermilov EA, **Kuzyniak W**, Höpfner M. Synthesis and characterization of novel zinc phthalocyanines as potential photosensitizers for photodynamic therapy of cancers. Photochem Photobiol Sci. 2014; 13(6):963-70

IF: 2.235

Share in publication: handling the cell culture, obtaining and analyzing the biological data – *in vitro*: proliferation study with Photofrin

Publication 2:

Kuzyniak W, Ermilov EA, Atilla D, Gürek AG, Nitzsche B, Derkow K, Hoffmann B, Steinemann G, Ahsen V, Höpfner M. Tetra-triethyleneoxysulfonyl substituted zinc phthalocyanine for photodynamic cancer therapy. Photodiagnosis Photodyn Ther. 2016; 13:148-57

IF: 2.412

Share in publication: planning the project; handling the cell culture; obtaining and analyzing the biological data - *in vitro*: PDT treatment, proliferation study, determination of cell viability, determination of cytotoxicity, detection of apoptotic specific changes e.g. caspase-3 activity, cell cycle analysis, Western blotting; *in vivo* - CAM assay: antiangiogenic study; writing the text and preparing the figures (except of synthetic pathway of ZnPc, fluorescence excitation and emission spectra)

Publication 3:

Kuzyniak W, Schmidt J, Glac W, Berkholz J, Steinemann G, Hoffmann B, Ermilov EA, Gürek AG, Ahsen V, Nitzsche B, Höpfner M. Novel zinc phthalocyanine as a promising photosensitizer for photodynamic treatment of esophageal cancer. Int J Oncol. 2017; 50(3):953-63

IF: 3.018

Share in publication: planning the project; handling the cell culture; obtaining and analyzing the biological data – *in vitro*: PDT treatment, proliferation study, detection of free radical formation, detection of apoptotic specific changes e.g. caspase-3 activity, Western blotting; *in vivo* - CAM assay: antineoplastic and antiangiogenic study, biotolerability; animal study – daily animal observation with weight monitoring, organ dissection and examination); writing the text and preparing the figures

Publication 4:

Kuzyniak W, Adegoke O, Sekhosana K, D'Souza S, Tshangana SC, Hoffmann B, Ermilov EA, Nyokong T, Höpfner M. Sythesis and characterization of quantum dots designed for biomedical use. Int J Pharm. 2014; 466(1-2):382-9

IF: 3.994

Share in publication: planning the project; handling the cell culture; obtaining and analyzing biological data – *in vitro*: proliferation study, determination of cytotoxicity, detection of intracellular uptake of QDs; *in vivo*: CAM assay – embryotoxicity; writing the text and preparing the figures (except of 2D structure of QDs, luminescence maxima and quantum yields of QDs, powder XRD spectra, UV/vis absorption and fluorescence emission)

Signature, date and stamp of the supervising University teacher

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Signature of the doctoral candidate

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Moeno S, Krause RW, Ermilov EA, **Kuzyniak W**, Höpfner M. Synthesis and characterization of novel zinc phthalocyanines as potential photosensitizers for photodynamic therapy of cancers. Photochem Photobiol Sci. 2014; 13(6):963-70. http://dx.doi.org/10.1039/c3pp50393c

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Curriculum vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

Complete list of publications

Publications:

1. **Kuzyniak W**, Adegoke O, Sekhosana K, D'Souza S, Tshangana SC, Hoffmann B, Ermilov EA, Nyokong T, Höpfner M. Synthesis and characterization of quantum dots designed for biomedical use. Int J Pharm. 2014; 466(1-2): 382-9.

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3. Berkholz J, **Kuzyniak W**, Hoepfner M, Munz B. Overexpression of the skNAC gene in human rhabdomyosarcoma cells enhances their differentiation potential and inhibits tumor cell growth and spreading. Clin Exp Metastasis. 2014; 31(8): 869-79.

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5. **Kuzyniak W**, Schmidt J, Glac W, Berkholz J, Steinemann G, Hoffmann B, Ermilov EA, Gürek AG, Ahsen V, Nitzsche B, Höpfner M. Novel zinc phthalocyanine as a promising photosensitizer for photodynamic treatment of esophageal cancer. Int J Oncol. 2017; 50(3):953-63

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Abstracts:

1. E. Ermilov, **W. Kuzyniak,** E. Sekhosana, B. Hoffmann, C. Litwinski, A.R. Pries, M. Höpfner: Semiconductor quantum dots for innovative approaches in photodynamic diagnosis and therapy. Frontiers in cardiovascular biology (FCVB 2012), London, UK, 30 March 30 - 1 April 2012.

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3. **W. Kuzyniak**, E. Ermilov, D. Atilla, A. Gürek, B. Nitzsche, K. Derkov, B. Hoffmann, A.R Pries, M. Höpfner: Antitumor and antiangiogenic potency of photodynamic therapy with tetra-triethyleneoxysulfonyl substituted zinc phthalocyanine as a novel photosensitizer. 3rd Joint Meeting of the German Society for Microcirculation and Vascular Biology (GfMVB) and the Swiss Society for Microcirculation and Vascular Research (SSMVR), Münster, Germany, 30 September - 2 October 2014.

4. **W. Kuzyniak**, E. Ermilov , D. Atilla, A. Gürek, B. Nitzsche, K. Derkow, B. Hoffmann, G. Steinemann, A.R. Pries, V. Ahsen, M. Höpfner: Tetra-triethyleneoxysulfonyl substituted zinc phthalocyanines are promising new photosensibilisators for cancer treatment with Photodynamic Therapy (PDT). Experimental Biology (EB 2015), Boston, USA, 28 March - 1 April 2015.

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