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# PHOTODYNAMIC THERAPY ACTIVITIES OF PHTHALOCYANINES ON CANCER

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MASTER THESIS
DEPARTMENT OF MEDICAL BIOTECHNOLOGY

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This thesis is my work, from the planning to writing of the thesis that I have no unethical behavior at all stages; all the information and interpretations not obtained by this thesis. That I have cited sources and included these sources in the list of references. And declare that I am not infringing any patents or copyrights at the time of writing.

08/01/2020

Şeyma Işık

## FOREWORDAND ACKNOWLEDGMENTS

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## LIST OF SYMBOLS

| | |
|---|---|
| % | Percentage |
| $-O_2$ | Singlet Oxygen |
| µg | Microgram |
| µm | Micrometer |
| µl | Microliters |
| µs | Microsecond |
| C° | Celsius |
| Al | Aluminum |
| $cm^2$ | Centimeter Square |
| $CO_2$ | Carbon Dioxide |
| Fe | Iron |
| g | Gram |
| $H_2O$ | Water |
| $H_2O_2$ | Hydrogen Peroxide |
| In | Indium |
| kcal | Kilo Calorie |
| kDa | Kilo Daltons |
| *l* | Liter |
| M | Molar |
| mg | Milligram |
| Mg | Magnesium |
| ml | Milliliter |
| mM | Millimolar |
| mV | Millivolt |
| ng | Nanograms |

| | |
|---|---|
| nm | Nanometer |
| ns | Nanoseconds |
| $O_2$ | Molecular Oxygen |
| pH | Potential of Hydrogen |
| rpm | Revolutions per minute |
| SD | Standard Deviation |
| SOD | Superoxide Dismutase |
| Sn | Tin |
| Zn | Zinc |

# LIST OF ABBREVIATIONS

| | |
|---|---|
| 3D | Three-Dimensional |
| ALA | 5-Aminolevulinic acid |
| AMD | Age-related macular degeneration |
| ATCC | American Type Culture Collection |
| Ce6-PVP | Chlorin e-6-polyvinpyrrolidone |
| DCFDA | 2′,7′-Dichlorodihydrofluorescein diacetate |
| DMSO | Dimethylsulfoxide |
| DNA | Deoxyribonucleic Acid |
| DNase | Deoxyribonuclease |
| DPBS | Dulbecco's phosphate-buffered saline |
| EDTA | Ethylenediaminetetraacetic Acid |
| FBS | Fetal Bovine Serum |
| FDA | The Food and Drug Administration |
| HBSS | Hank's Balanced Salt Solution |
| HpD | Hematoporohyrin derivative |
| HPPH | 2-(1-hexyloxyethyl)-2devinyl pyropheophorbide-a |
| IR | Infrared radiation |
| LED | Light-emitting diode |
| MACE | Mono-(L)-aspartylchlorşn-e6 |
| min. | Minute |
| mTHPC | m-tetrahydroxyphenylchlorin |
| PBS | Phosphate-Buffered Saline |
| Pc | Phthalocyanine |
| PDT | Photodynamic Therapy |
| PS | Photosensitizer |

| | |
|---|---|
| RNase | Ribonuclease |
| ROS | Reactive Oxygen Species |
| TUBITAK | Scientific and Technological Research Council of Turkey |

## SUMMARY

Cancer is one of the most death-causing health problems in the world. There are many treatment approaches to cure this disease. Photodynamic therapy is one of the most promising cancer treatments since their advantages including being less invasive than radiotherapy, surgery, and have the ability to bypass the mechanisms that make the cell resistant to chemotherapeutic drugs and ionizing radiation. Photodynamic therapy is based on light excitation of a photosensitizer molecule that transfers its energy/photon to nearby molecular oxygen. This mechanism leads to the formation of harmful singlet oxygen and reactive oxygen species, by the way, cellular damage. Phthalocyanine molecules, synthetic macrocyclic chemical compounds with absorption spectrum and fluorescent characteristic and capability to create energy to form singlet oxygen that leads to cellular death by triggering mechanisms within the cell, are suitable to be used in photodynamic therapy as a photosensitizer agent.

In this thesis, in-vitro photodynamic therapy effects of nine specifically synthesized tetra-substituted symmetric water-soluble phthalocyanine molecules were investigated in many terms. By using their self-fluorescent features, cellular uptake of the molecules was revealed through proper channels of flow cytometry without any need to label the phthalocyanine molecules. Biocompatibility of the molecules was studied on mouse L929 cell line considering prescription of International Organization for Standardization, ISO10993. After revealing biocompatibility of the molecules, firstly dark cytotoxicity experiments were carried out to determine the non-toxic concentration range to be used for PDT studies. Among different light fluence, proper light fluence was determined, by the way, at the proper Pc concentrations and light fluence, in-vitro photodynamic therapy was applied on different cancer cell lines, salivary gland carcinoma, pharynx carcinoma, colorectal adenocarcinoma, and breast carcinoma. Further, whether the PDT-mediated cellular damage is triggered through reactive oxygen species formation or not, for each treatment group, oxidative stress was evaluated.

It has been shown that all the molecules used in the study are biocompatible, have cellular uptake properties, do not have cytotoxicity in the dark. Moreover, each phthalocyanine molecule except non-metallo phthalocyanine with morpholinyl moieties, HQH2Pc, causes significant cell death due to photodynamic therapy and this cell death is related to ROS production. The highest PDT effect was determined in the salivary gland carcinoma cells and in the cationic zinc phthalocyanine, EH1. In conclusion, these molecules are powerful candidates that can be used as photosensitizer agents in photodynamic therapy for further studies.

**Keywords**: Cancer, Photodynamic therapy, Phthalocyanines, Reactive oxygen species, Singlet oxygen

# ÖZET

**Ftalosiyanin Moleküllerinin Kanser Üzerindeki Fotodinamik Tedavi Etkileri**

Kanser, dünyada ölüme en çok sebebiyet veren hastalıklardan bir tanesidir. Kanser tedavisi için farklı yaklaşımlar söz konusudur. Fotodinamik tedavi, radyoterapi ve cerrahi yöntemlerinden daha az invaziv olması, ayrıca hücreyi kemoterapötik ilaçlara ve iyonlaştırıcı radyasyona dirençli hale getiren mekanizmaları elimine edebilmesi, kıyasla ucuz bir tedavi yöntemi olması gibi pek çok avantaja sahip olmasından dolayı ümit vaat eden kanser tedavilerinden biridir. Fotodinamik tedavi temelde, enerjisini / fotonunu yakındaki moleküler oksijene aktaran ışığa duyarlılaştırıcı bir molekülün belirli dalga boyunda ışık ile uyarılmasına dayanır. Bu mekanizma, zararlı singlet oksijenin ve reaktif oksijen parçacıklarının oluşumuna neden olarak hücresel hasara yol açar. Ftalosiyanin molekülleri sentetik makrosiklik kimyasal bileşiklerdir. Absorpsiyon spektrum karakteristikleri ve hücresel hasara yol açan singlet oksijeni oluşturmak için gerekli olan enerjiyi üretebilme kapasiteleri sayesinde, fotodinamik tedavide bir ışığa duyarlılaştırıcı madde olarak kullanılabilmektedirler.

Bu tez çalışmasında, dokuz tane özel olarak sentezlenmiş simetrik yapıda olan suda çözünür ftalosiyanin molekülünün in vitro fotodinamik tedavi etkileri birçok açıdan incelenmiştir. Floresan özellikleri olan bu moleküller, bu özellikleri kullanılarak, hücrelere bağlanma kapasiteleri, floresan başka bir madde ile boyanmaya gerek duyulmaksızın akış sitometrisinin uygun kanalları ile gösterilmiştir. Moleküllerin biyouyumluluğu, fare fibroblast L929 hücreleri üzerinde çalışılmıştır. Moleküllerin biyouyumluluğunun gösterilmesinden sonra, ilk önce PDT çalışmaları için kullanılacak toksik olmayan konsantrasyon aralığının belirlenmesi için karanlıkta sitotoksisite deneyleri yapılmıştır. Belirlenen uygun Pc konsantrasyonu ve ışık dozunda, farklı kanser hücre hatlarında fotodinamik tedavi uygulaması gerçekleştirilmiştir. Ayrıca, fotodinamik tedavi kaynaklı hücresel hasarın, reaktif

oksijen parçacıklarının oluşumu ile tetiklenip tetiklenmediğini göstermek amacıyla, her tedavi grubu için oksidatif stres değerlendirilmesi yapılmıştır.

Çalışmada kullanılan tüm moleküllerin biyouyumlu oldukları, hücresel alım özelliklerine sahip oldukları, karanlıkta sitotoksisitelerinin bulunmadığı gösterilmiştir. Ayrıca, merkez metali bulundurmayan morfolinil gruplu ftalosiyanin molekülü olan $HQH_2Pc$ molekülü hariç her molekül, fotodinamik tedaviye bağlı hücre ölümüne neden olmuştur ve bu hücre ölümünün reaktif oksijen parçacığı üretimine bağlı olduğu gösterilmiştir. En yüksek PDT etkisi, tükürük bezi karsinom hücrelerinde ve katyonik çinko ftalosiyanin molekülü olan EH1 molekülünde belirlenmiştir. Sonuç olarak, bu moleküller ileri çalışmalar için fotodinamik tedavide ışığa duyarlılaştırıcı ajanlar olarak kullanılabilecek güçlü adaylardır.

**Anahtar Kelimeler**: Fotodinamik tedavi, Ftalosiyanin, Kanser, Reaktif oksijen parçacığı, Singlet oksijen.

# 1. INTRODUCTION

In order to meet the demands of developing technology, hybrid compounds formed by combining two or more compounds are important among high technology materials. The absorption spectrum characteristics of the synthetic macrocyclic compounds, phthalocyanines, make them used photosensitizers and the molecules have become interesting materials for photodynamic therapy (PDT) applications in recent years (1, 2). Photodynamic therapy is one of the most promising treatment modalities due to its low side effects. The basis of photodynamic cancer treatment is light, specific photo sensor, photosensitizer, and oxygen that can absorb light in the near IR region (3). The first step in treatment begins with the administration of the photosensitizer (PS) agent into the body. The agent is absorbed by all the cells of the body, but it is held in cancer cells more than normal cells. 24-72 hours after the injection, the agent is completely excreted from normal cells but remains retained by cancerous cells (4). When the light exposure at a specific wavelength occurs, the PS molecules are stimulated and change their energy state from the baseline to the excited state. As it returns from the excited level to the basic state, the released energy is transferred to molecular oxygen within the cell, causing singlet oxygen and reactive oxygen species (ROS) formation (4, 5). The resulting singlet oxygen and ROS causes damage and death to cancerous cells. Since this reaction is very short-lived (10-350 ns) and local (approximately 10-55 nm in the cell), tissues other than cancerous tissues are not damaged. In addition to these instant effects of PDT application, there are two other important advantages that show its effect for a long time. The first is that the vascularization in the tumor is disrupted after therapy, and the structure is left without food, and the second is that T cells attack cancer cells as a result of activation of the immune system (6).

The response to PDT at the cellular level depends on the internal and external parameters. External parameters are including factors such as PS concentration, light dose, incubation conditions. Internal parameters are including factors such as metabolic properties of the cell, the oxygen level in tissue, cell genotype, and cell cycle

phase (7). The mechanism of PDT is also directly related to the intracellular localization of the PS molecule. This is because the life of singlet oxygen, the most effective photoproduct in PDT, is shorter than 0.05 μs. Owing to its high reactive property, -$O_2$ can diffuse only 0.02 μm from the region of release (8, 9). Therefore, the localization of PS within the cell determines which organelle is primarily damaged. Because most PSs are localized outside the nucleus and have a short lifespan of singlet oxygen, the mutagenic effect of PDT is quite low. PDT mediated direct cell death occurs in the form of apoptosis, necrosis or combination. In many cases, PDT has been shown to be highly effective in inducing apoptosis (10, 11).

In this thesis study, in-vitro PDT effects of nine specifically synthesized cationic water-soluble phthalocyanine (Pc) derivatives (12, 18) on Salivary gland carcinoma, A253 cell line, Pharynx carcinoma, FaDu, Colorectal adenocarcinoma, HT29, Breast carcinoma, T47D cell lines has been aimed. Respectively, the binding status of the molecules to the cells, their biocompatibility, cytotoxic effects in the dark, the fluency of the light to be used, and photodynamic therapy-induced cell death, and the ability to trigger the formation of reactive oxygen species due to photodynamic therapy were investigated and reported.

# 2. BACKGROUND

## 2.1. Cancer

Cancer is a branch of a group of diseases with the capacity of abnormal cell growth and invading and spreading surrounding parts of the body (20).

## 2.2. Hallmark of Cancer

In 2000, Douglas Hanahan and Robert A Weinberg reported 6 hallmarks of cancer that are acquired during the progression of tumorigenesis. One of the hallmarks is that resistance to cell death where healthy cells are induced cell death by different mechanisms including apoptosis, autophagy, and necrosis. Also, cancer cells have the capacity to limitless growth due to evading growth suppression where healthy cell growth is restricted and suppressed. They activate cellular proliferation signaling pathways by producing an excessive amount of growth factors and also producing abnormal growth factor receptors that do not require ligand to trigger cascade in order to proliferate unstoppably. Additionally, cancer cells have the ability to replicate limitless due to their genetic material alteration where healthy cells can replicate for only an amount of time. To address the oxygen and nutrition needs due to their rapid growth capacities, cancer cells trigger angiogenesis that is the formation of new blood vessels through angiogenic growth factors. Further, Cancer cells have the ability to invade other local tissues and metastasize to the distant sites on the body by blood and lymph circulation (21). In addition to these 6 hallmarks of cancer, in 2011 Douglas Hanahan and Robert A Weinberg expand their understanding of cancer by hallmarks of cancer: generation. They have put in 4 additional hallmarks of cancer in their previous reports. One of these hallmarks is genome instability and mutation. Instability of genetic material can lead to mutations that can be normally altered by check mechanism, in this case, these gathered mutation results in the formation of cancer

cells by triggering unrestricted cell growth. Also, they reported that the destruction of this immune system is one of the hallmarks to progress cancer where cancer progression normally restricted by natural immunity of the body. Moreover, in contrast to healthy cells, cancer cells regulate their glucose metabolism to fulfill their excessive energy needs by limiting it mostly to glycolysis even in the presence of oxygen, called aerobic glycolysis. Lastly, they stated that cancer cell proliferation and progress is also supported by tumor promoted inflammatory cells (22).

## 2.3. Photodynamic Therapy

There is various cancer treatment currently used with FDA approval such as chemotherapy, radiotherapy, immunotherapy, targeted drug therapy, etc. (national cancer institute). Among cancer treatments, photodynamic therapy is one of the most promising therapy due to its low side effect, selectivity, long term efficiency, and eliminating possible drug-resistant (23).

PDT application is based on 3 elements that are a light source, a photosensitizer agent, and molecular oxygen. The first step of this treatment begins with the administration of tumor localizing photosensitizer agent that is activated by a light source of an appropriate wavelength (3, 10).

The photosensitizer changes its energy state to short-lived singlet excited stated as a result of the absorption of a photon of light. When changing its state from singlet to longer-lived triplet state, exchange its energy with nearby molecular oxygen yielding singlet oxygen as well as reactive oxygen species that trigger cell damage and death (10).

## 2.4. History of Photodynamic Therapy

The history of PDT underlies before the century, however first recognition of cell death triggered by a chemical compound and light was reported 100 years ago, in 1900 by a medical student Oscar Raab following his study on malaria-causing protozoa. As a change, he found that acridine red and light combination had a lethal effect on Infusoria. Following his studies, he found that the lethal effect was a result of energy transfer from light to chemical compound as like photosynthesis in plants (7, 24).

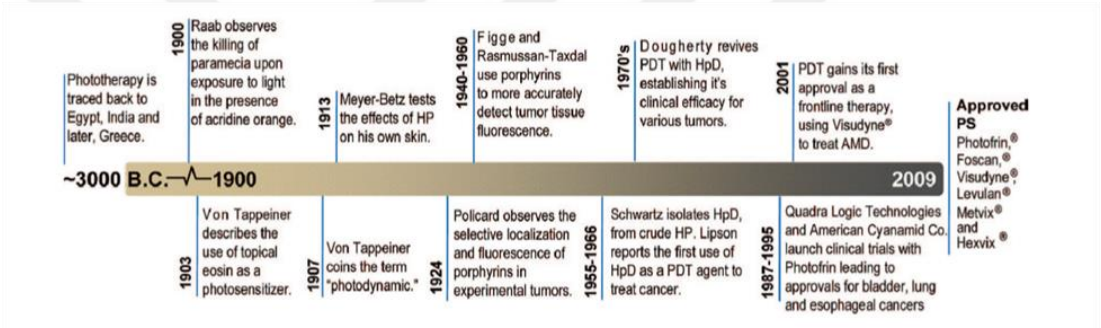



**Figure 1** Milestones of Photodynamic Therapy.

Reference: Reprinted with permission from (Celli JP, Spring BQ, Rizvi I, Evans CL, Samkoe KS, Verma S, et al. Imaging and photodynamic therapy: Mechanisms, monitoring, and optimization. Chem Rev. 2010; 110(5): 2795–2838.)

In 1903, the first usage to treat skin tumors, eosin and the white light combination were used by von Tappeiner. Then, von Tappeiner with Jodlbauer revealed oxygen requirement in photoreaction in 1907, and they described this phenomenon as ‘‘photodynamic action’’ (25). In 1913, Freiedrich Meyer-Betz injected himself with hematoporphyrin to see the effects in humans. In 1924 Frenchman Policard reported the localization of porphyrin in a malignant tumor. In 1948, Figge and Weiland studied on porphyrin localization in each tumor type to the application of porphyrin in diagnosis and treatment and reported that in normal tissues, excepted to lymph nodes, fetal and placental tissue and healing wounds,

fluorescent was not monitored (26). In 1955, Schwartz showed hematoporphyrin was composed of various porphyrin with different features. After some purification and dissolving procedures, he produced hematoporphyrin derivative (HpD) that had a much more cytotoxic effect than crude hematoporphyrin (27). The first treatment with photo induced HpD on a tumor was in 1975 as a result of the studies of Dougherty and coworkers. In the 2001 verteporfin, menzoporphyrin derivative monoacid ring A, commercially named as Visudyne, was approved by FDA to primarily treat age-related macular degeneration (7).

## 2.5. Component of Photodynamic Therapy

The working principle of photodynamic therapy includes three components: light source, photosensitizer, and oxygen (23).

### 2.5.1. Photosensitizer

Photosensitizers are chemical molecules that induce a photochemical reaction in other molecules through photosensitization. Photosensitizers are one of the main components of photodynamic therapy.

Currently, various photosensitizer agents with Food Drug Administration approval are used in the clinic (Table 1) to treat different types of diseases including cancer. Some of them are Photofrin, Foscan, Visudyne®, Levulan®, and Metvix®. On the other hand, there are many photosensitizer candidates are currently under

clinical trials including but not limited to HPPH, motexafin lutetium, NPe6, SnET2 (28).

**Table 1** Currently used photosensitizer molecules with FDA approval, and their commercial names.

| Photosensitizer | Structure | Wavelenght (nm) | Approved | Trials | Cancer type |
|---|---|---|---|---|---|
| Porfimer sodium (Photofrin) (HpD) | Porphrin | 630 | Worldwide | | Lung, esophagus, bile,duct, bladder, brain, ovarian |
| ALA | Porphrin precursor | 635 | Worldwide | | Skin, bladder, brain, esophagus |
| ALA esters | Porphrin precursor | 635 | Europe | | Skin, bladder |
| Temoporfin (Foscan) (mTHPC) | Chlorin | 652 | Europe | United States | Head and neck, lung, brain, skin, bile,duct |
| Verteporfin | Chlorin | 690 | Worldwide (AMD) | United Kingdom | Ophthalmic, pancreatic,skin |
| HPPH | Chlorin | 665 | | United States | Head and neck, esophagus, lung |
| SnEt2 (Purlytin) | Chlorin | 660 | | United States | Skin, breast |
| Talaporfin (LS11, MACE, NPe6) | Chlorin | 660 | | United States | Liver, colon, brain |
| Ce6-PVP (Fotolon) | Chlorin | 660 | | Belarus, Russia | Nasopharyngeal, sarcoma, brain |
| Silicon phthalocyanine (Pc4) | Phthalocyanine | 675 | | United States | Cutaneous T-cell lymphoma |
| Padoporfin (TOOKAD) | Bacteriochlorin | 762 | | United States | Prostate |
| Motexafin lutetium (Lutex) | Texaphyrin | 732 | | United States | Breast |

Reference: Reprinted with permission (lisence number, 4724860266940) from (Agostinis P, Berg K, Cengel KA, Foster TH, Girotti AW, Gollnick SO, et al. Photodynamic therapy of cancer: An update. CA Cancer J Clin. 2011; 61(4): 250–281.)

#### 2.5.1.1. Selective tumor distribution of photosensitizer

Although tumor-specific localization of photosensitizers is not fully understood, some features of tumor tissues can be used to understand these selective distributions. Excessive low-density protein receptors, the presence of macrophage (29, 30) and low pH are some of the differences between tumor tissues and normal tissues. Tumor tissues due to their lower pH and higher lactic acid content of interstitial fluid (31) can preferentially uptake photosensitizers compared to normal tissues (32, 33). Furthermore, the large interstitial space of tumor tissues, a leaking vessel, damaged lymphatic drainage, a large amount of newly synthesized collagen (34, 35), and a high amount of lipids are also effective for the preferential distribution of photosensitizers (36).

#### 2.5.1.2. Phthalocyanines

Phthalocyanines that are synthetic macrocyclic compounds have been used as photosensitizers and become interesting materials for photodynamic therapy (PDT) applications in recent years due to their absorption spectral characteristics with high molecular extinction coefficient in the red Q band. PCs that have to produce 26-30 kcal/mole energy at their triplet state can give yield appropriate energy for efficient photodynamic therapy where singlet oxygen formation requires 23 kcal/mole energy (37, 38).

Moreover, PCs have some properties that make them useful in research and clinical including tumor selectivity, non-toxicity, effective as a photosensitizer, fluorescence capacities, and long-lived triplet state production efficiency upon irradiation, photochemical and chemical stability (38, 40). PCs can be altered by adding groups to the surrounding of their ring structure. Also, different metal ions can be inserted on the central ring of non-metallo PCs. Blue and green colors and photo energy transfer features of PCs allow them used in different industries. Photo reduction rates of PCs depend on the metal group on the central ring of metallo-PCs respectively as Zn > Al > Mg > Sn > Fe (37, 41).

### 2.5.2. Light

In photodynamic therapy, currently, a large range of light source has been used. One of them is laser light source with having some disadvantages including powerful irradiation, being highly expensive compared to alternatives, and requiring an optical system that widens the beam of laser in order to be effective in a large area. As a non-laser source, conventional lambs lead to overheating of the cell during photo illumination by its high irradiance capacities. In addition to these light sources with limitations, light-emitting diodes (LEDs) have been also used in photodynamic

therapy application as an appropriate source due to being safe, irradiation features on large areas, wide emission wavelength range, and thermally being non-destructive (42, 43).

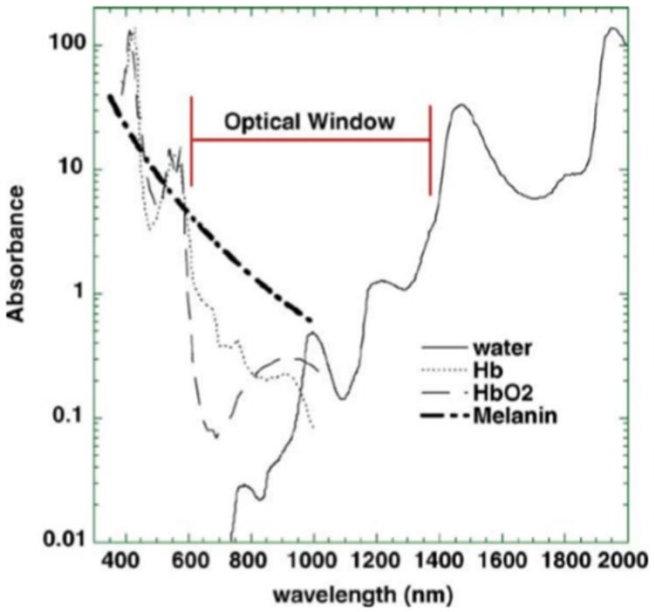



**Figure 2** Optical window in tissue. In tissue, water, de-oxy/oxyhemoglobin ($Hb/HbO_2$), cytochromes and melanin are the most important chromophores, and their absorbance spectrums describe the optical window for photodynamic therapy.

Reference: Reprinted with permission (lisence number, 4724840999158) from (Castano AP, Demidova TN, Hamblin MR. Mechanisms in photodynamic therapy: Part one - Photosensitizers, photochemistry and cellular localization. Photodiagnosis Photodyn Ther. 2004; 1(4): 279–93.)

### 2.5.3. Oxygen

The role of molecular oxygen in photodynamic therapy is absolute, the oxygen concentration is one of the key factors that affect photodynamic therapy efficiency. However, it is one of the limiting factors. Hypoxia through the rapid proliferation of cancer cells, further vascular damage, and ROS production by PDT limit efficiency of PDT on solid tumors (44).

Peroxisome and metabolic activities of cancer cells make them produce a higher amount of ROS compared to the non-cancerous cell. Among ROS produced by cancer cells, hydrogen peroxide is predominantly more abundant. Decomposition capacity of hydrogen peroxide to molecular oxygen by Fenton reaction enhances PDT efficiency by increasing oxygen concentration in the presence of metal ions (44, 45). Additionally, hydroxyl radicals can trigger cancer cell apoptosis by oxidizing and damaging organelles. Singlet oxygen has a short lifetime(<40ns) with low diffusion rate(20nm) limits the area effected by PDT, therefore non-cancerous cells where the photosensitizers are cleaned up after a time scale are protected from PDT induced damage (46).

## 2.6. Mechanism of PDT

After activation through a light of specific wavelengths, PS pass from ground energy state to triplet energy state that leads to two types of photochemical response in nearby molecules, mostly molecular oxygen in PDT. Photo physical activation of ground-state oxygen takes place in two different reactions (43, 47). During type-I reaction, electrons or photons transfer to molecular oxygen to reactive oxygen formation, however in type-II reaction, rather than the electron, energy transfer takes place. Electron transfer by type I reaction results in reactive oxygen species, superoxide anions formation. Due to its short lifetime of superoxide anion in biological systems, at the end of the reaction hydrogen peroxide is formed (5, 47).

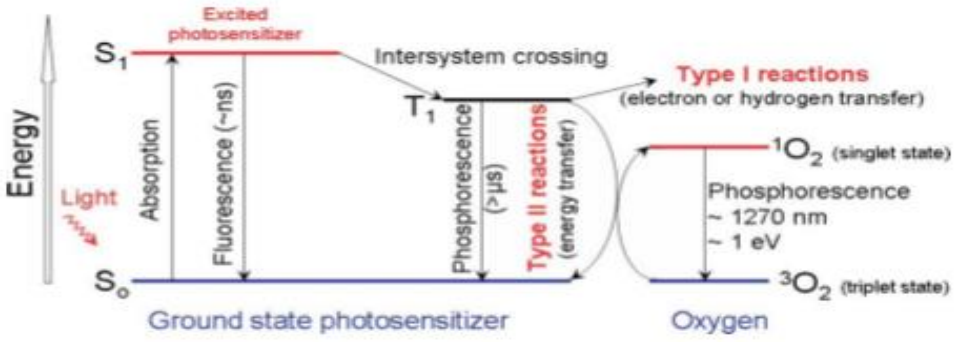



**Figure 3** Photophysical changes of photosensitizer agent shown by Jablonski diagram.

Reference: Reprinted with permission (lisence number, 4724860651423) from (Agostinis P, Berg K, Cengel KA, Foster TH, Girotti AW, Gollnick SO, et al. Photodynamic therapy of cancer: An update. CA Cancer J Clin. 2011; 61(4): 250–281.)

The damage by hydrogen peroxide is not limited to cellular component due to its capacity to diffusion through the membrane. Further, the higher concentration of hydrogen peroxide causes it to react with superoxide anions and the formation of hydroxyl radicals that can also be formed by Fenton reaction in presence of metal ions (5, 43). On the other hand, Energy transfer by type 2 reaction end up with very reactive singlet oxygen formation from molecular ground state oxygen. Both reactions can take place together following PDT, however, type two reaction takes place predominantly (43, 49). The damage by hydrogen peroxide is not limited to cellular component due to its capacity to diffusion through the membrane. Further, the higher concentration of hydrogen peroxide causes it to react with superoxide anions and the formation of hydroxyl radicals that can also be formed by Fenton reaction in the presence of metal ions (5).

Symbols of Figure 6 are $H_2O_2$, hydrogen peroxide; $O_2$ ($1\Delta g$) $O_2$ ($1\Delta g$), singlet oxygen (excited state); $O_2$ ($3\sum -g$) $O_2$ ($3\sum g-$), triplet oxygen (ground state); $O-\cdot 2O_2-\cdot$, superoxide anion; $OH-\cdot OH-\cdot$, hydroxyl radical; SOD, superoxide dismutase; X - / - ++X - / - ++, anion/cation species; X·X·, radical species (43).

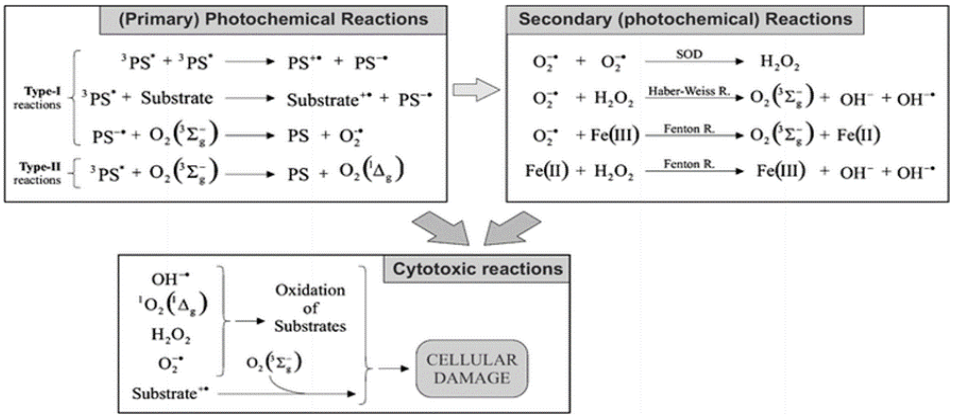



**Figure 4** Schematic represent of photochemical reactions through PDT in order to cause reactive oxygen species formation and cellular damage.

Reference: Reprinted with permission from (Plaetzer K, Krammer B, Berlanda J, Berr F, Kiesslich T. Photophysics and photochemistry of photodynamic therapy: Fundamental aspects. Lasers Med Sci. 2009; 24(2): 259–68.)

## 2.7. Cell Death Mechanisms Triggered by PDT: Apoptosis or Necrosis

Cell death following photo irradiation in photodynamic therapy takes place either necrosis or apoptosis due to some factors that are light dose, subcellular localization of photosensitizer, and cell type (11). Normally, lower light doses trigger apoptotic cell death while higher doses cause more necrotic cell death as a result of that apoptosis-related enzymes and components such as caspases can be inactivated by high doses PDT (50). After PDT, 70 percent or less cell death is a result of apoptosis where 99 percent of cell death generally occurs due to necrosis (51).

## 2.8. Subcellular Localization of Photosensitizer

Subcellular localization photosensitizer is one of the significant factors that determine the localization of primary cellular damage, type of cell death whether apoptotic or necrotic and rescue response including the changes in gene and protein expression levels (6).

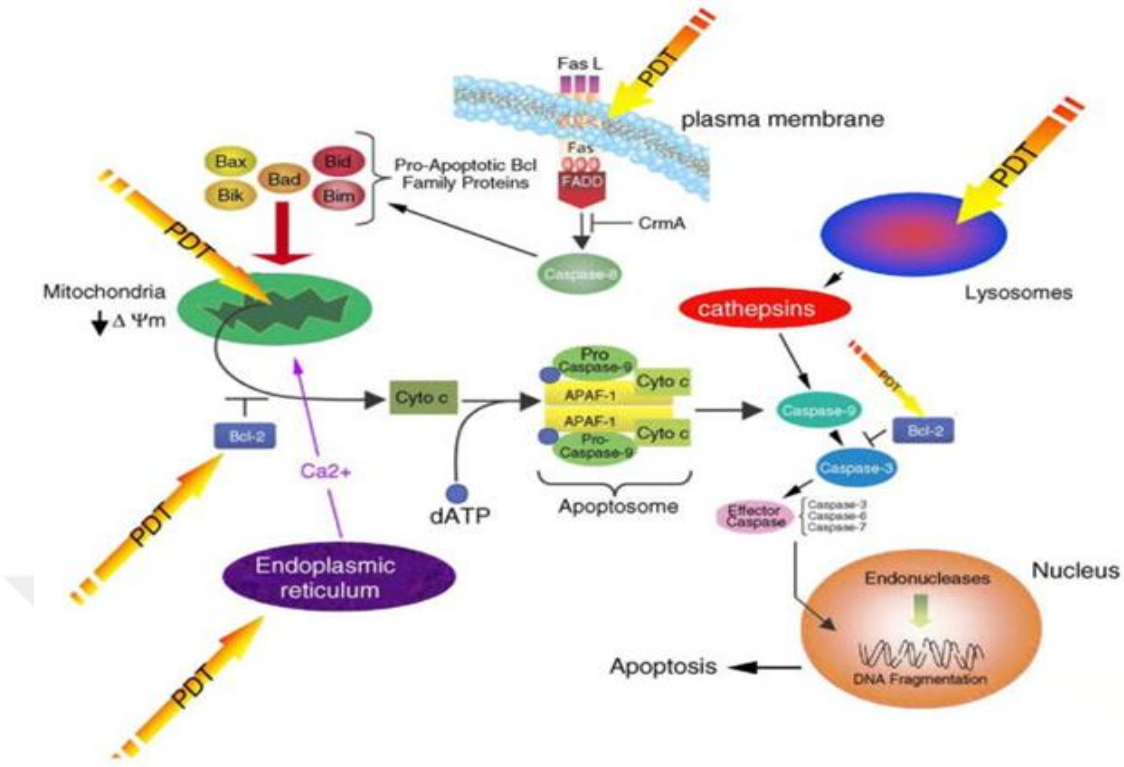



**Figure 5** Cellular signaling cascade triggered through ROS generation following PDT, leads to programmed cell death depend on subcellular localization of PS.

Reference: Reprinted with permission (lisence number, 4724850332368) from (Castano AP, Demidova TN, Hamblin MR. Mechanisms in photodynamic therapy: Part two - Cellular signaling, cell metabolism and modes of cell death. Photodiagnosis and Photodynamic Therapy. 2005; 2(1): 1–23.)

PS have a cationic charge as well as hydrophobic residues can localize mitochondria in the cell through mitochondrial membrane potential and membrane lipid bilayer (20, 54). Mitochondrial localization of photosensitizer during photo illumination can trigger such signaling pathways that are responsible for programmed cell death (55). Firstly, PDT with this subcellular localization causes releasing of pro-apoptotic proteins, such as cytochrome c and apoptosis-inducing factor from mitochondria into the cytosol, also rapid membrane potential loss (56, 57). Cytochrome c release from mitochondria induces caspase 3 activity which is the key compartment in triggering the apoptotic pathway. Plasma membrane localization of PS can trigger necrotic cell death by causing integrity loss of plasma membrane, and depletion of intracellular ATP (58).

# 3. MATERIALS AND METHODS

## 3.1. Materials

List of cell lines used in this study is given in Table 2.

**Table 2** List of cell lines

| Cell line | Cat No, Brand, Country |
|---|---|
| A253, Human salivary gland epidermoid carcinoma | HTB-41, ATCC, USA |
| FaDu, Human pharynx squamous cell carcinoma | HTB-43, ATCC, USA |
| HT29, Human colon colorectal adenocarcinoma | HTB-38, ATCC, USA |
| T47D, Human breast infiltrating ductal carcinoma | HTB-133, ATCC, USA |
| L929, Mouse connective tissue fibroblast | CCL-1, ATCC, USA |

List of equipment used in this study is given in Table 3.

**Table 3** List of equipment

| Name of Equipment | Cat No, Brand, Country |
|---|---|
| Class II Biological Safety Cabinets | AC2-4L1, ESCO, Southeast Asia |
| Vacuum glass | BORO 3.3, Isolab, Germany |
| Inverted light microscope | CKX41, Olympus, USA |
| Inbubator (for mouse cell lines) | BINDER, USA |
| Incubator (for human cell lines) | Sanyo, Japan |
| Imaging reader | Cytation 5, BioTek, USA |
| Flow Cytometer | Novocyte, Acea, USA |
| Water bath | Benchmark Scientific, USA |
| Heating and Cooling Thermostat | Ecoline RE206, Lauda, USA |
| Smart Personal Centrifuge | SPROUT, Heathrow Scientific, USA |
| Personal Centrifuge | M08, ThermoFisher, USA |
| Automated cell counter | LunaII, ThermoFisher, USA |
| Pipette | Easypet, Eppendorf, Germany |

| | |
|---|---|
| Pipette (10, 100, 200, 1000 uL) | Research Plus, Eppendorf, Germany |
| Centrifuge | 5702R, Eppendorf, Germany |
| Freezer (+4) | Bosch, Germany |
| Freezer (-20) | Bosch, Germany |
| Freezer (-80) | U570, New Brunswick Scientific, USA |
| LED array | CELED 96, CETONI, Germany |
| LED controller | CETONI, Germany |
| Cryogenic vial | 430488, Corning, USA |
| Liquid nitrogen tank | |
| Lucetta Luminometer | Lonza, Switzerland |
| XCELLigence | Acea, USA |
| E-plate (16 well) | Acea, USA |

List of buffer, solution and kits used in this study is given in Table 4.

**Table 4** List of Buffer, Solution, and Kits

| **Name of Buffer, Solution and Kits** | **Cat No, Brand, Country** |
|---|---|
| McCoy5A Modified medium | 30-2007, Gibco, USA |
| Minimum Essential Medium Eagle with jocklic modification | M0518, Sigma, USA |
| RPMI 1640 medium | 61870044, Gibco, USA |
| Bovine insulin | Humulin N, Lilly |
| Heat-inactivated fetal bovine serum | 16140071, Gibco, USA |
| Penicillin-streptomycin | 15140122, Gibco, USA |
| Sodium bicarbonate | 25080094, Gibco, USA |
| DPBS | 14190250, Gibco, USA |
| Trypsin-EDTA 0,25% | 25200056, Gibco, USA |
| alamarBlue cell viability reagent | DAL1025, Gibco, USA |
| 2′,7′-Dichlorofluorescin diacetate | D6883, Sigma Aldrich, USA |
| HBSS | 14175129, Gibco, USA |
| WST-1 | 11 644 807 001, Roche, USA |
| DMSO cell culture grade | 4-X, ATCC, USA |
| UltraPure™ DNase/RNase-Free Distilled Water | 10977035, ThermoFisher, USA |

| | |
|---|---|
| Trypan Blue | T8154, Sigma Aldrich, USA |
| MycoAlert PLUSTM | Lonza, Switzerland |

### 3.1.1. Light-emitting diode system

The basis of photodynamic therapy is the stimulation of photosensitizer molecules by light at certain wavelengths (5). To achieve this, a light source is needed which can provide the desired wavelength in a controlled manner and is compatible with the plates used in cell culture. As a stimulating light source, CETONI 96 ceLED 96 LEDs were used. ceLED 96 is a standard LED array. Optimized for irradiation of all wells of 96 well plates at desired wavelengths (624-755 nm) and in the desired configuration. There is an external liquid cooling system to facilitate excessive power density in a small space. The ceLED Led Array is controlled by the QmixElements software, which enables simultaneous control of multiple irradiation zones. The software consists of a system kernel that provides basic functions and services, such as an application window, event log, or toolbar. Flexible grouping of multiple LED channels with irradiation groups allows simultaneous control of all channels of a group, rapid activation, deactivation of all LEDs, and simultaneous control of multiple LED arrays.

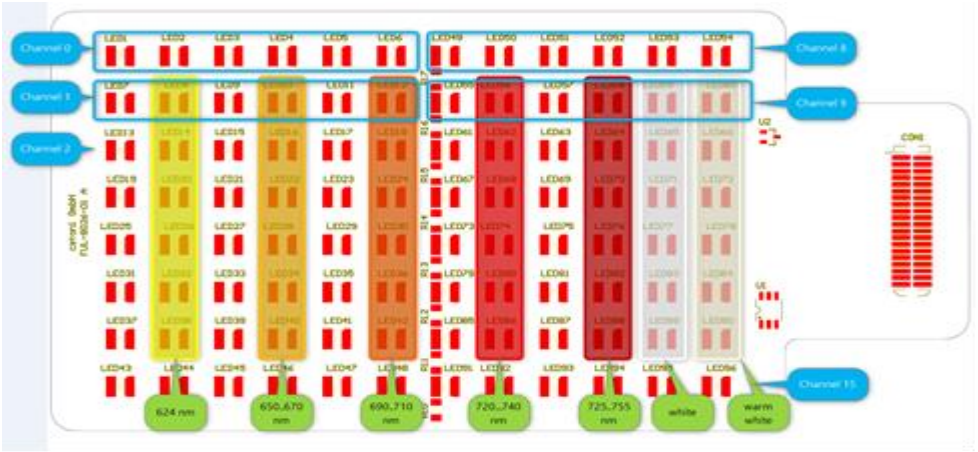



**Figure 6** LED configuration of CETONI 96 led array.

## 3.2. Methods

### 3.2.1. Phthalocyanine molecules

Nine water-soluble cationic tetra-substituted symmetric phthalocyanines (47-52) were obtained from the Chemistry Department of Istanbul Technical University. They were suspended in RPMI 1640 medium (Gibco, Cat No 61870044) as the final concentration is 10 mg/ml. Structures, molecular weights (Mw), and UV-Vis spectrums of the Pc molecules used in the thesis was listed in Table 5, Table 6, and Table 7.

**Table 5** List of EH Series Phthalocyanines

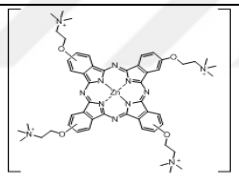
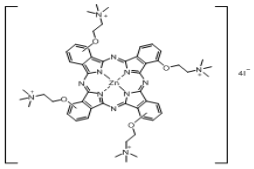
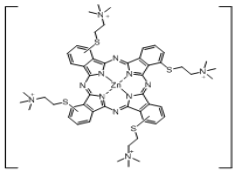
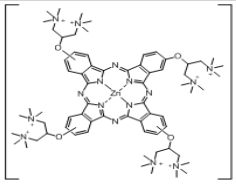
| Pc Code | Molecular Structure | Mw | UV-Vis (nm) |
|---|---|---|---|
| EH1 |  | 1494,16 g/mol | In water: 340, 636, 670 nm |
| EH2 |  | 1494,16 g/mol | In water: 327, 653, 694 nm |
| EH3 |  | 1558,41 g/mol | In water: 329, 651, 693 nm |
| EH4 |  | 2290,30 g/mol | In water: 340, 633, 677 nm |

**Table 6** List of HQ Series Phthalocyanines

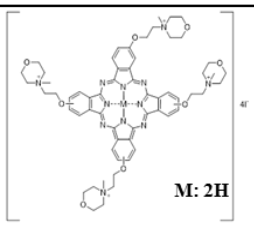
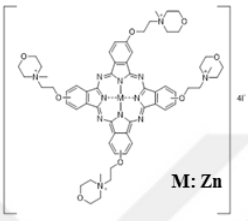
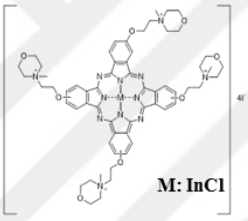
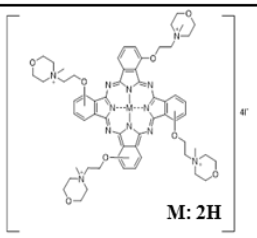
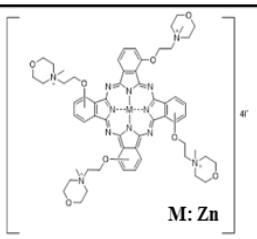
| Pc Code | Molecular Structure | Mw | UV-Vis (nm) |
|---|---|---|---|
| HQH2Pc |   | 1598,95 g/mol | In water: 341, 623 nm |
| HQZnPc |   | 1662,31 g/mol | In water: 345, 634, 655 nm |
| HQInPc |   | 1747,20 g/mol | In water: 346, 650 nm |

**Table 7** List of SQ Series Phthalocyanines

| Pc Code | Molecular Structure | Mw | UV-Vis (nm) |
|---|---|---|---|
| SQH2Pc |   | 1598,95 g/mol | In water: 314, 700 nm |
| SQZnPc |   | 1662,31 g/mol | In water: 320, 701 nm |

### 3.2.2. Cell culture

#### 3.2.2.1. Culture condition of cell lines

Cells that were used in this study were purchased from ATCC. A253 (ATCC, HTB-41), human submaxillary salivary gland epidermoid carcinoma cells were cultured in McCoy's 5a medium modified (Gibco, Cat No 30-2007) supplemented with final concentration 10% heat-inactivated fetal bovine serum (Gibco, Cat No 16140071) and 1% penicillin (10,000 Units/ml) - streptomycin (10,000 Units/ml) (Gibco, Cat No 15140122). FaDu (ATCC, HTB-43), human pharynx squamous cell carcinoma were cultured in Minimum Essential Medium Eagle with jocklic modification (Sigma Aldrich, Cat No M0518) supplemented with final concentration 2.0 g/L sodium bicarbonate (Gibco, Cat No 25080094), 10% heat-inactivated fetal bovine serum (Gibco, Cat No 16140071) and 1% penicillin (10,000 Units/ml) - streptomycin (10,000 Units/ml) (Gibco, Cat No 15140122). HT29 (ATCC, HTB-38), human colon colorectal adenocarcinoma cells cultured in McCoy's 5a Medium Modified (Gibco, Cat No 30-2007) supplemented with final concentration 10% heat-inactivated fetal bovine serum (Gibco, Cat No 16140071) and 1% penicillin (10,000 Units/ml) - streptomycin (10,000 Units/ml) (Gibco, Cat No 15140122). T47D (ATCC, HTB-133), human mammary gland ductal carcinoma cell were cultured in RPMI 1640 medium (Gibco, Cat No 61870044) supplemented with final concentration 0.2 Units/ml bovine insulin (Lilly, Cat No Humulin N), 10% heat-inactivated fetal bovine serum (Gibco, Cat No 16140071) and 1% penicillin (10,000 Units/ml) - streptomycin (10,000 Units/ml) (Gibco, Cat No 15140122). L929 (ATCC, CCL-1) mouse subcutaneous connective tissue fibroblast cells were cultured in Minimum Essential Medium Eagle with alpha modification supplemented with final concentration 10% heat-inactivated fetal bovine serum (Gibco, Cat No 16140071) and 1% penicillin-streptomycin (Gibco, Cat No 15140122).

#### 3.2.2.2. Cell cryopreservation

The cell stocks were kept inside liquid nitrogen tank. Cells were detached from the flask through incubation with 0.25% trypsin-EDTA for around 5 minutes at 37°C. Then, trypsin was neutralized with 3 times more amount of serum-containing complete medium and centrifuged at 1300 rpm for 5 minutes to get rid of waste components. After discarding the supernatant, the cell pellet was suspended with cryopreservation media, and cell aliquots was prepared in Corning cryogenic vial with 0-ring. The vials were placed into Mr. Frost that provides decreasing temperature 1°C per minutes and stored at -80 °C for min overnight max a week, and the vials were transferred into liquid nitrogen tank. Cryopreservation media for each cell line was 95% complete media with 5% DMSO (cell culture grade, ATCC).

#### 3.2.2.3. Continuous culture of cell lines

Cell passage was carried out two to three times per week, considering their population doubling time. Firstly, the serum-containing medium was removed by washing with DPBS (Gibco, Cat No 14190250). Cells were detached from the flask through incubation with 0.25% trypsin-EDTA (Gibco, Cat No 25200056) for around 5 minutes at 37°C. Then, trypsin was neutralized with three times more amount of serum-containing complete medium and centrifuged at 1300 rpm for 5 minutes to get rid of waste components. After discarding the supernatant, the cell pellet was suspended with complete medium and seeded appropriate aliquots in new cell culture flasks.

During cell culture studies, cell counting was carried by trypan blue dye which is not cell permeable and could not pass from the membrane of healthy cells while can stain dead cells. Therefore, living cells appears white, and dead cells takes the color of

the dye, blue. Counting was performed using automated cell counting system, Luna II that also demonstrates percent viability.

#### 3.2.2.4. Determination of population doubling time

The population doubling time of the cell lines was determined by XCELLigence equipment. The cells were detached from cell culture flask by 0.25% trypsin and centrifuged at 1300 rpm for 5 min when cell confluence around 70-80%. With cell viability higher than 90%, cells were suspended in their complete media as final concentration 5x104 cells/ml. On the other hand, complete media of each cell lines without cells was placed into the wells of E-plates to measure blanks. After blanking, the cells were seeded into the wells and the plates were placed on the XCELLigence equipment. The program was started, and the data was collected for 125 hours with intervals of 15 minutes.

The xCELLigence® RTCA device uses non-invasive electrical impedance monitoring to measure cell proliferation and morphology change in an unlabeled, real-time manner. The instrument is placed in a standard CO2 cell culture incubator and fed and controlled by a cable connected to the control unit located outside the incubator. Real-time cell analysis (RTCA) software includes real-time data display and analysis functions and allows real-time interface creation. Real-time cell analysis with xCELLigence® RTCA greatly improves efficiency, bypassing estimates associated with endpoint analyzes.

The operating principle of the device is based on cellular impedance. The practical unit of this cellular impedance assay is a series of gold microelectrodes fused to the well base of a microtiter plate. When immersed in an electrically conductive solution, an electric capacity is applied alongside those electrodes, the electrons exit the negative terminal, pass via the solution, and then collect on the positive terminal, thereby

completing the circuit. The gold microelectrode biosensors inside the wells of ACEA's digital microtiter plates (E-Plate®) cowl 70-80% of the surface area. The electrodes in each well of the E-Plate are related to the cords forming a sequence of interdigitates. This arrangement permits for simultaneous monitoring of populations of cells, thereby providing exquisite sensitivity for the number of cells introduced to the plate, size/morphology of cells, and cell-substrate binding quality

#### 3.2.2.5. Mycoplasma detection

In cell culture studies, mycoplasma contamination disrupts the physiology and metabolism of the cells and negatively affects the efficiency and results of the researches. All the cell lines were periodically tested for mycoplasma by MycoAlert PLUSTM kit from Lonza using the Lucetta Luminometer that is a single tube luminometer developed for the detection of bioluminescence and chemiluminescence.

Firstly, 2 ml of the cell culture samples to be tested were centrifuged at 200 xg for 5 min. After centrifugation, 100 µl of the supernatant was transferred to the luminometer (Lucetta) tube. 100 µl of MycoAlert PLUSTM reagent was added to each sample, and incubated for 5 minutes. At the end of the period, the tubes were placed in the luminometer reader and the program was started (Reading A). Then 100 µl of MycoAlert TM PLUS substrate was added and incubated for 10 minutes. The program was started by placing the cuvette in the luminometer reader (Reading B). Ratio of the readings (Rate = Reading B / Reading A) was taken to detect mycoplasma.

### 3.2.3. Cellular uptake of Pc

Phthalocyanines are self-fluorescent molecules, and this feature allows them to be detected through the specific filters of flow cytometry without any need for extra labeling when binds to a cell. Therefore, cellular uptake of different Pc molecules was

evaluated by flow cytometry analysis. Cells were detached from cell culture plate by trypsinization, then suspended in DPBS as final cell concentration of $10x10^6$ cell/ml. Each cell suspension including 100 µg/ml Pc molecules was incubated at room temperature to binding of Pc molecules to the cells. Fluorescence of Pc molecules that binds to the cells was determined through determined cytometer settings as excitation at 637 nm and emission acquired 780/60 nm filter in Acea Novocyte Flow Cytometer.

A laser-based technology, flow cytometry is used to analyze and characterize cells in a fluidic system. Cells in a suspension are separated from each other through hydrodynamic focusing in a fluid stream and pass through lasers in the cytometer system. The lasers provide lights of specific wavelengths that excite fluorochrome on the cell, and this excitation causes light scattering that is detected by photomultiplier tubes. Through the tubes, detected light is amplified and converted to a voltage which can be analyzed digitally. Self-fluorescent features of phthalocyanine molecules make them possible to be detected by flow cytometry analysis without any labeling.

Cancer cell lines, A253, FaDu, HT29, and T47D, were incubated with phthalocyanines, then detection was done under cytometer settings as excitation at 637 nm and emission acquired 780/60 nm filter in Acea Novocyte Flow Cytometer. Firstly, forward and side scatters of all cell population was obtained. From all populations, singlet cells were selected to get rid of excessive fluorescence signaling of double cells and non-binding molecules. A fluorescence signaling histogram was created from the singlet cell population. As a control, untreated cells were used, and Pc containing samples was compared relative to untreated control.

### 3.2.4. Biocompatibility

To evaluate the biocompatibility of the nine phthalocyanine molecules, dark and photo-irradiated cytotoxicity of the molecules was analyzed on the mouse L929 cell

line. Firstly, dark cytotoxicity studies were carried out. In the biocompatible concentration range of the Pc molecules where control-related cytotoxicity is less than 30%, photo-irradiated cytotoxicity's were evaluated.

L929 cells were detached from cell culture flask by 0.25% trypsin and centrifuged at 1300 rpm for 5 min when cell confluence around 70-80%. With cell viability higher than 90%, cells were suspended in alpha mem media supplemented with 10% FBS and 1% penicillin-streptomycin as final concentration $5x10^4$ cells/ml. Into 96 well plates, the cells were seeded($5x10^3$ cells/well) and incubated in a 37°C, 5% $CO_2$ humidified incubator for 24 hours.

For the dark cytotoxicity group, after 24 hours incubation, phthalocyanine molecules in different concentrations (100 μg/mL, 10 μg/mL, 1 μg/mL, 0,1 μg/mL) that were prepared in complete cell culture media were applied onto the cells, and incubated in a 37°C, 5% $CO_2$ humidified incubator as covered with aluminum foil for 24 hours.

For the photo-irradiated cytotoxicity group, after 24 hours incubation, phthalocyanine molecules in determined concentration according to results of dark cytotoxicity were applied onto the cells and incubated in a 37°C, 5% $CO_2$ humidified incubator for an hour in order to cellular uptake of Pc molecules. After cellular uptake of Pc molecules, the cells were irradiated through different wavelengths for 5 minutes at 70 mV using Cetoni-LED. As a dark group, cells then were incubated in a 37°C, 5% $CO_2$ humidified incubator for 24 hours.

Post-24 hours of the treatment, cell viability evaluation was carried out by colorimetric WST-1 assay that is based on the cleavage of tetrazolium salts into formazan dye by mitochondrial dehydrogenases in viable cells. WST-1 solution is diluted 1:10 in complete media, and the media in each well was replaced with a 1:10

WST-1 solution. The cells were incubated to be avoided from light in a 37°C, 5% $CO_2$ humidified incubator for 3 hours. After incubation, endpoint absorbance of the wells was taken at 450 nm; references 650 nm through Cytation 5 multi-plate reader. References values were distracted from an actual value. As a control, non-treated cells and cells treated with 10% DMSO were used. Percentage values were calculated respected to non-treated cells.

### 3.2.5. Dark cytotoxicity

In photodynamic therapy, photosensitizer molecules are non-toxic unless they are irradiated with light of specific wavelengths. In order to determine the non-toxic concentration range of phthalocyanine molecules on different cancer cell lines, cytotoxic assay with the nine phthalocyanine molecules and 4 cancer cell lines (A253, FaDu, HT29, and T47D) was set up in dark conditions.

The cells were detached from cell culture flask by 0.25% trypsin and centrifuged at 1300 rpm for 5 min when cell confluence around 70-80%. With cell viability higher than 90%, cells were suspended in their complete media as final concentration $5x10^4$ cells/ml. Into 96 well plates, the cells were seeded ($5x10^3$ cells/well) and incubated in a 37°C, 5% $CO_2$ humidified incubator for 24 hours.

After 24 hours incubation, phthalocyanine molecules in different concentrations (100 µg/mL, 10 µg/mL, 1 µg/mL, 0,1 µg/mL) that were prepared in complete cell culture media were applied onto the cells, and incubated in a 37°C, 5% $CO_2$ humidified incubator as covered with aluminum foil for 24 hours.

Post-24 hours of the treatment, cell viability evaluation was carried out by colorimetric WST-1 assay that is based on the cleavage of tetrazolium salts into formazan dye by mitochondrial dehydrogenases in viable cells. WST-1 solution is

diluted 1:10 in complete media, and the media in each well was replaced with a 1:10 WST-1 solution. The cells were incubated to be avoided from light in a 37°C, 5% $CO_2$ humidified incubator for 3 hours. After incubation, endpoint absorbance of the wells was taken at 450 nm; references 650 nm through Cytation 5 multi-plate reader. References values were distracted from an actual value. As a control, non-treated cells and cells treated with 10% DMSO were used. Percentage values were calculated respected to non-treated cells. Respect to the dark cytotoxicity results, the concentration range of the nine phthalocyanine molecules for photodynamic therapy was determined.

### 3.2.6. Photo-irradiated cytotoxicity

Cells were prepared as mentioned in the dark cytotoxicity section and administered with Pc molecules with the concentration that determined as non-toxic respect to dark cytotoxicity studies. Following the administration of Pc molecules with different concentrations (10 µg/mL, 1 µg/mL), the cells were incubated in a 37°C, 5% $CO_2$ humidified incubator for an hour in order to cellular uptake of Pc molecules. After cellular uptake of Pc molecules, the cells were irradiated through different wavelengths for 5 minutes at 70 mV using Cetoni-LED. As a dark group, cells then were incubated in a 37°C, 5% $CO_2$ humidified incubator for 24 hours. As control non-treated cells were used. Beside each photo-irradiated cytotoxicity assay, a dark cytotoxicity experiment was set up.

Post-24 hours of treatment, cytotoxicity evaluation was carried out with WST-1 as in the dark cytotoxicity section. Further, to support the reliability of the WST-1 results, another cytotoxicity assay ends up with resazurin alamarBlue cell viability reagent (Gibco, Cat No DAL1025) that working principle is based on giving a colorimetric response to metabolic reduction of healthy cells and yielding proportional fluorescence relative to the number of cells respiring.

### 3.2.7. Oxidative stress evaluation

In photodynamic therapy, one of the photo damage processes is triggered through reactive oxygen species formation. In order to carry out ROS formation, the cells were detached from cell culture flask by 0,25% trypsin-EDTA and centrifuged at 1300 rpm for 5 min when cell confluence around 70-80%. With cell viability higher than 90%, cells were suspended in their complete media as the final concentration of $10x10^4$ cells/ml. Into 96 well plates, the cells were seeded ($10x10^3$ cells/well) and incubated in a 37°C, 5% $CO_2$ humidified incubator for 24 hours. After 24 hours incubation, phthalocyanine molecules in different concentrations (10 μg/mL, 1 μg/mL) that were prepared in complete cell culture media were administrated into the cells. Following treatment with Pc molecules, the photo-irradiated group was irradiated with 2 joules/$cm^2$ with different wavelengths using CETONI-LED equipment after 1-hour incubation with Pc molecules. Following irradiation, dark and photo-irradiated groups incubated in a 37°C, 5% $CO_2$ humidified incubator as covered with aluminum foil for 30 minutes. At the end of the incubation, reactive oxygen species intensity was evaluated using 2′, 7′-Dichlorofluorescin diacetate, DCFDA (sigma, D6883). DCFDA is a non-fluorescent cell-permeable probe that undergoes esterase reaction in the presence of reactive oxygen species and yields highly fluorescent 2′, 7′-dichlorofluorescein. DCFDA that is used was prepared in ethanol as the final concentration is 20 mM. To carry out the experiment, old medium from treated and control groups were removed. Into the wells, 10 μM DCFDA in HBSS (Gibco, Cat No 14175129) were applied and incubated by avoiding light in a 37°C, 5% $CO_2$ humidified incubator for 30 minutes. After that, endpoint fluorescent intensity was read where the excitation wavelength is 504 nm and the emission wavelength are 529 nm. As a control to see the system was working, 50 μM $H_2O_2$ was used.

# 4. RESULTS

## 4.1. Determination of Population Doubling Time

Population doubling time of A253, FaDu, HT29, and T47D cell lines was determined using electrical impedance based XCELLigence device. Real time measurement for each cell line was collected for 125 hours with intervals of 15 min. After that, population doubling time was analyzed as shown in Table 8.

**Table 8** Population doubling time of A253, FaDu, HT29 and T47D cell lines.

| Cell line | Cell number seeded | Population doubling time (hours) |
|---|---|---|
| A253 | 5000 cells/well | 13,905±1,189 |
| FaDu | 5000 cells/well | 9,459±1,250 |
| HT29 | 5000 cells/well | 13,650±0,7432 |
| T47D | 5000 cells/well | 15,627±3,885 |

## 4.2. Mycoplasma Detection

Each cell line was perodically tested for mycoplasma detection by MycoAlert PLUSTM kit from Lonza using the Lucetta Luminometer. Luminesce readings that were taken before (Reading A) and after (Reading B) MycoAlert TM PLUS substrate addition. Mycoplama contamination was evaluated based on the ratio of the reading, and the results was shown in Table 9.

**Table 9** Mycoplasma detection on the cell lines

| Cell line | Ratio | Evaluation |
|---|---|---|
| A253 | 0,26 | Negative |
| FaDu | 0,43 | Negative |
| HT29 | 0,16 | Negative |
| T47D | 0,38 | Negative |

ratio of < 1 refers negative for mycoplasma, > 1.2 refers mycoplasma contamination

## 4.3. Cellular Uptake of Phthalocyanine Molecules

Cancer cell lines, A253, FaDu, HT29, and T47D, were incubated with phthalocyanines, then detection was done under cytometer settings as excitation at 637 nm and emission acquired 780/60 nm filter in Acea Novocyte Flow Cytometer. A fluorescence signaling histogram was created from the singlet cell population. As a control, untreated cells were used, and Pc containing samples was compared relative to untreated control.

Cellular uptake of the ftalocyanine molecules through the A253 cell line was shown in Figure 7.

Cellular uptake of the ftalocyanine molecules through the FaDu cell line was shown in Figure 8.

Cellular uptake of the ftalocyanine molecules through the HT29 cell line was shown in Figure 9.

Cellular uptake of the ftalocyanine molecules through the T47D cell line was shown in Figure 10.

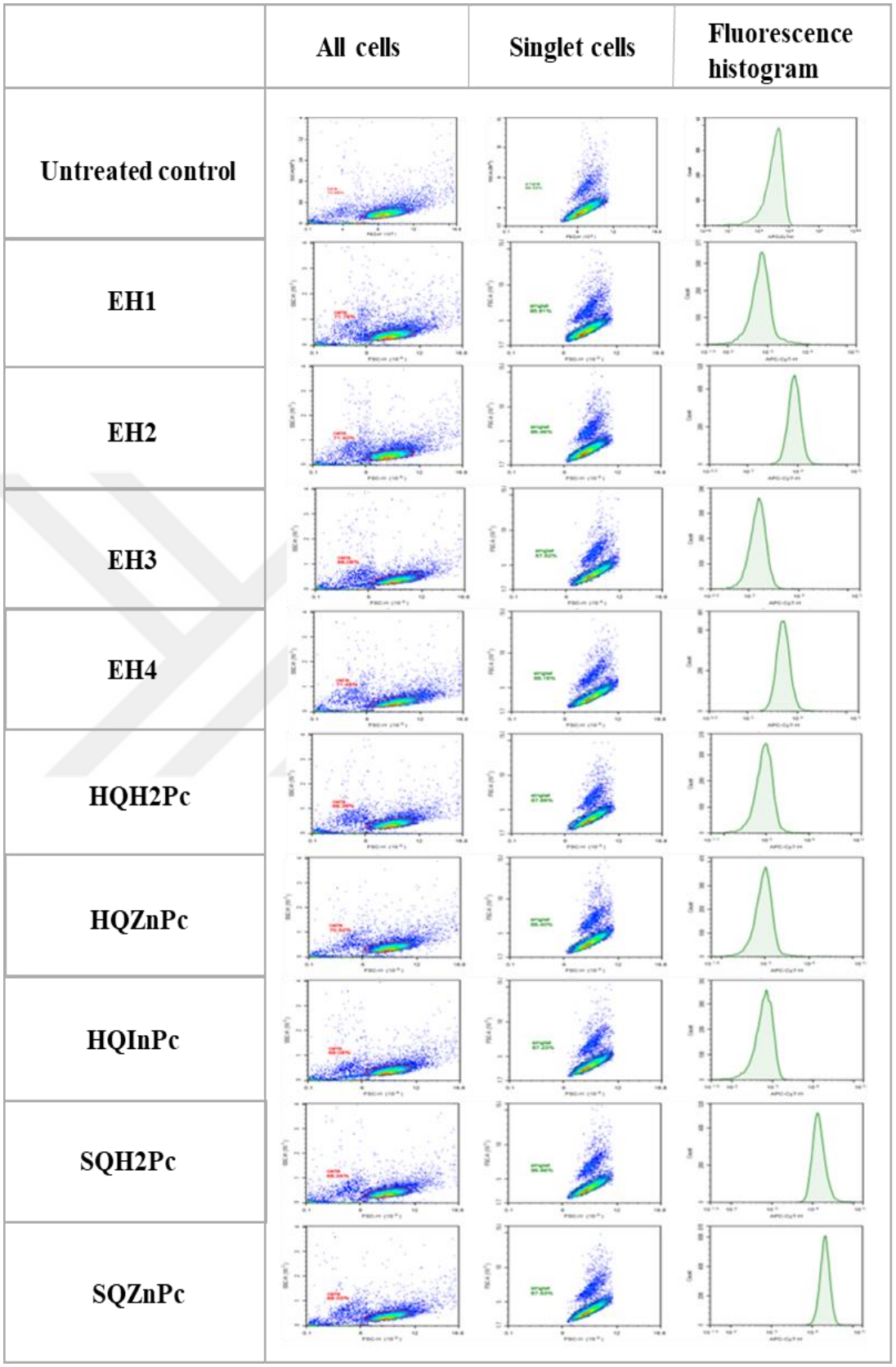



**Figure 7** Flow cytometry forward and side scatters, and fluorescence histograms to demonstrate cellular uptake of phthalocyanine molecules through A253 cell line.

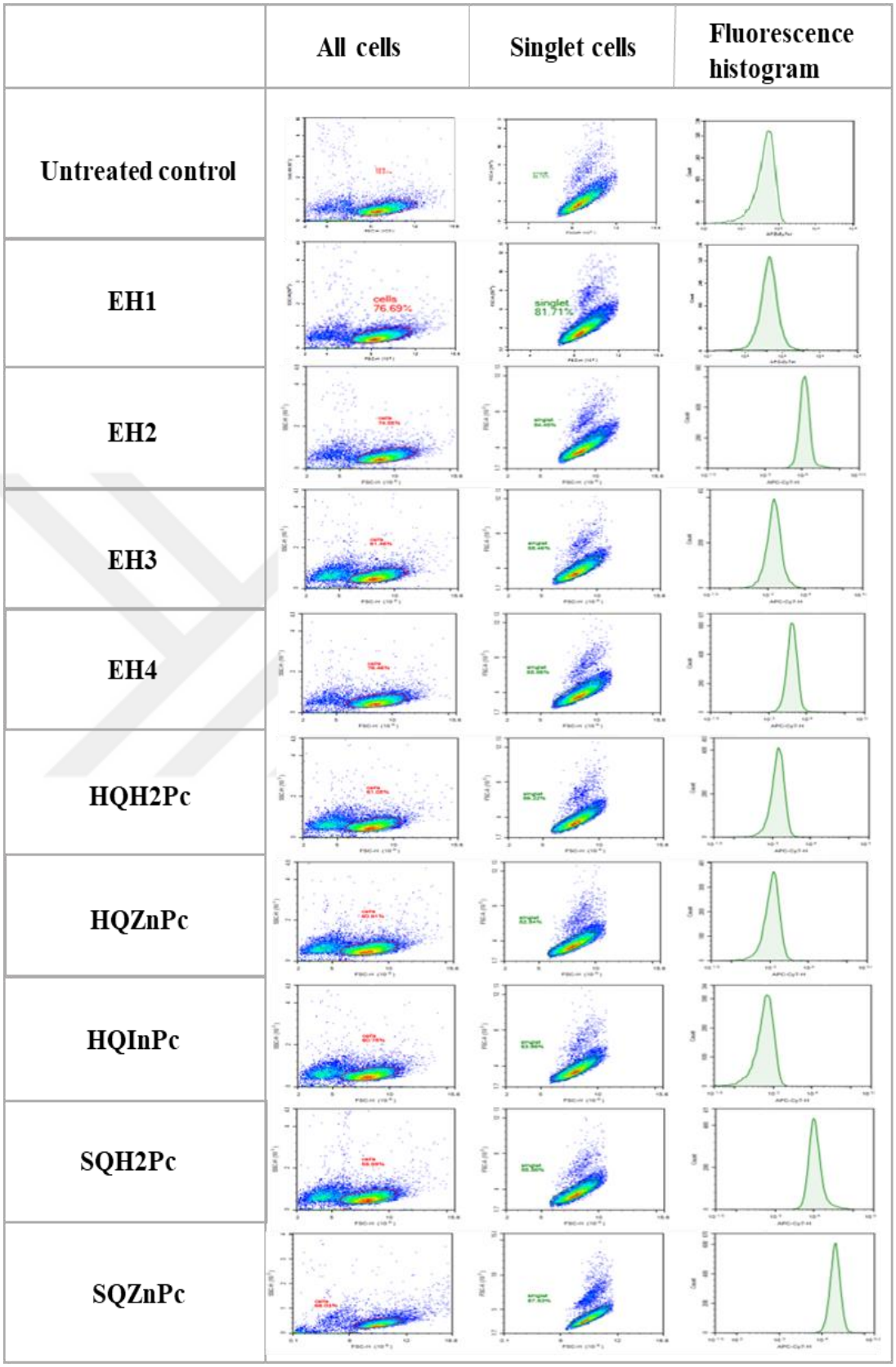



**Figure 8** Flow cytometry forward and side scatters, and fluorescence histograms to demonstrate cellular uptake of phthalocyanine molecules through FaDu cell line.

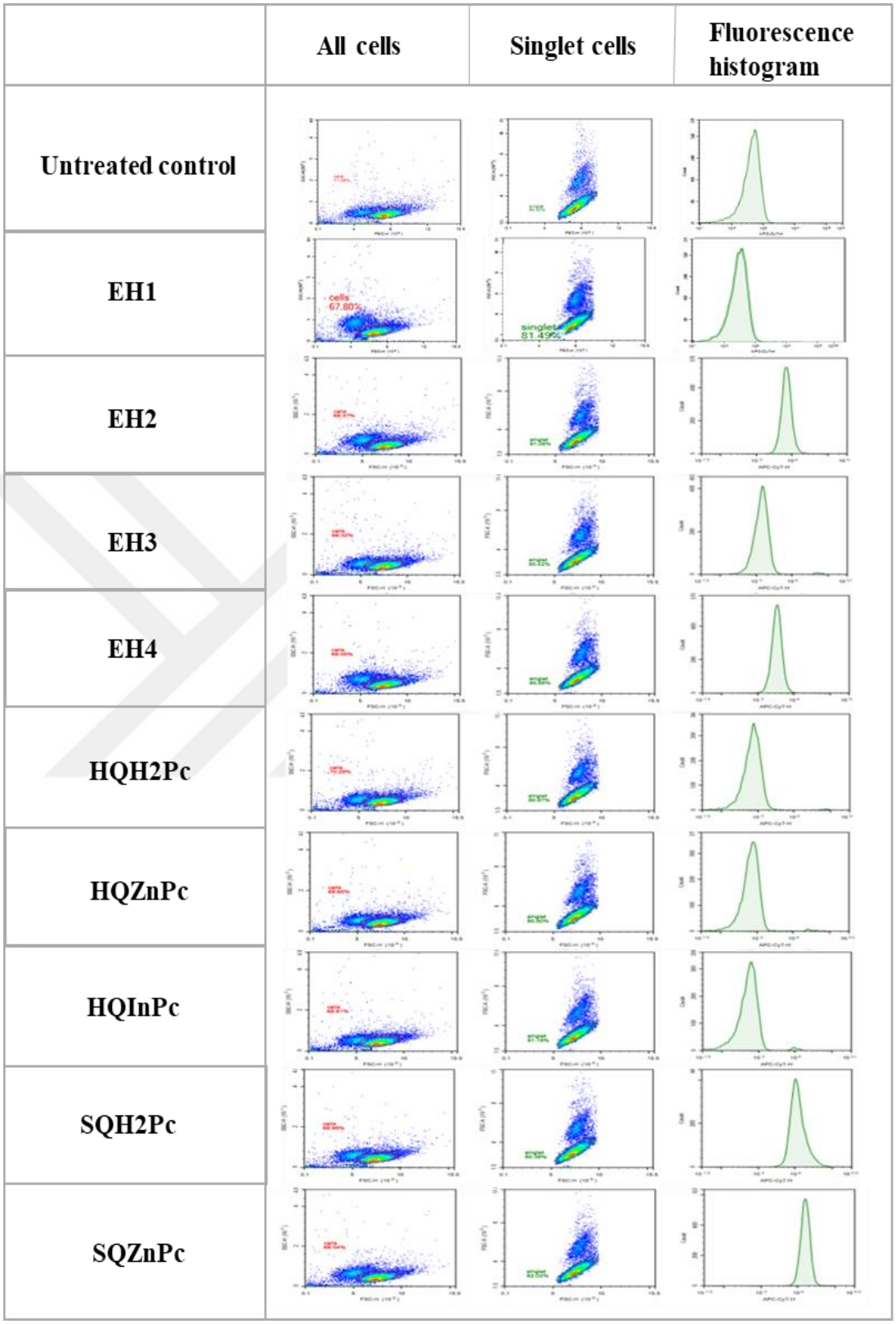



**Figure 9** Flow cytometry forward and side scatters, and fluorescence histograms to demonstrate cellular uptake of phthalocyanine molecules through HT29 cell line.

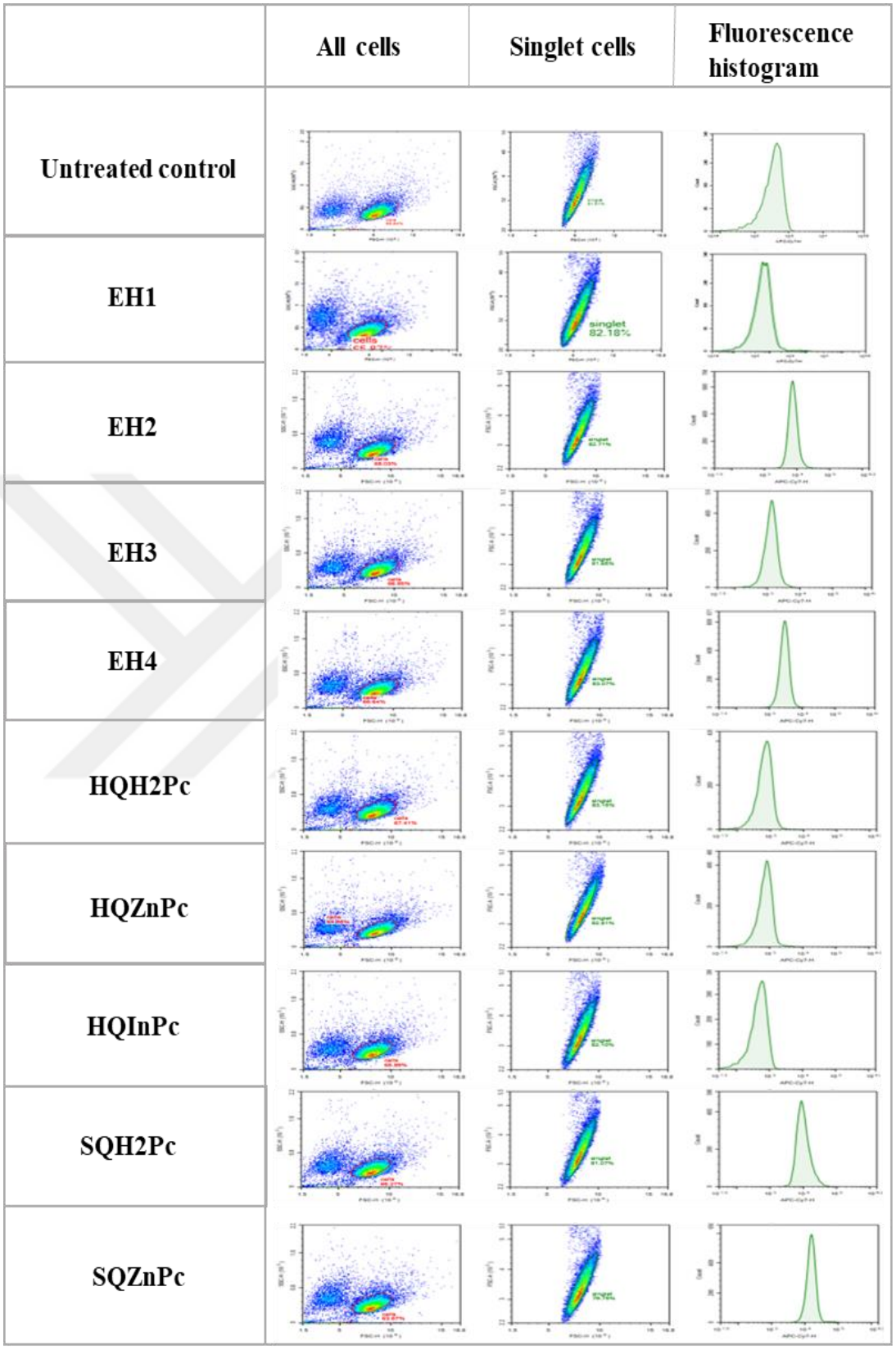



**Figure 10** Flow cytometry forward and side scatters, and fluorescence histograms to demonstrate cellular uptake of phthalocyanine molecules through T47D cell line.

Overlaying fluorescence histograms created from the side and forward scatters of singlet cells allows making a comparison between Pc molecules by comparing fluorescence signaling as seen in Figure 11.

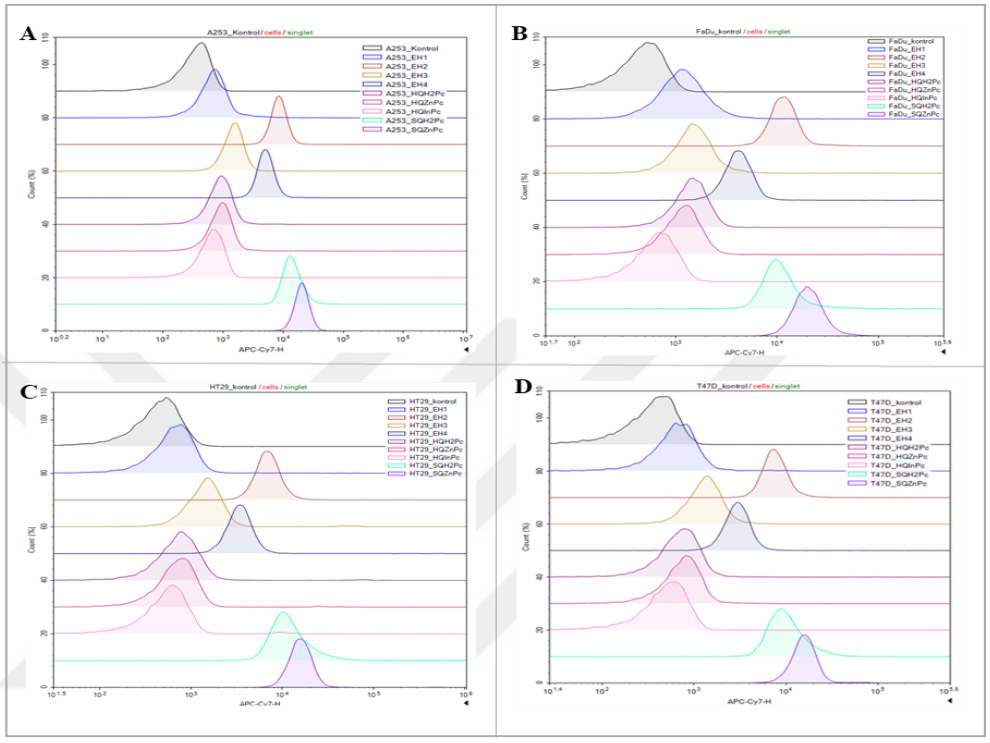



**Figure 11** Fluorescence histograms overlays of cell lines to compare cellular uptake of phthalocyanine molecules relative to untreated control and each other. A. A253 B. FaDu C. HT29 D. T47D.

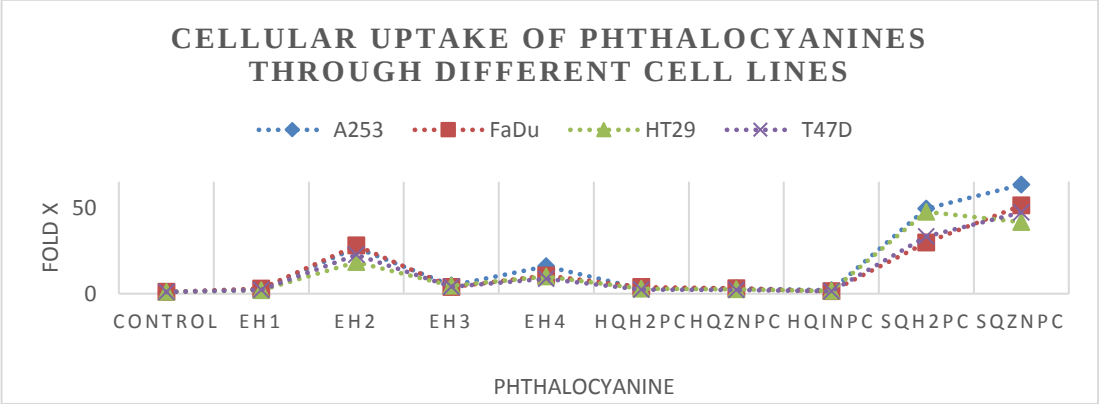



**Figure 12** Graph shows cellular uptake of Pc molecules based on how many times the signal was received by flow cytometry compared to the untreated control.

**Table 10** How many times Pc molecules was taken by cells relative to untreated control.

| | Control | EH1 | EH2 | EH3 | EH4 | $HQH_2PC$ | HQZnPc | HQInPc | $SQH_2Pc$ | SQZnPc |
|---|---|---|---|---|---|---|---|---|---|---|
| **A253** | 1 | 3 | 27 | 5 | 16 | 3 | 3 | 2 | 49 | 63 |
| **FaDu** | 1 | 3 | 28 | 4 | 10 | 4 | 3 | 2 | 30 | 51 |
| **HT29** | 1 | 2 | 18 | 5 | 10 | 3 | 3 | 2 | 48 | 42 |
| **T47D** | 1 | 2 | 23 | 4 | 9 | 2 | 2 | 1 | 33 | 47 |

Following flow cytometry application, overall results demonstrated that SQ group Pcs, $SQH_2Pc$, and SQZnPc have a higher affinity to each cell line, following EH2 and EH4 Pcs compared to other Pc molecules. Further, it can be said that each nine Pc molecules were taken through each cell lines at least 2 times more than control groups according to obtained fluorescence signals relative to untreated controls.

## 4.4. Biocompatibility of Phthalocyanine Molecules

To determine the biocompatible concentration range of Pc molecules, different concentrations of the molecules exposed to the mouse L929 cell line firstly in dark conditions for 24 h. As to meet the requirement of ISO standards, the samples that are equal or exceed 70% viability conditions were confirmed as non-toxic. Photodynamic therapy application normally does not cause damage to non-cancerous cells, therefore, at the determined concentration as non-toxic at dark, photodynamic therapy was carried out at different wavelengths that range between 624 and 755 nm with light dose 2 joules/$cm^2$. The survival percentage of L929 cells was determined relative to the non-treated control. Values are shown as the mean±SD of three independent experimental repeats.

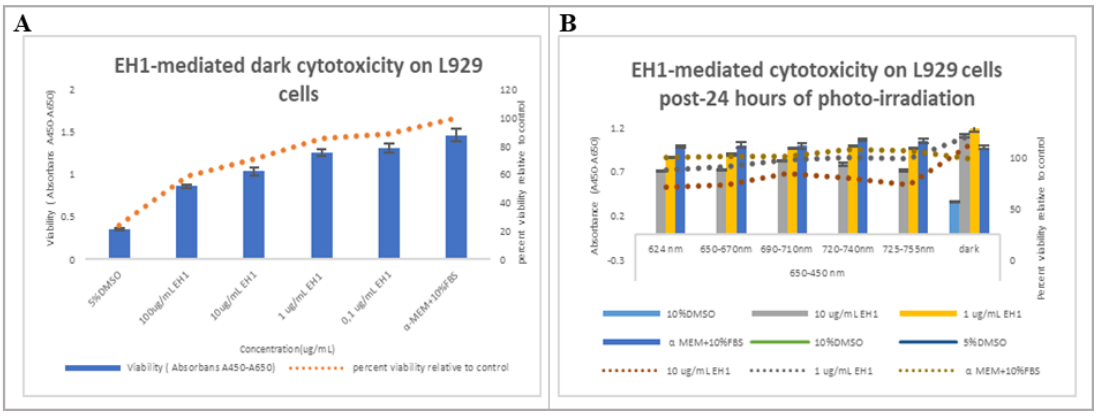



**Figure 13** Dose dependent A. dark and B. photo-induced cytotoxicity of EH1 phthalocyanine. Viability was measured by WST-1 assay following 24h of PDT.

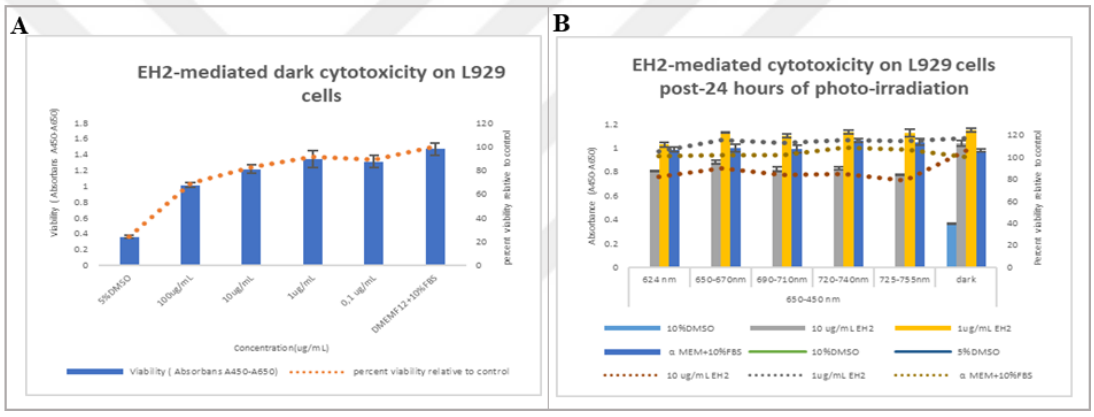



**Figure 14** Dose dependent A. dark and B. photo-induced cytotoxicity of EH2 phthalocyanine. Viability was measured by WST-1 assay following 24h of PDT.

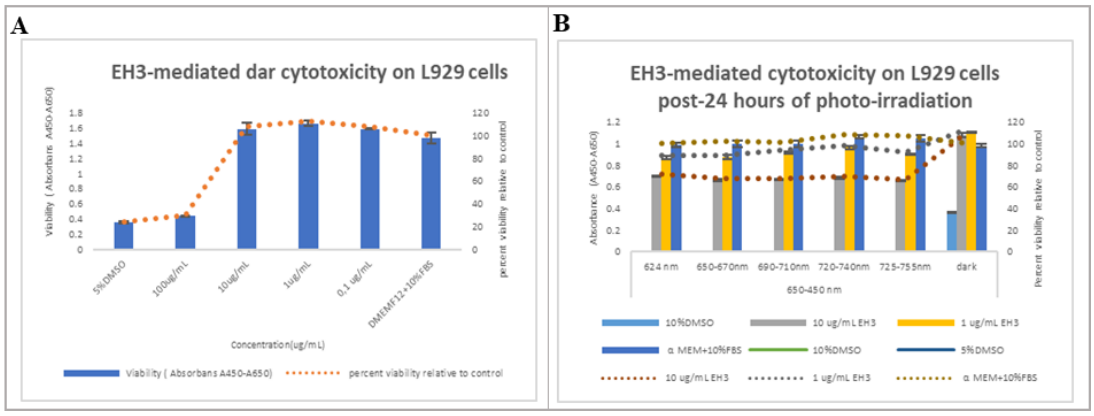



**Figure 15** Dose dependent A. dark and B. photo-induced cytotoxicity of EH3 phthalocyanine. Viability was measured by WST-1 assay following 24h of PDT.

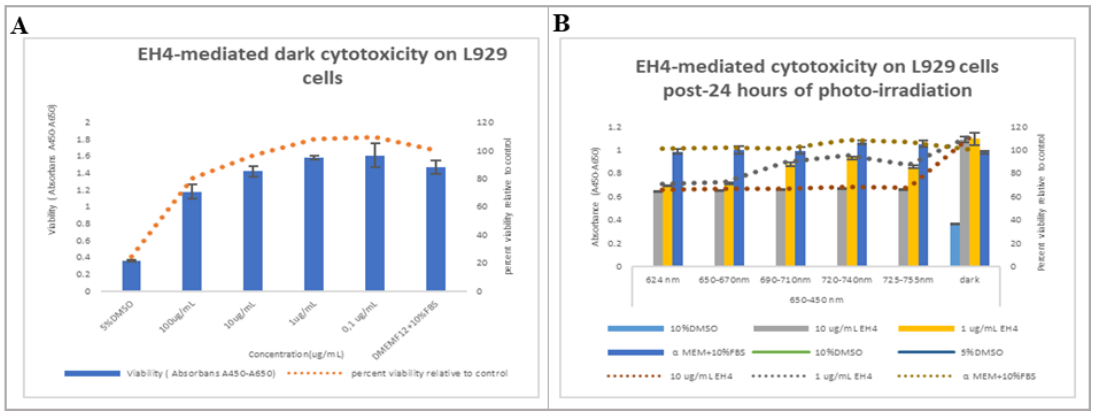



**Figure 16** Dose dependent A. dark and B. photo-induced cytotoxicity of EH4 phthalocyanine. Viability was measured by WST-1 assay following 24h of PDT.

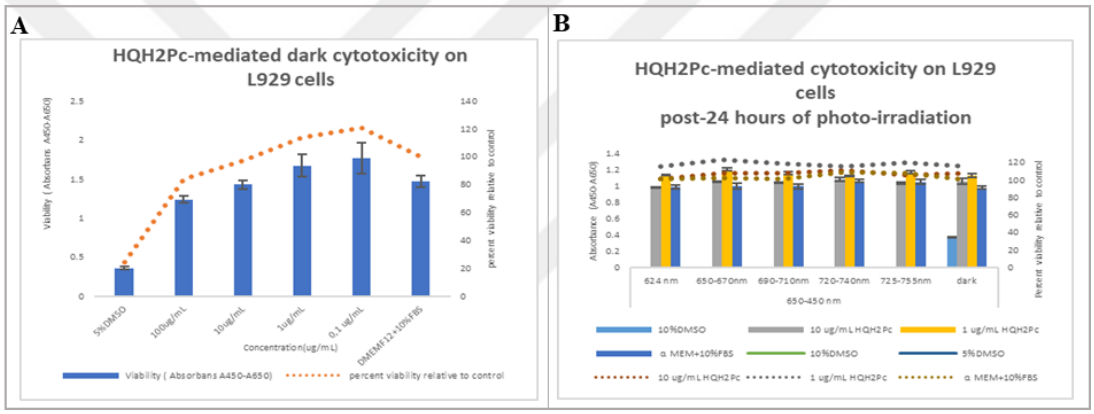



**Figure 17** Dose dependent A. dark and B. photo-induced cytotoxicity of $HQH_2Pc$ phthalocyanine. Viability was measured by WST-1 assay following 24h of PDT.

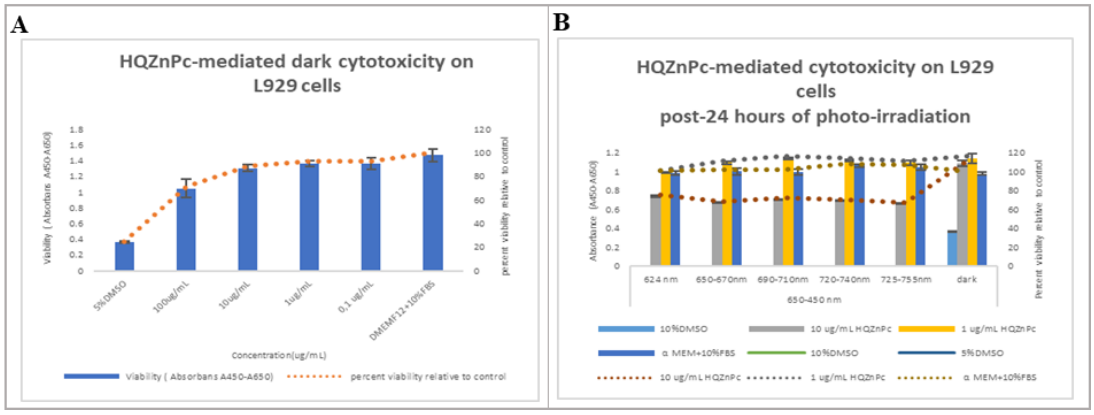



**Figure 18** Dose dependent A. dark and B. photo-induced cytotoxicity of HQZnPc phthalocyanine. Viability was measured by WST-1 assay following 24h of PDT.

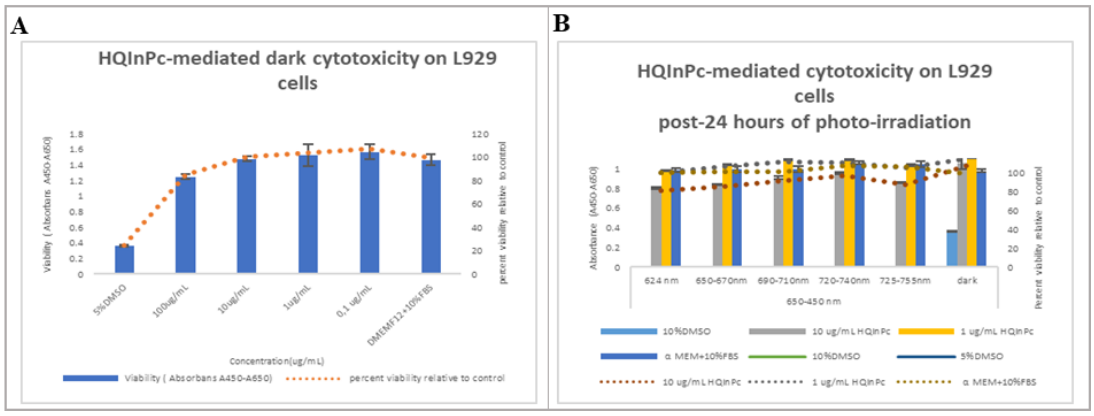



**Figure 19** Dose dependent A. dark and B. photo-induced cytotoxicity of HQInPc phthalocyanine. Viability was measured by WST-1 assay following 24h of PDT.

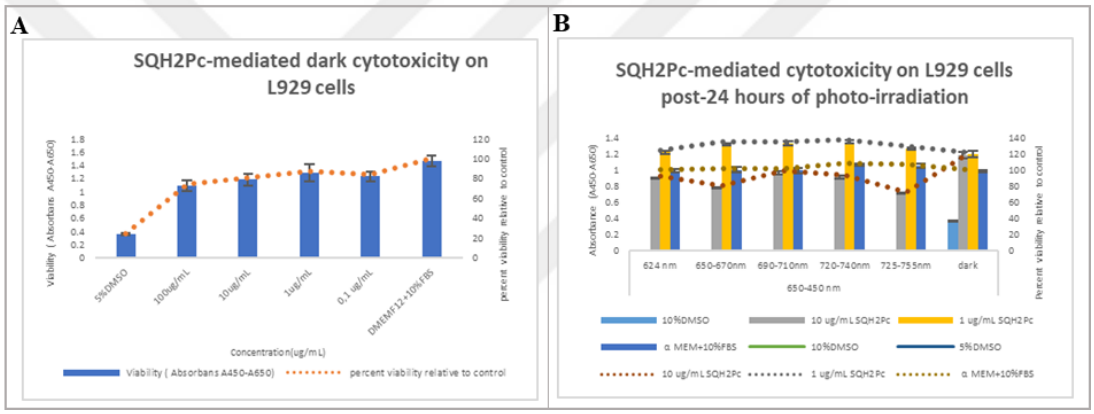



**Figure 20** Dose dependent A. dark and B. photo-induced cytotoxicity of $SQH_2Pc$ phthalocyanine. Viability was measured by WST-1 assay following 24h of PDT.

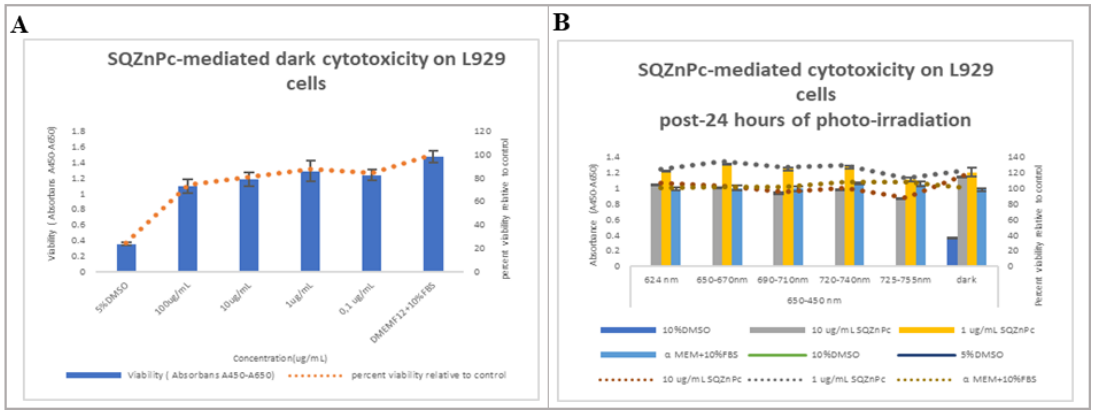



**Figure 21** Dose dependent A. dark and B. photo-induced cytotoxicity of SQZnPc phthalocyanine. Viability was measured by WST-1 assay following 24h of PDT.

The phthalocyanine molecules without photo-exposure led to cytotoxicity where concentration is 100 µg/mL or higher. Below this point, two concentrations, 10 µg/ml and 1 µg/ml were determined to be used for PDT. At the light dose of any wavelengths, both high and low doses of each Pc molecules did not cause a reduction in cell viability below 70% relative to the non-treated control. Based on this information, concentration <10 µg/mL of each Pc molecules was selected as photosensitizers to be used for PDT studies for carcinoma cells.

## 4.5. Pc-induced Dark Cytotoxicity on Cancer Cell Lines

Photodynamic therapy only has cytotoxic effect when photosensitizer agent is irradiated with a proper wavelength of a light source so that it is not expected a cytotoxic response in dark condition. It should be well defined non-toxic concentration range of the Pc molecules to be used in photo-induced treatments. The survival percentage of the cells was determined relative to the non-treated control. Values are shown as the mean±SD of three independent experimental repeats.

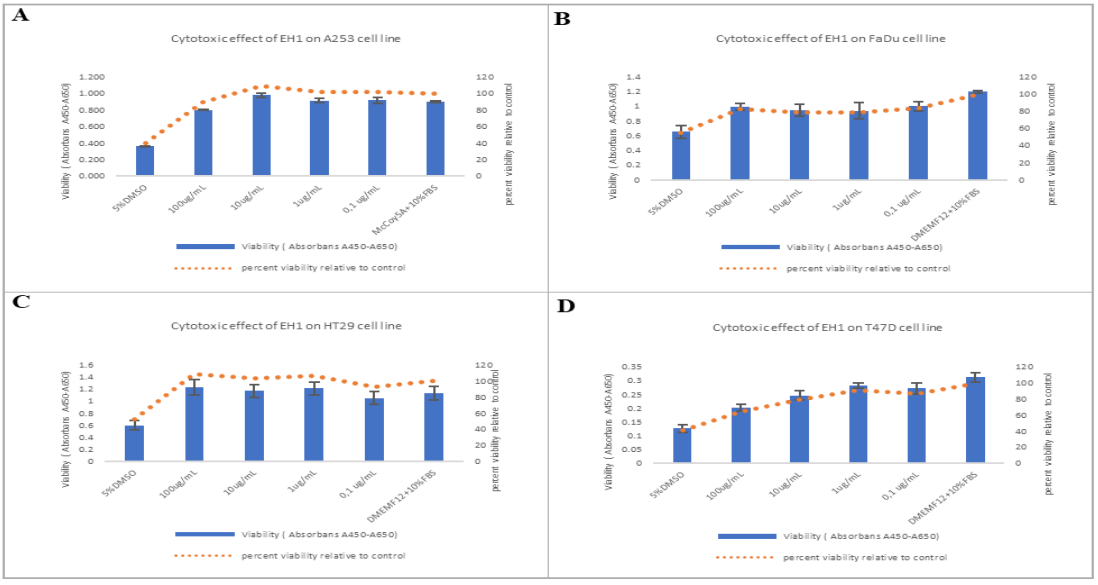



**Figure 22** Dose dependent dark cytotoxicity of EH1 phthalocyanine on A. A253 B FaDu C. HT29 D. T47D cell lines. Viability was measured by WST-1 assay following 24h of drug administration.

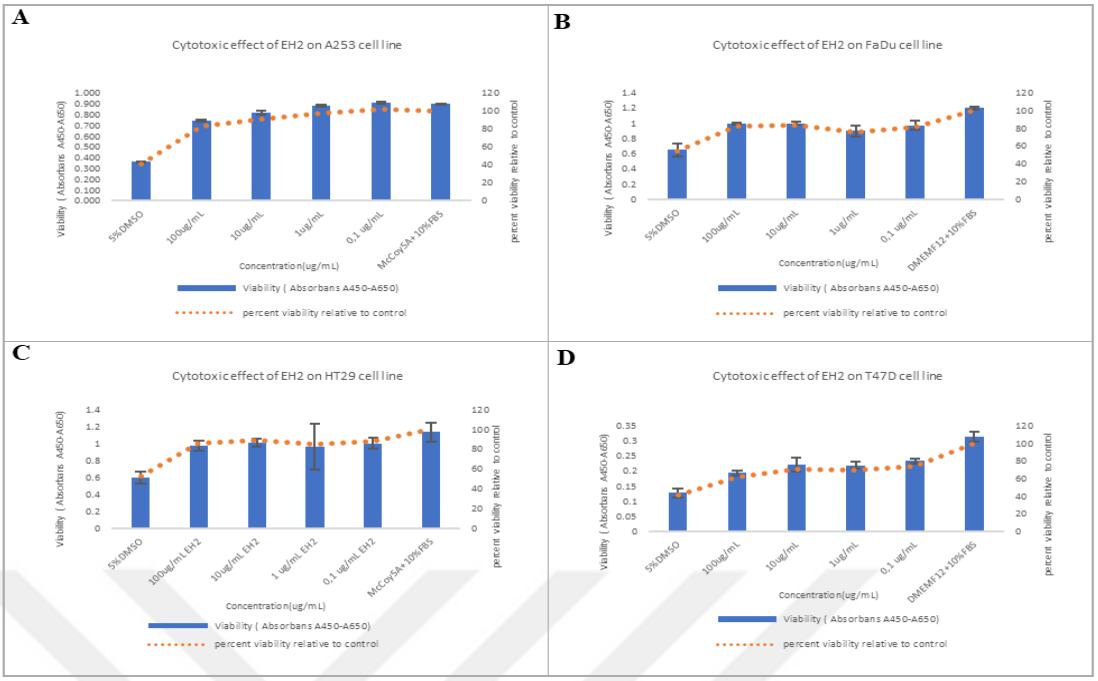



**Figure 23** Dose dependent dark cytotoxicity of EH2 phthalocyanine on A. A253 B FaDu C. HT29 D. T47D cell lines. Viability was measured by WST-1 assay following 24h of drug administration.

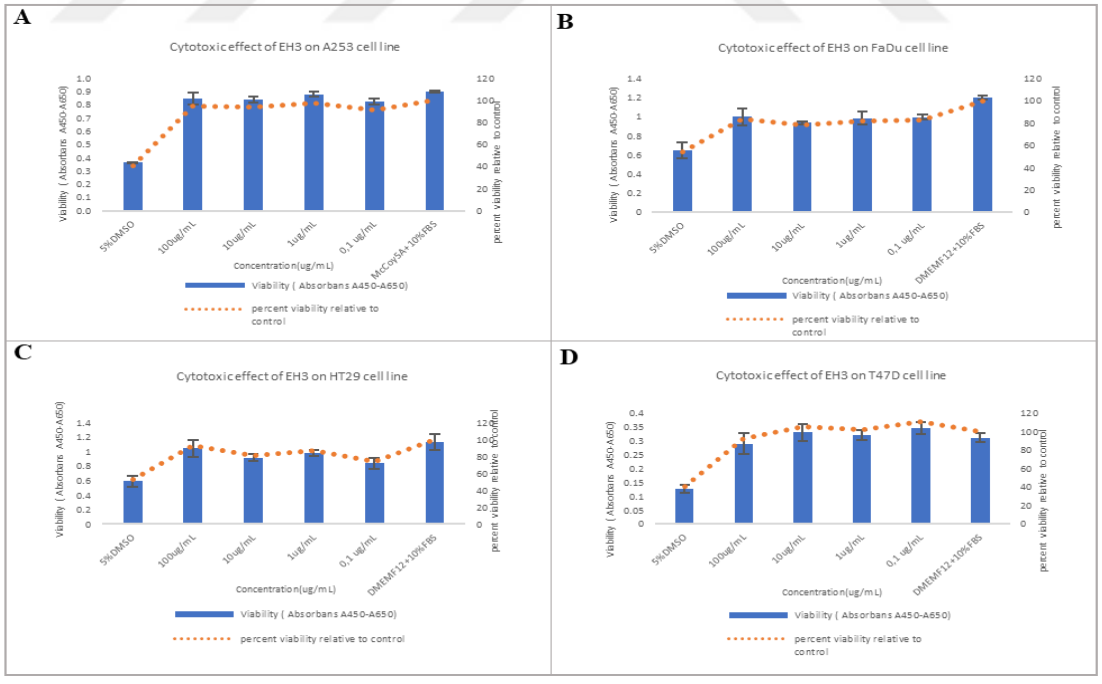



**Figure 24** Dose dependent dark cytotoxicity of EH3 phthalocyanine on A. A253 B FaDu C. HT29 D. T47D cell lines. Viability was measured by WST-1 assay following 24h of drug administration.

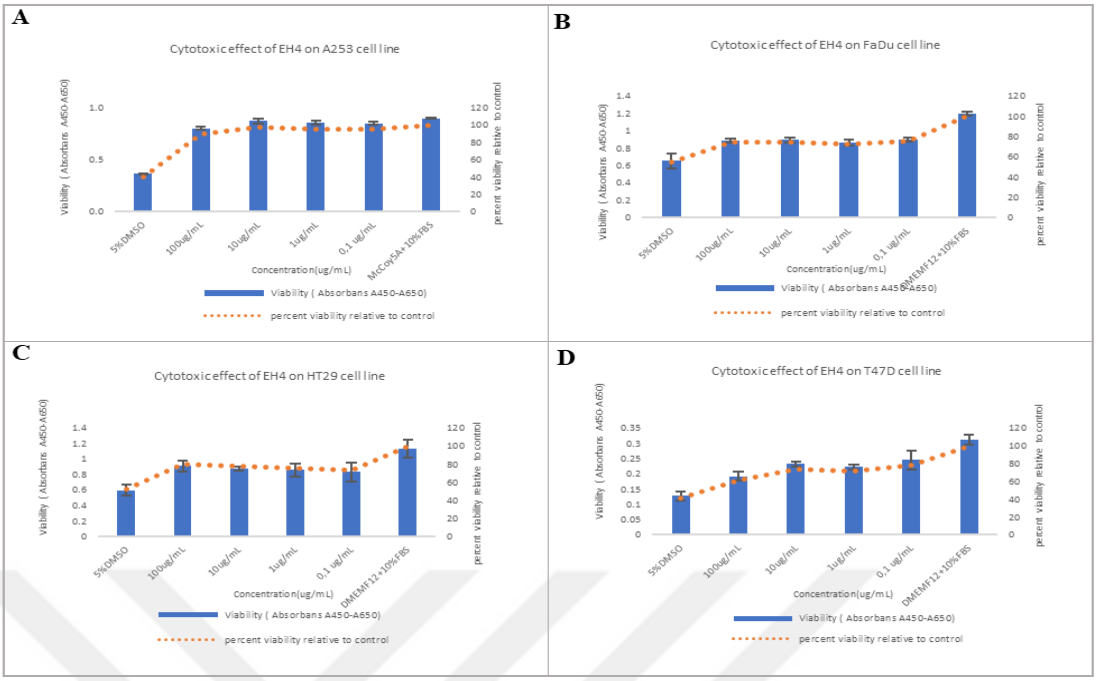



**Figure 25** Dose dependent dark cytotoxicity of EH4 phthalocyanine on A. A253 B FaDu C. HT29 D. T47D cell lines. Viability was measured by WST-1 assay following 24h of drug administration.

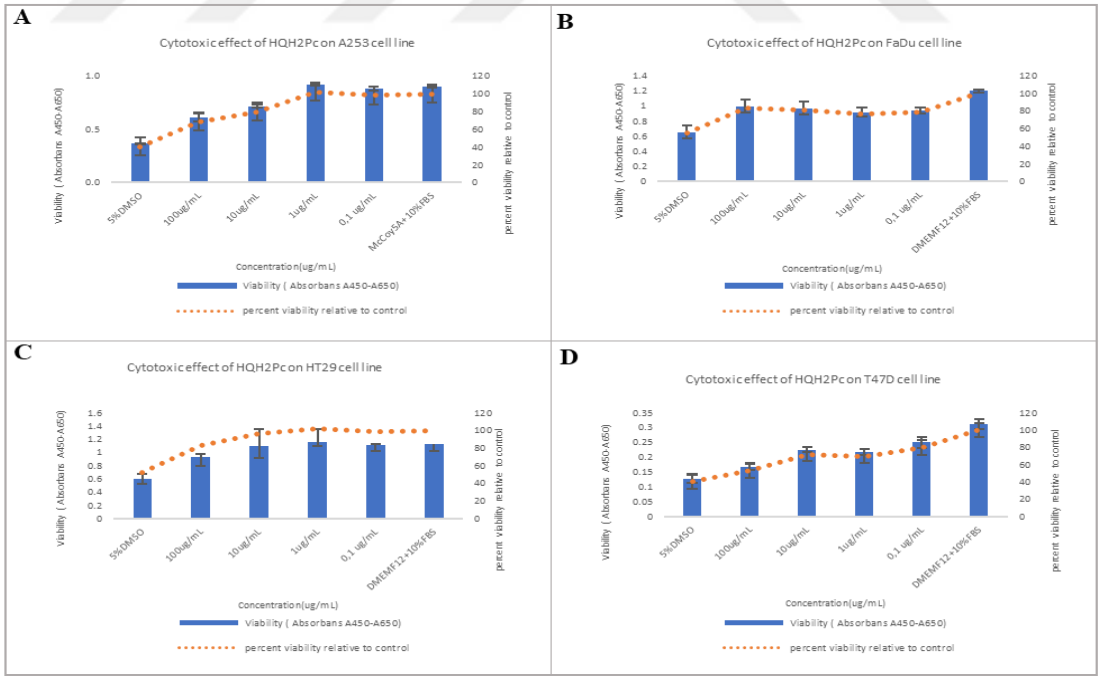



**Figure 26** Dose dependent dark cytotoxicity of $HQH_2Pc$ phthalocyanine on A. A253 B FaDu C. HT29 D. T47D cell lines. Viability was measured by WST-1 assay following 24h of drug administration.

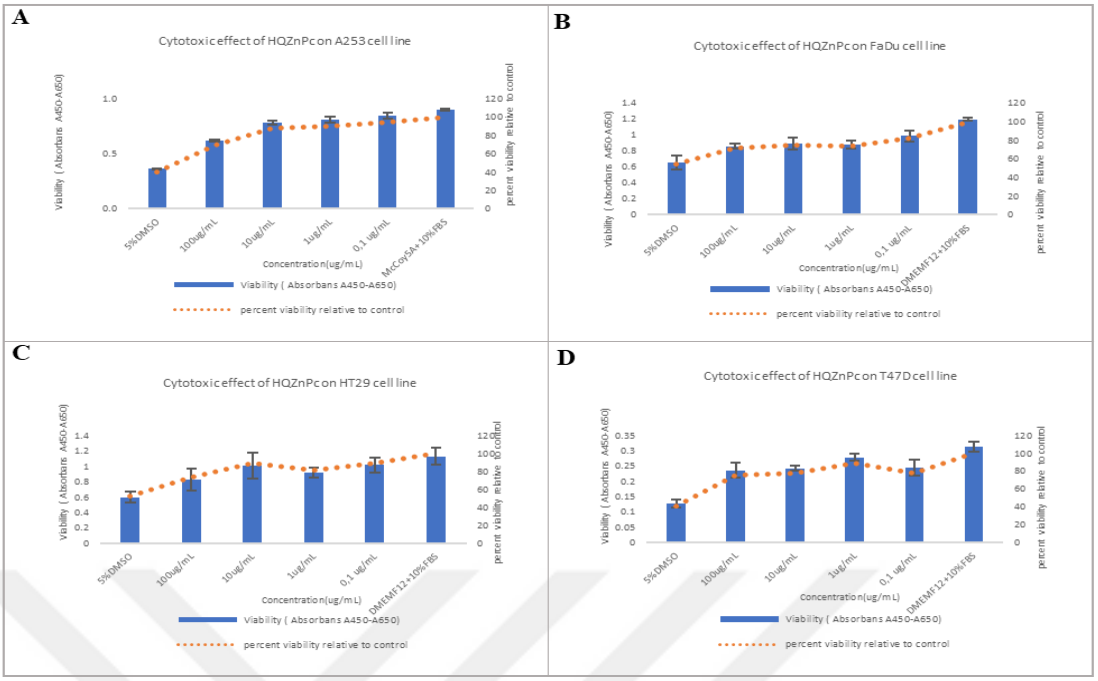



**Figure 27** Dose dependent dark cytotoxicity of HQZnPc phthalocyanine on A. A253 B FaDu C. HT29 D. T47D cell lines. Viability was measured by WST-1 assay following 24h of drug administration.

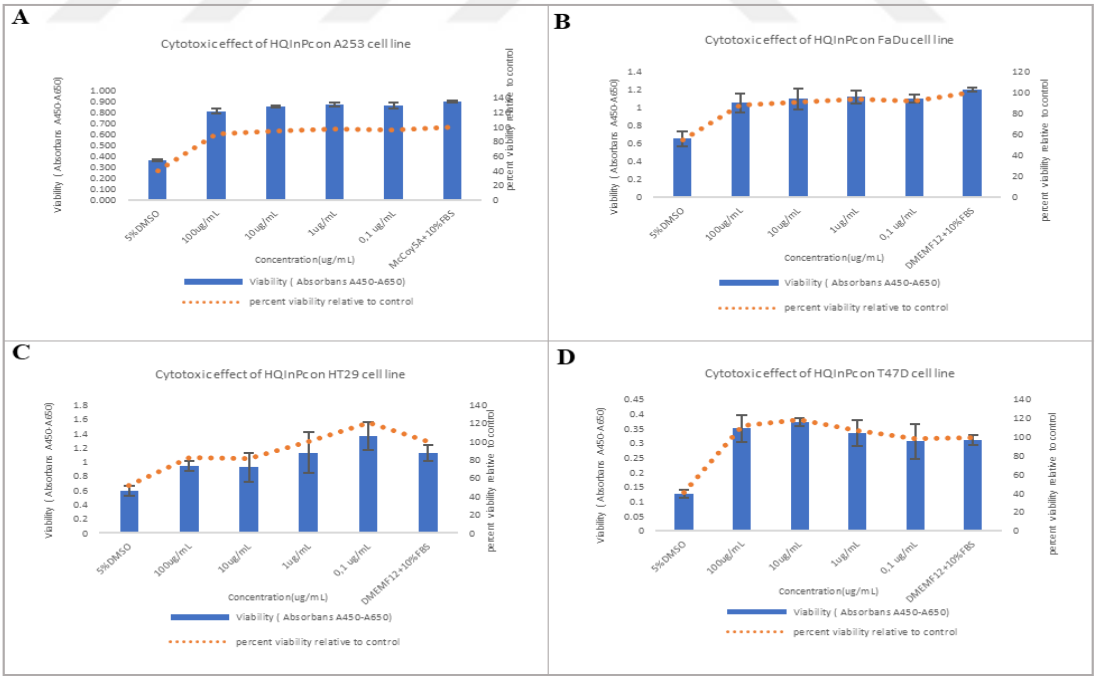



**Figure 28** Dose dependent dark cytotoxicity of HQInPc phthalocyanine on A. A253 B FaDu C. HT29 D. T47D cell lines. Viability was measured by WST-1 assay following 24h of drug administration.

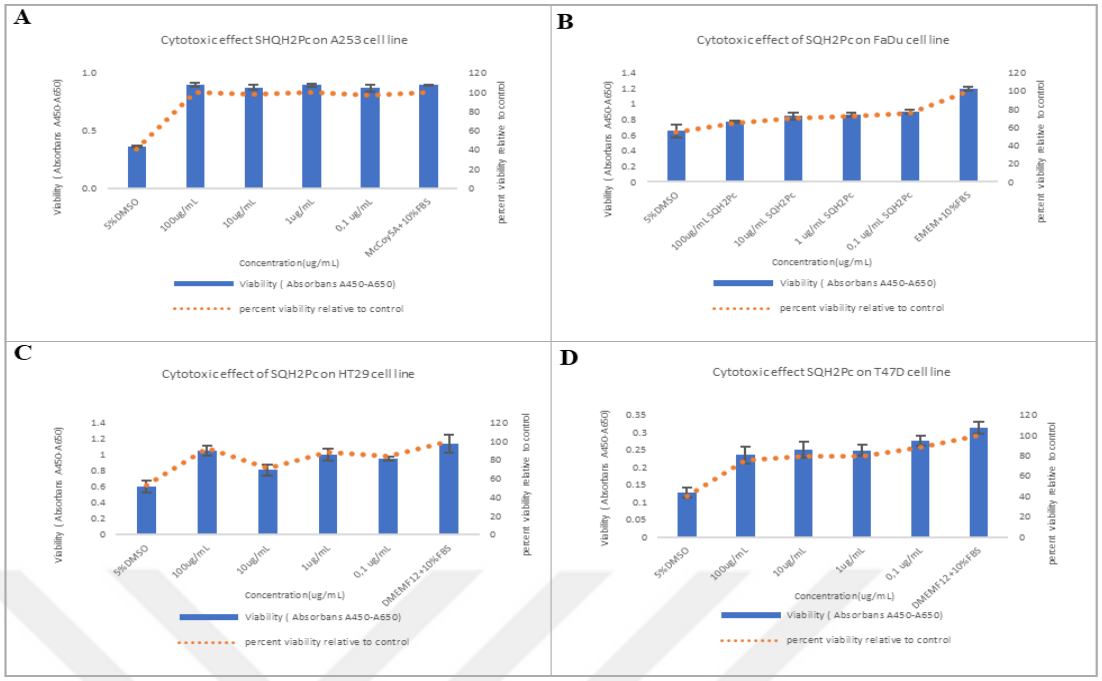



**Figure 29** Dose dependent dark cytotoxicity of $SQH_2Pc$ phthalocyanine on A. A253 B FaDu C. HT29 D. T47D cell lines. Viability was measured by WST-1 assay following 24h of drug administration.

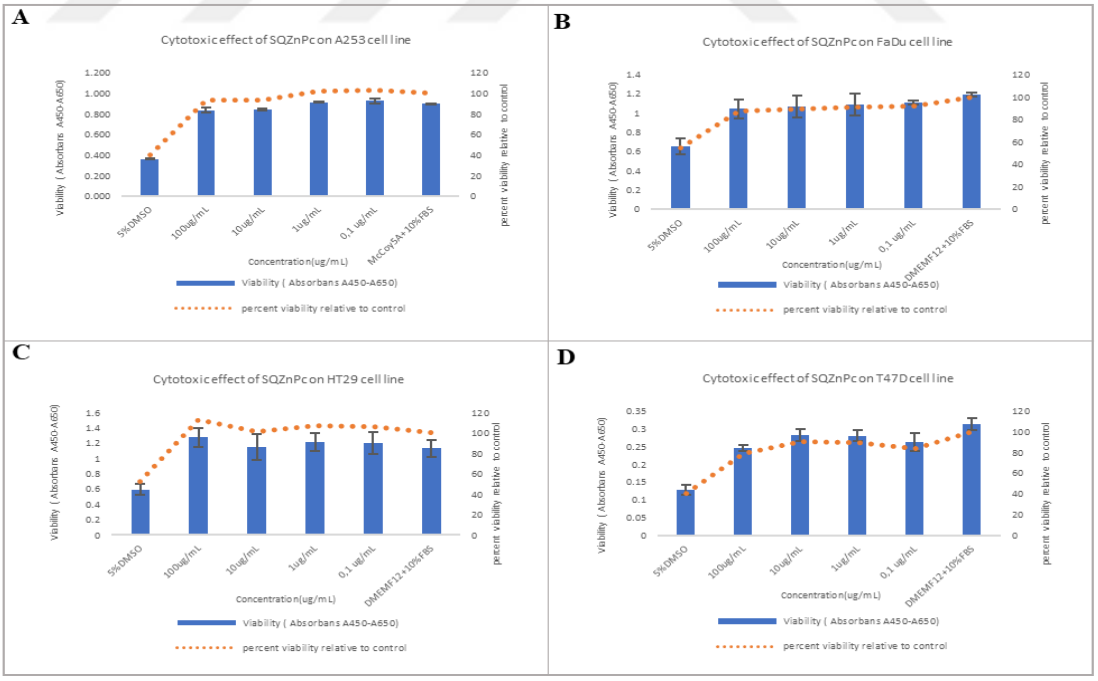



**Figure 30** Dose dependent dark cytotoxicity of SQZnPc phthalocyanine on A. A253 B FaDu C. HT29 D. T47D cell lines. Viability was measured by WST-1 assay following 24h of drug administration.

At four different cancer cell lines, the Pc molecules were administrated, and the cytotoxic response was measured after 24h. Concentration, where the Pc molecules did not have any cytotoxic response which causes reduction cell viability below 70% in each cell line, was determined as 10 μg/ml and 1 μg/ml.

### 4.6. Photo-induced Cytotoxicity of the Phthalocyanine Molecules on Cancer Cell Lines

Two concentrations, 10 μg/ml and 1 μg/ml that was selected according to dark cytotoxicity studies of Pc molecules were administrated on the cell lines. The cytotoxic response that was created through energy/photon transfer from the Pc molecules to molecular oxygen was measured post 24h of photo-irradiation of 2 joules/$cm^2$ of the wavelength range of 624 nm-755 nm.

Viability was measured by both WST-1 and resazurin assays following 24h of PDT with an hour drug administration at 37 C˚ and 5% $CO_2$ humidified environment. Survival percentage of A253, FaDu, HT29, and T47D cells was determined relative to the non-treated control. At the different range of wavelength, constant light fluence was applied for each group. The temperature during light treatment was kept constant to 37 C˚ to avoid temperature-related damages. Values are shown as the mean±SD of three independent experimental replicates.

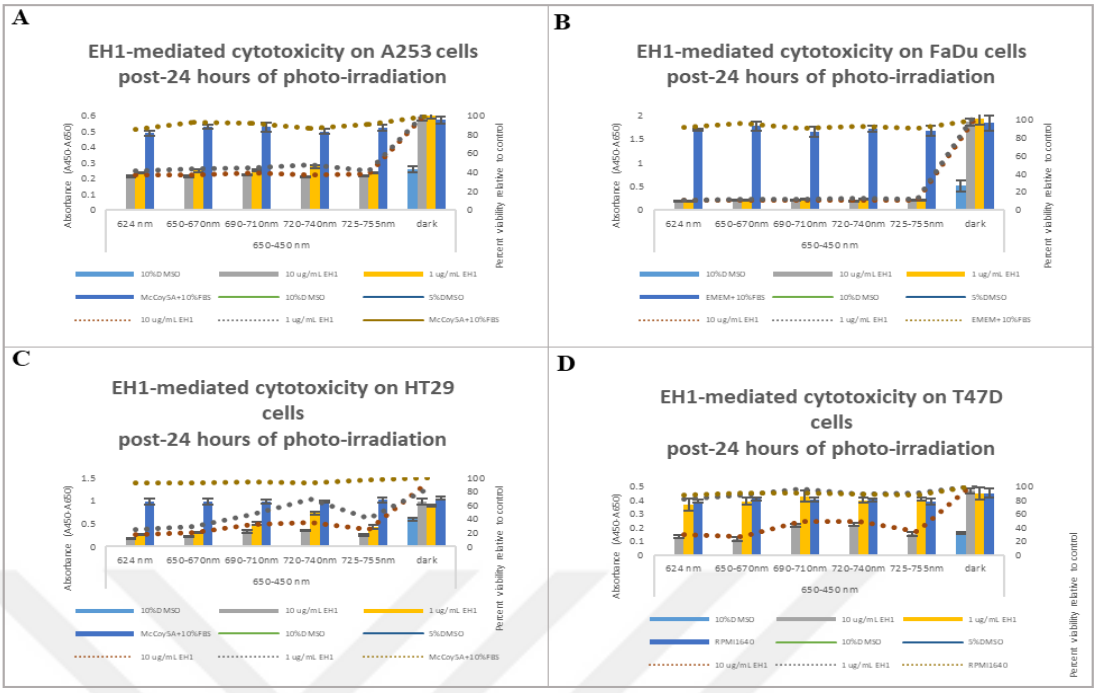



**Figure 31** Dose dependent photo-induced cytotoxicity of EH1 phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines by WST-1 assay.

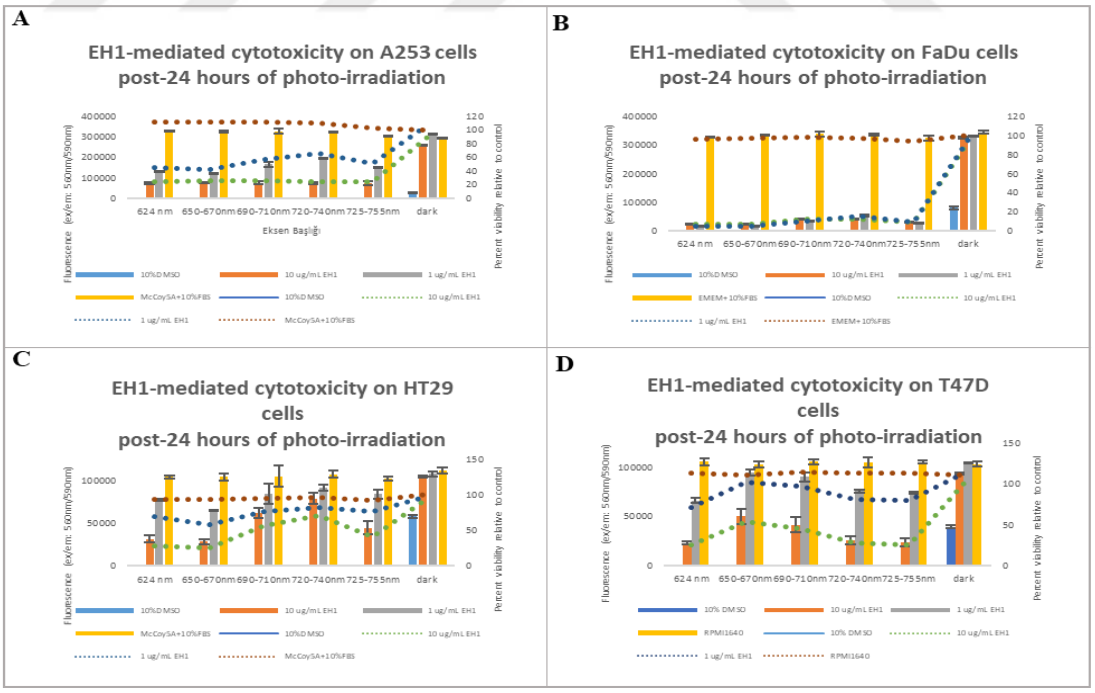



**Figure 32** Dose dependent photo-induced cytotoxicity of EH1 phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines by resazurin assay.

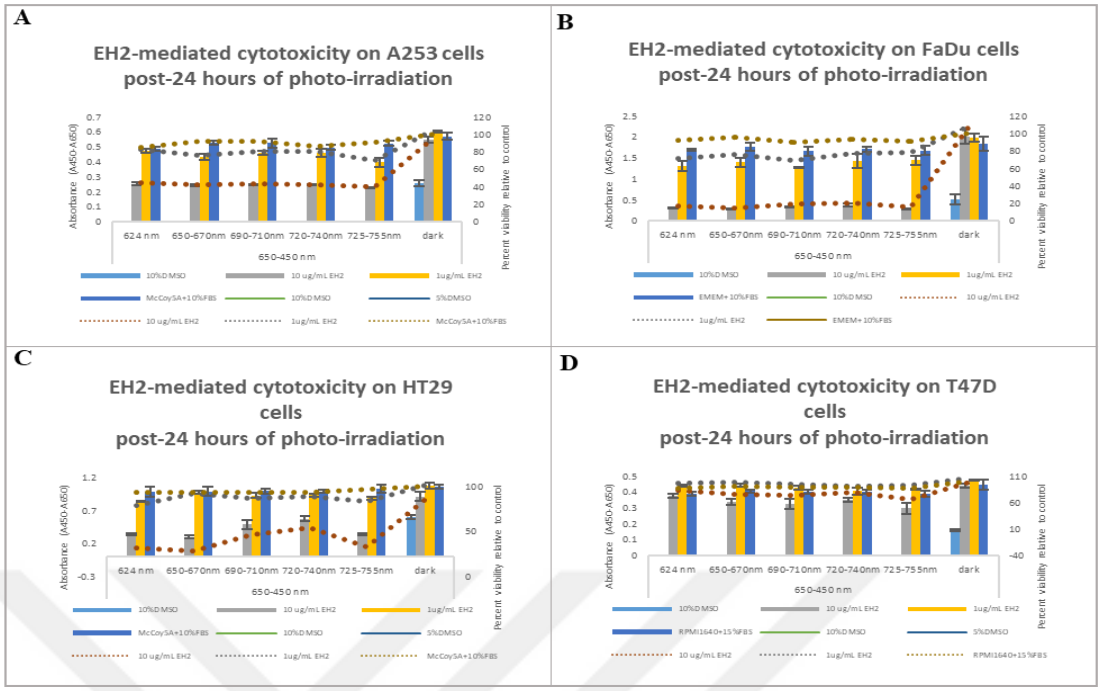



**Figure 33** Dose dependent photo-induced cytotoxicity of EH2 phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines by WST-1 assay.

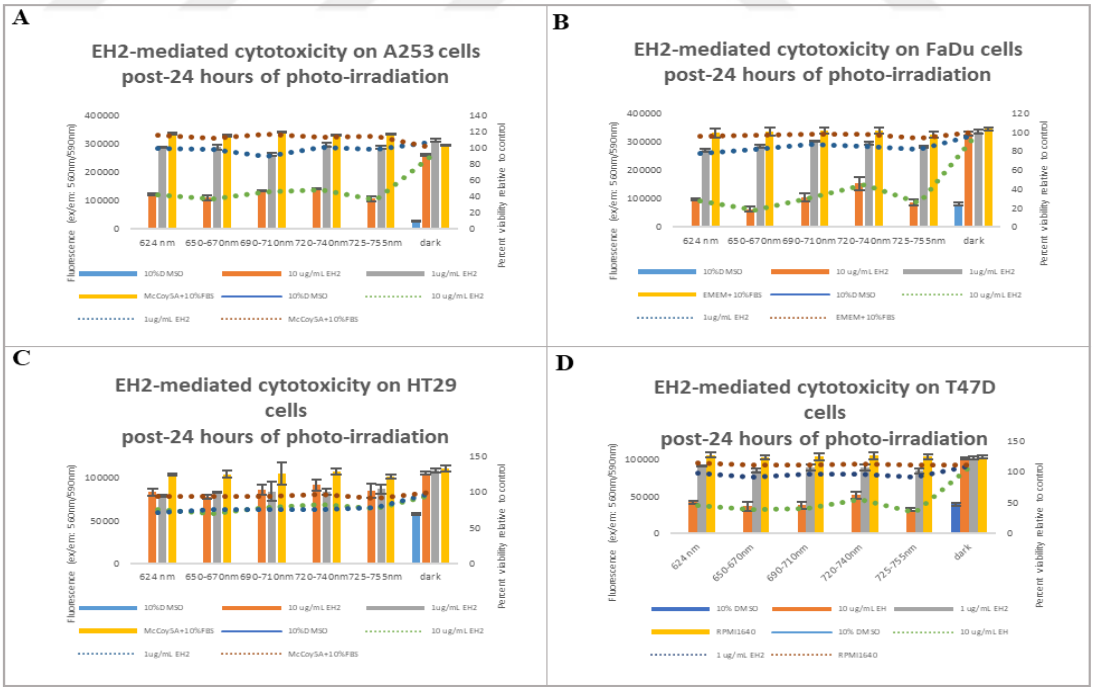



**Figure 34** Dose dependent photo-induced cytotoxicity of EH2 phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines by resazurin assay.

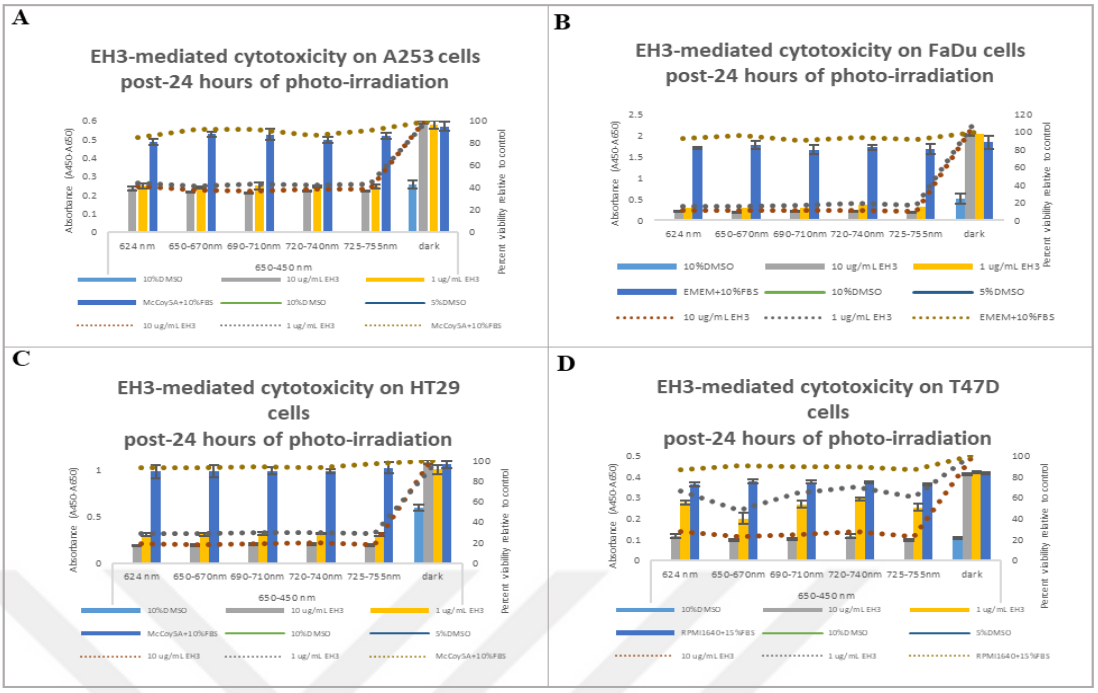



**Figure 35** Dose dependent photo-induced cytotoxicity of EH3 phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines by WST-1 assay.

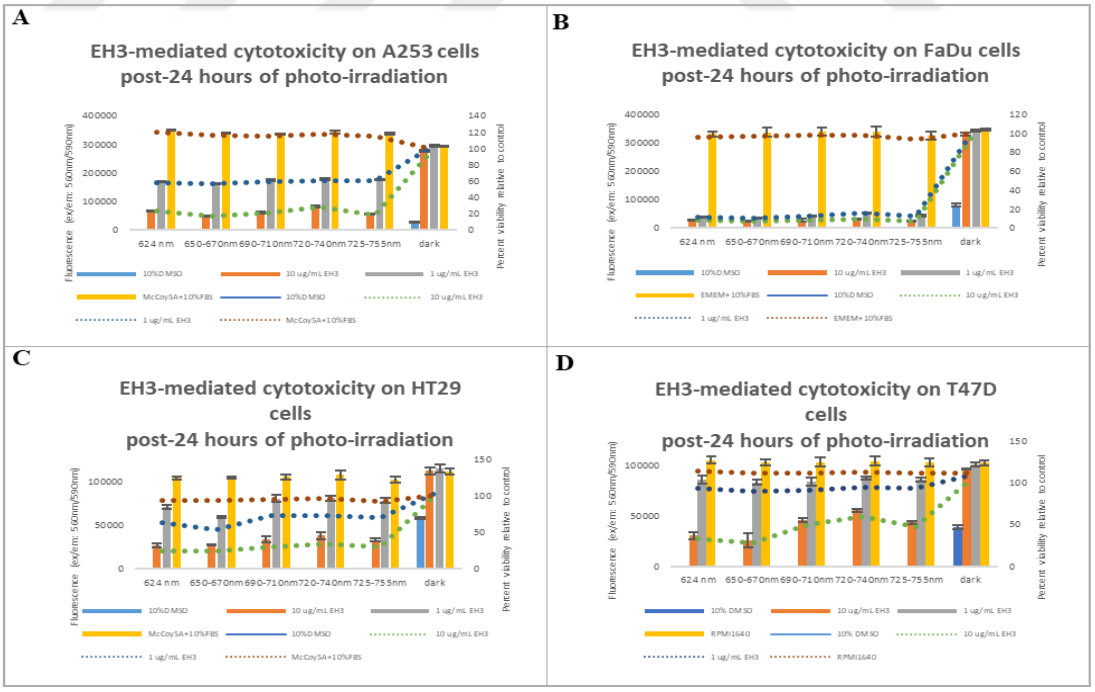



**Figure 36** Dose dependent photo-induced cytotoxicity of EH3 phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines by resazurin assay.

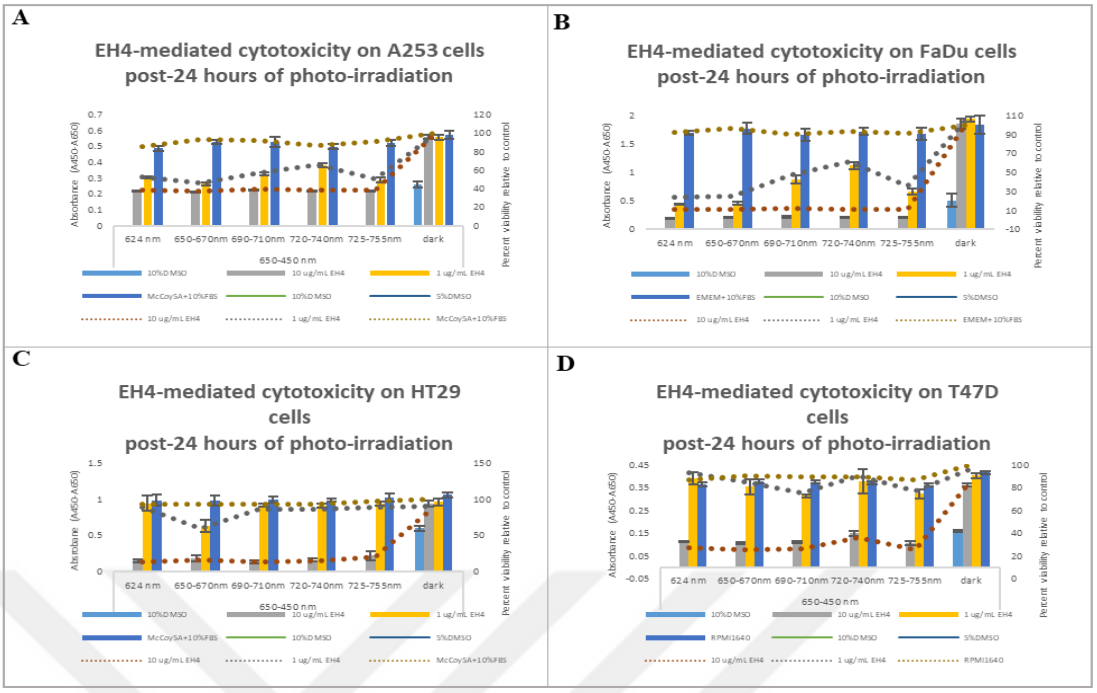



**Figure 37** Dose dependent photo-induced cytotoxicity of EH4 phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines by WST-1 assay.

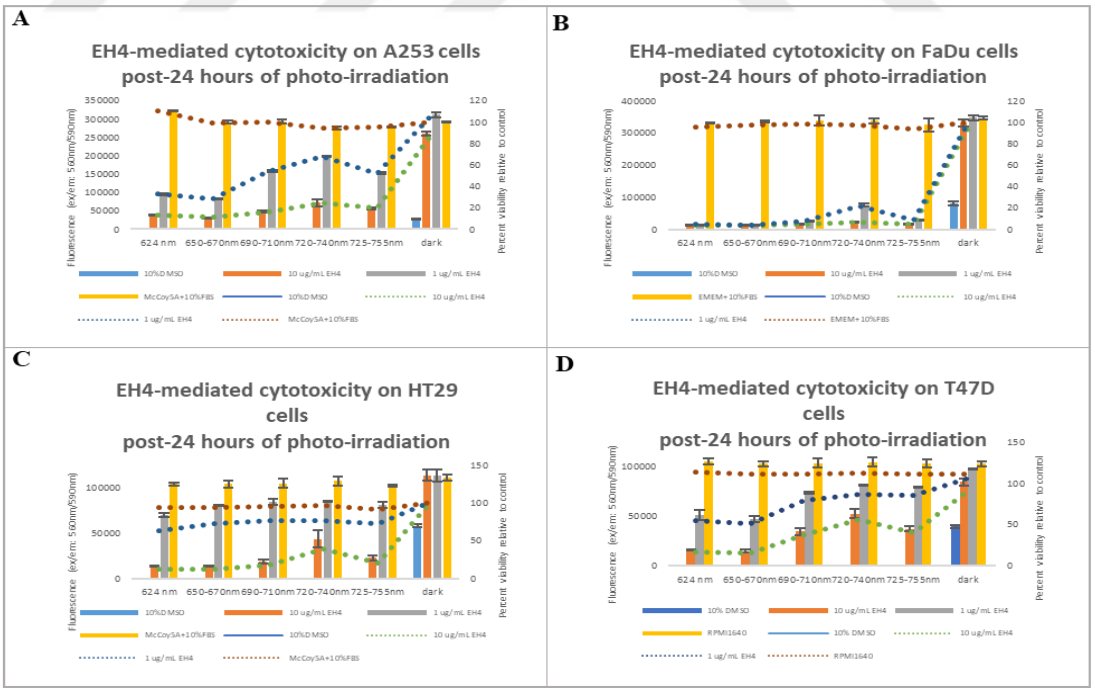



**Figure 38** Dose dependent photo-induced cytotoxicity of EH4 phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines by resazurin assay

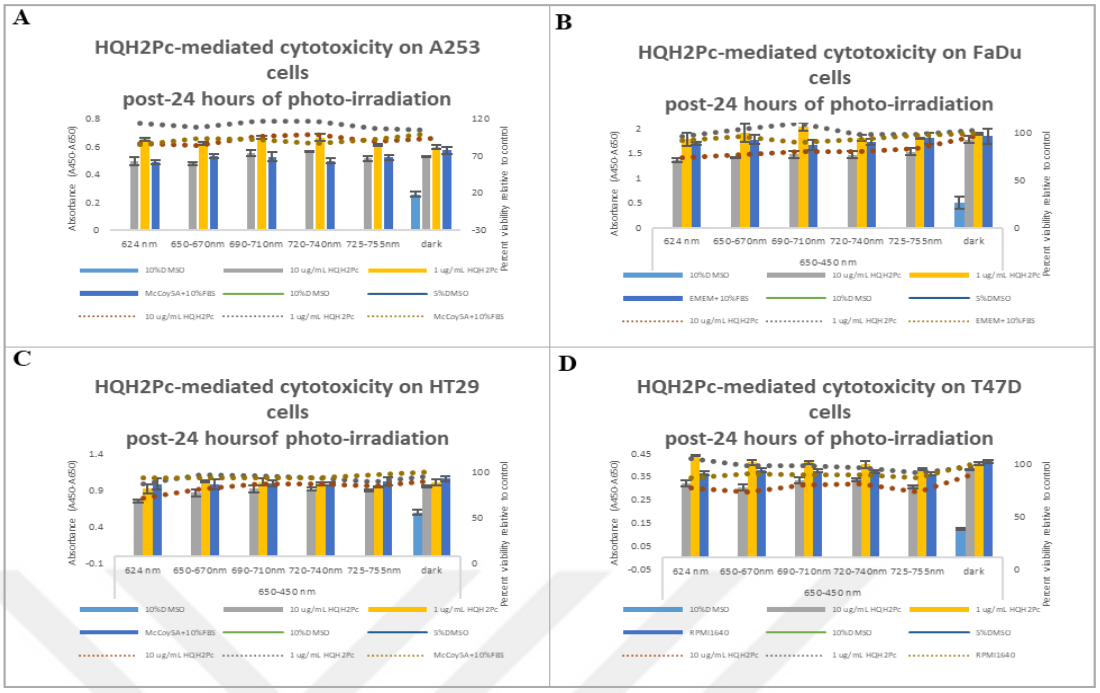



**Figure 39** Dose dependent photo-induced cytotoxicity of $HQH_2Pc$ phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines by WST-1 assay.

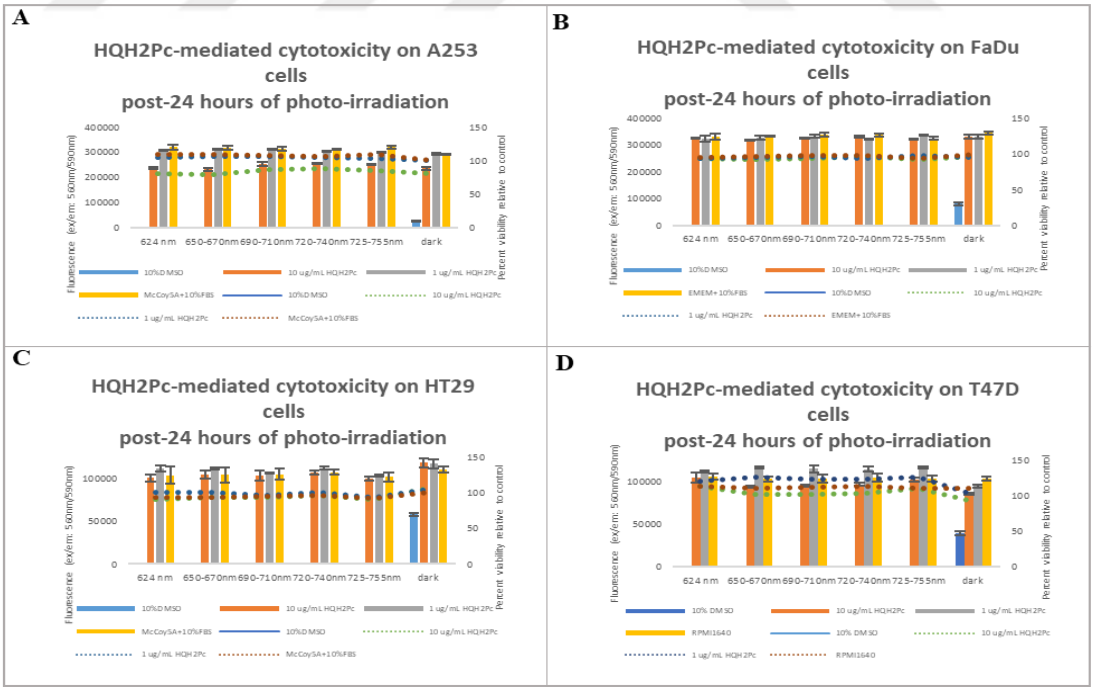



**Figure 40** Dose dependent photo-induced cytotoxicity of $HQH_2Pc$ phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines by resazurin assay.

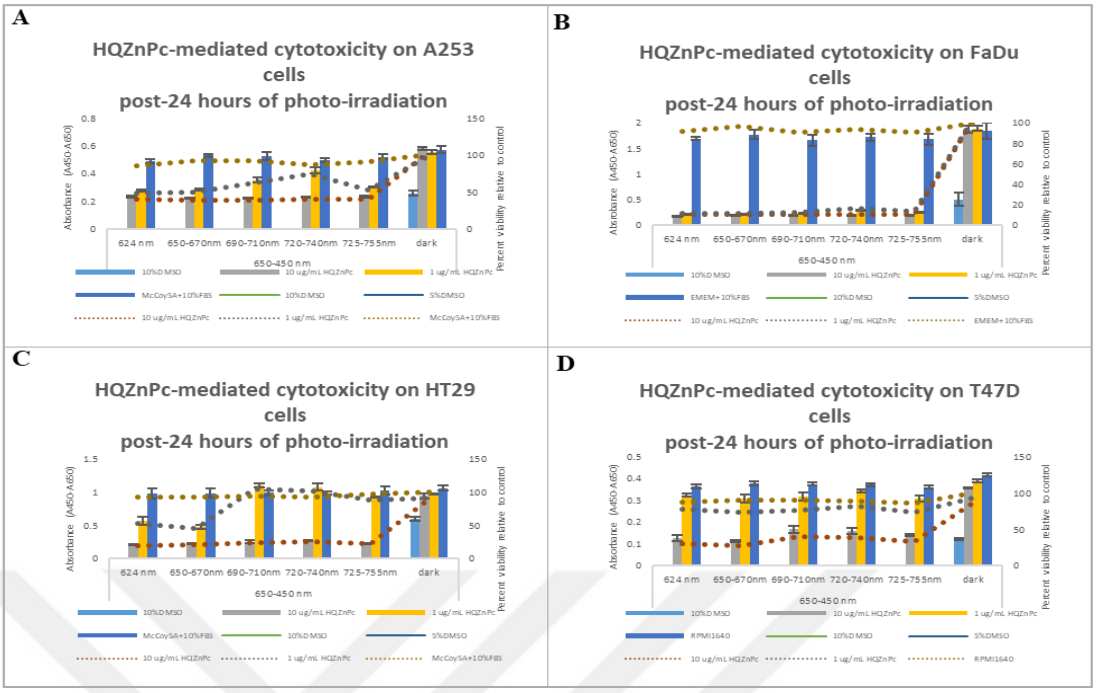



**Figure 41** Dose dependent photo-induced cytotoxicity of HQZnPc phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines by WST-1 assay.

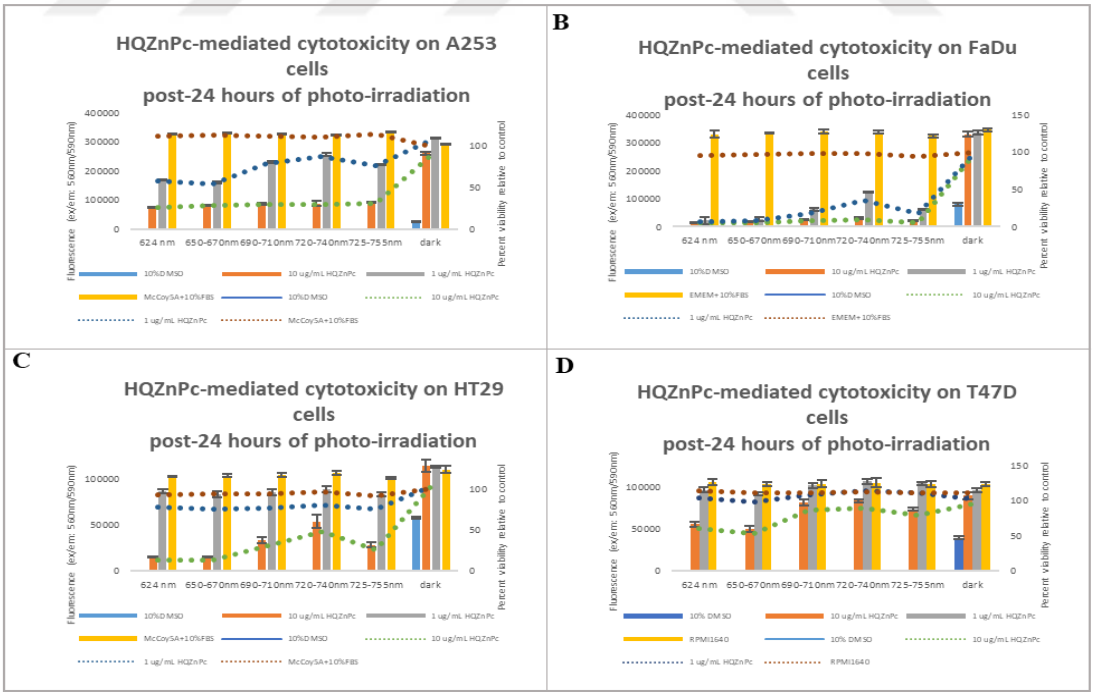



**Figure 42** Dose dependent photo-induced cytotoxicity of HQZnPc phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines by resazurin assay.

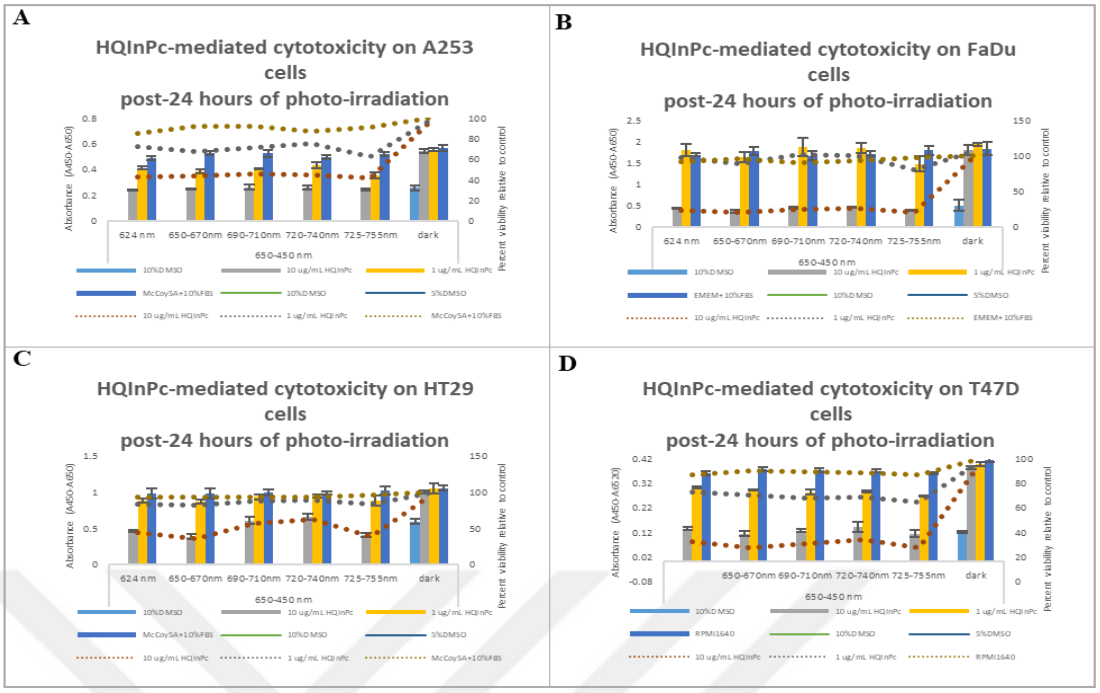



**Figure 43** Dose dependent photo-induced cytotoxicity of HQInPc phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines by WST-1 assay.

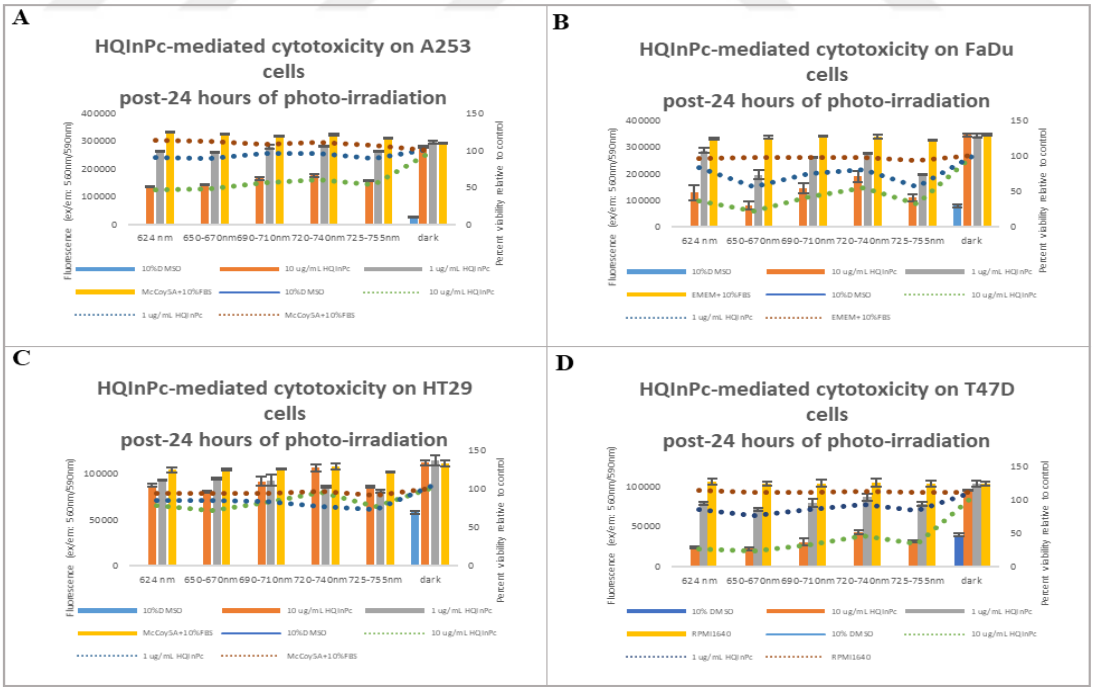



**Figure 44** Dose dependent photo-induced cytotoxicity of HQInPc phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines by resazurin assay.

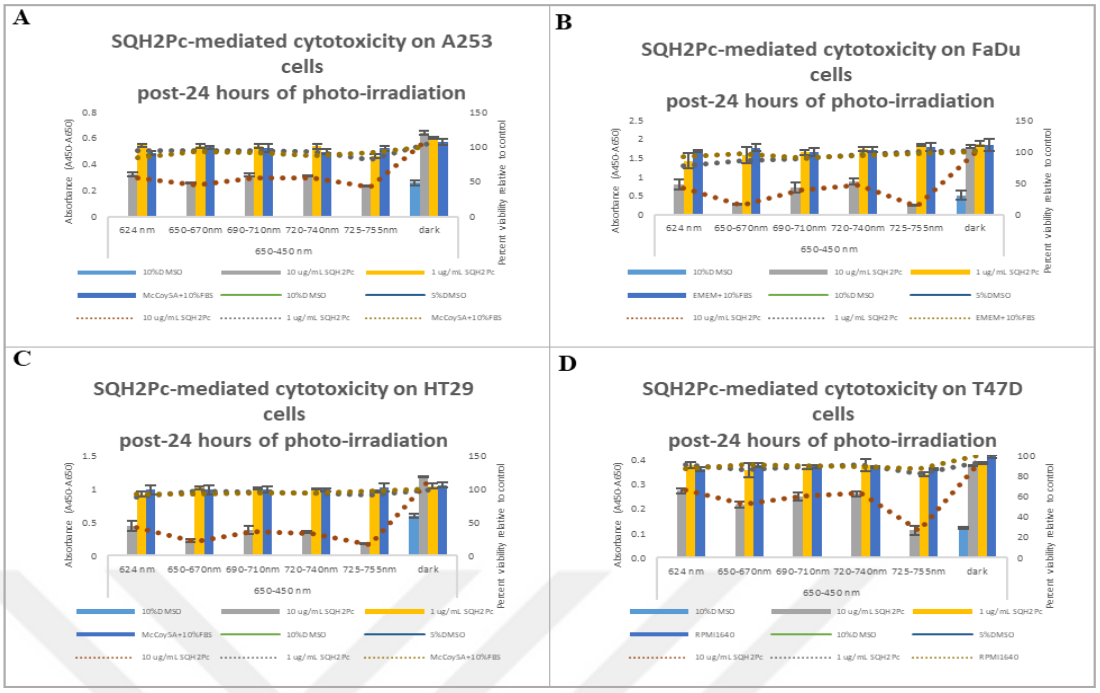



**Figure 45** Dose dependent photo-induced cytotoxicity of $SQH_2Pc$ phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines by WST-1 assay.

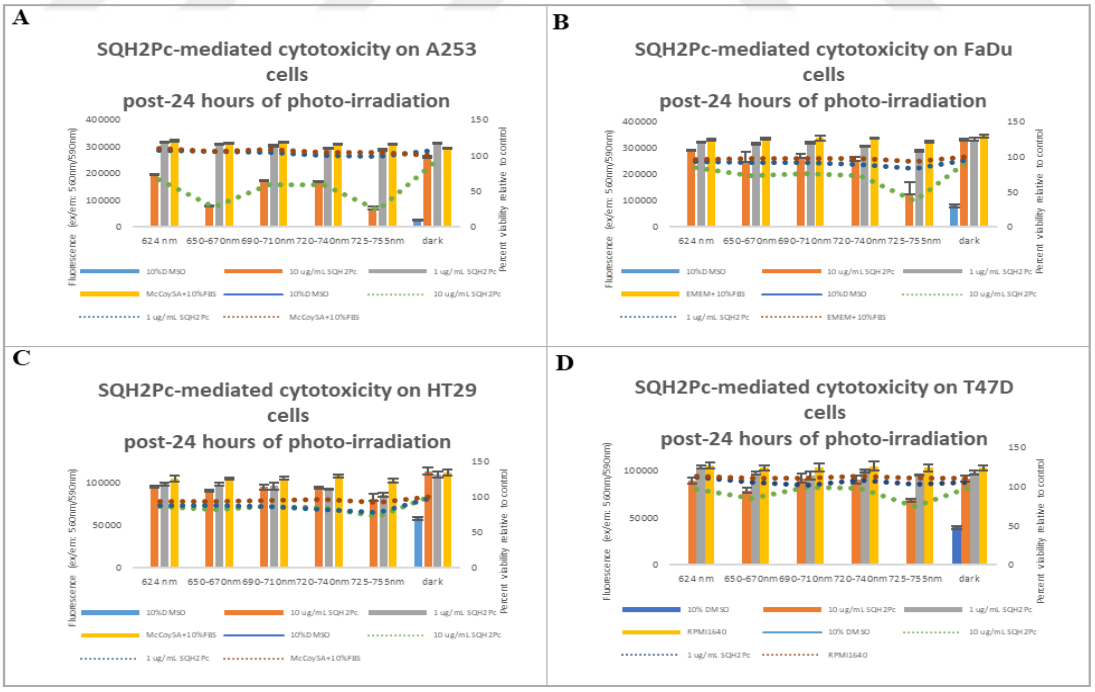



**Figure 46** Dose dependent photo-induced cytotoxicity of $SQH_2Pc$ phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines by resazurin assay.

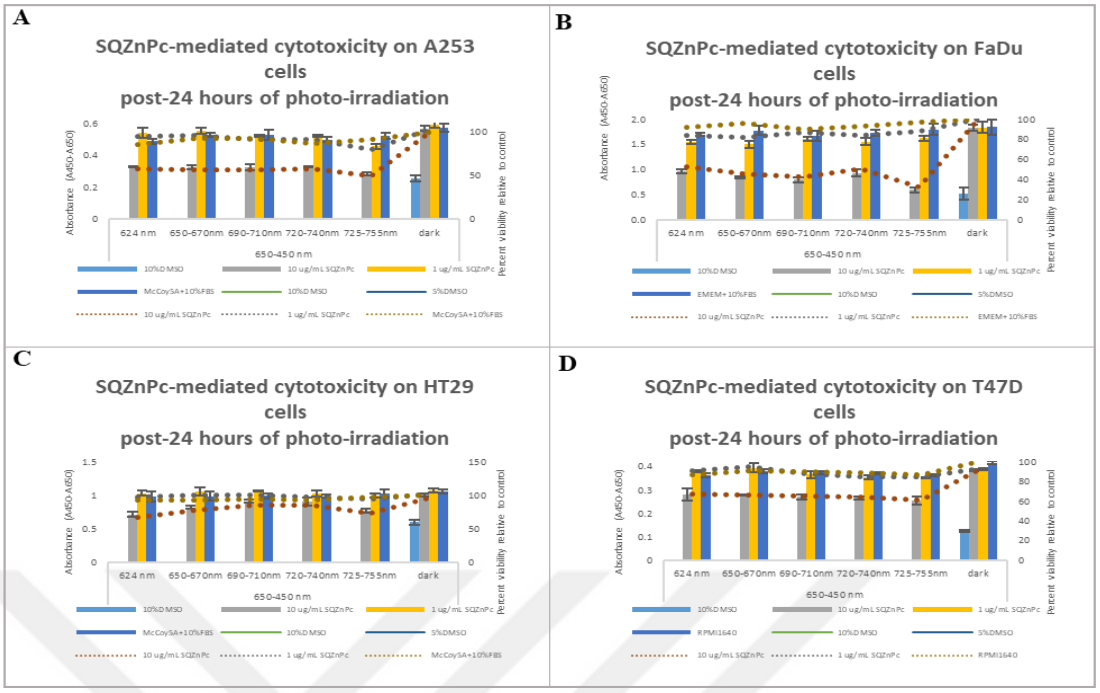



**Figure 47** Dose dependent photo-induced cytotoxicity of SQZnPc phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines by WST-1 assay.

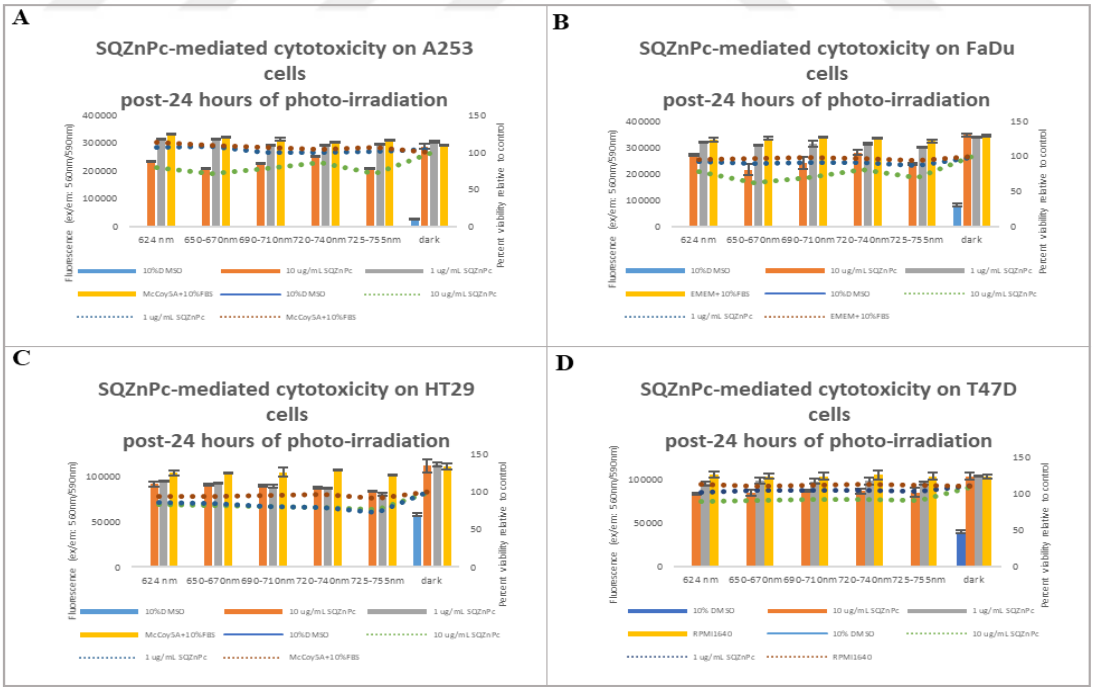



**Figure 48** Dose dependent photo-induced cytotoxicity of SQZnPc phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines by resazurin assay.

### 4.7. PDT-mediated Oxidative Stress Evaluation

In photodynamic therapy, cellular damage mostly occurs due to reactive oxygen species formation through energy transfer from Pc molecules to nearby molecular oxygen. To link the reason for the cell PDT-induced cell damage, ROS evaluation following post-30 minutes (immediate) and post-24 hours (late) of PDT was carried out using non-fluorescent DCFDA that forms fluorescent DCF in presence of ROS.

Following 30 minutes and 24 hours of PDT with an hour drug administration at 37 C˚ and 5% CO2 humidified environment, the relative ROS amount was measured by DCFDA that yields fluorescent DCF. At the different range of wavelength and constant light fluence was applied for each group. The temperature during light treatment was kept constant to 37 C˚ to avoid temperature-related oxidative stress. Values are shown as the mean±SD of three independent experimental replicates.

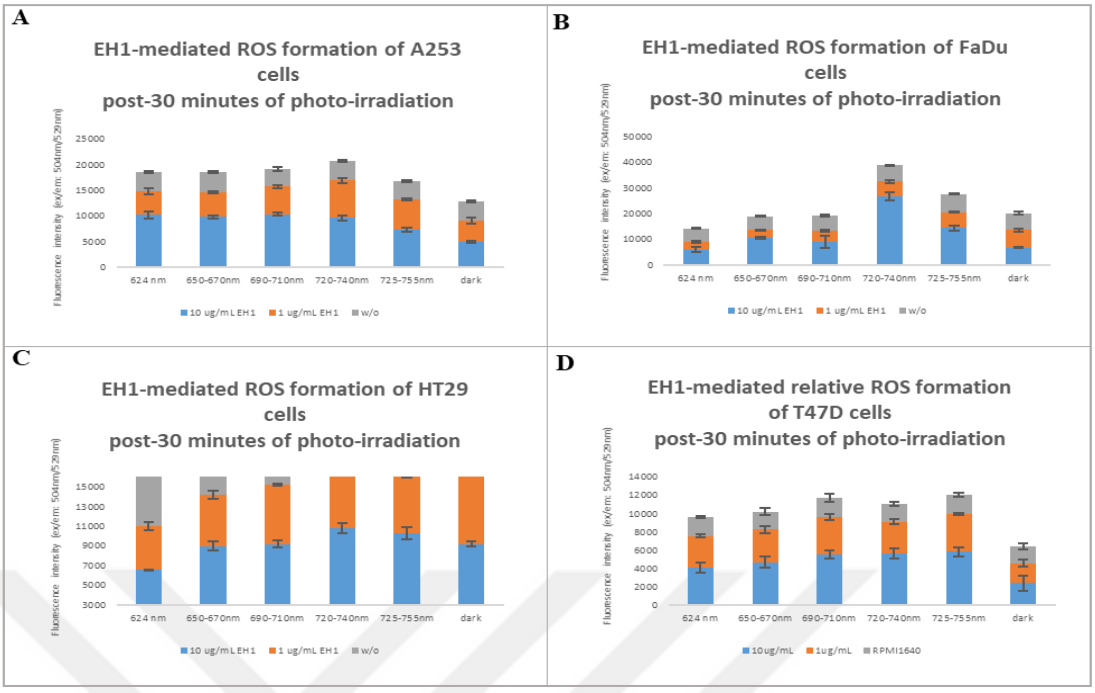



**Figure 49** Dose dependent post-30 minutes photo-induced ROS formation of EH1 phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines.

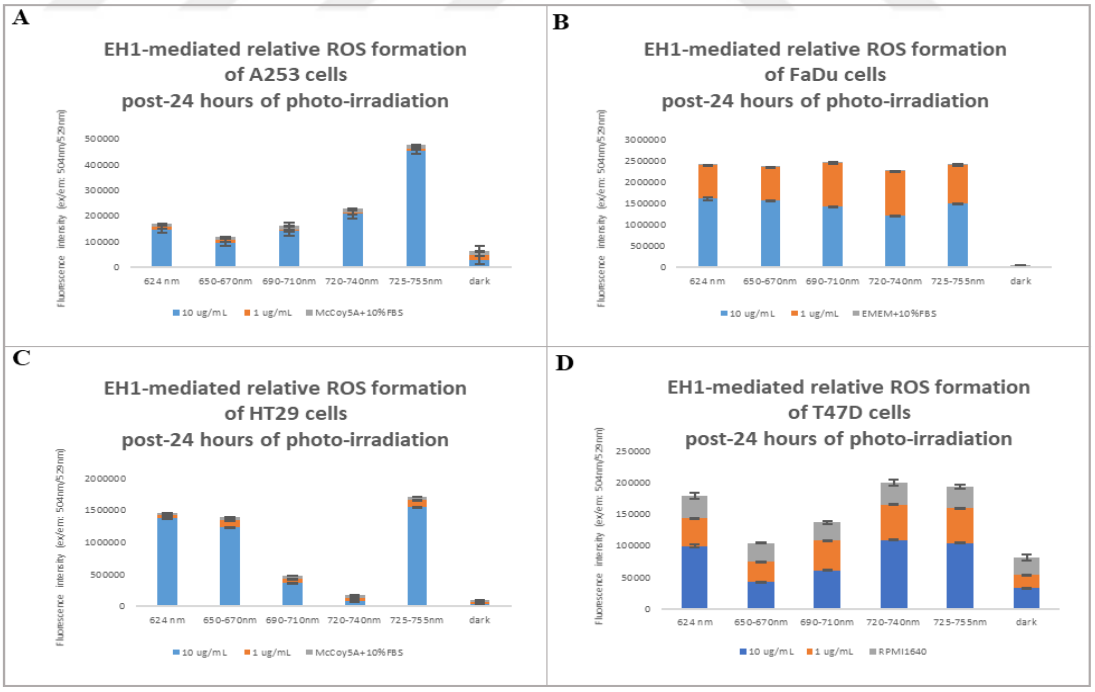



**Figure 50** Dose dependent post-24 hours photo-induced ROS formation of EH1 phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines.

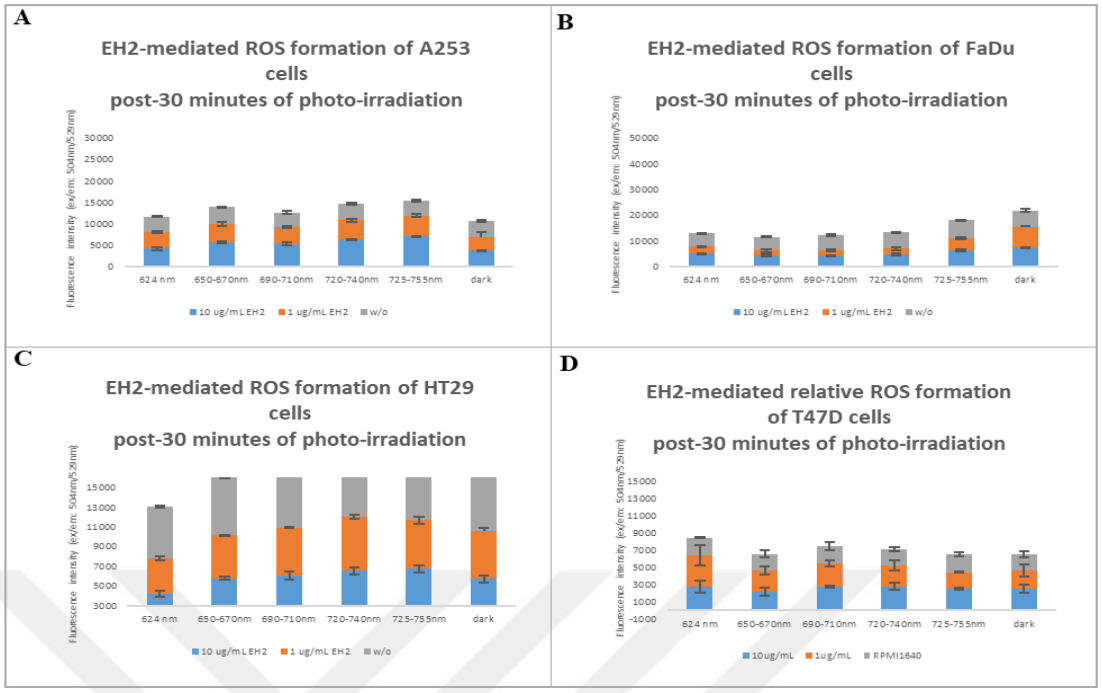



**Figure 51** Dose dependent post-30 minutes photo-induced ROS formation of EH2 phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines.

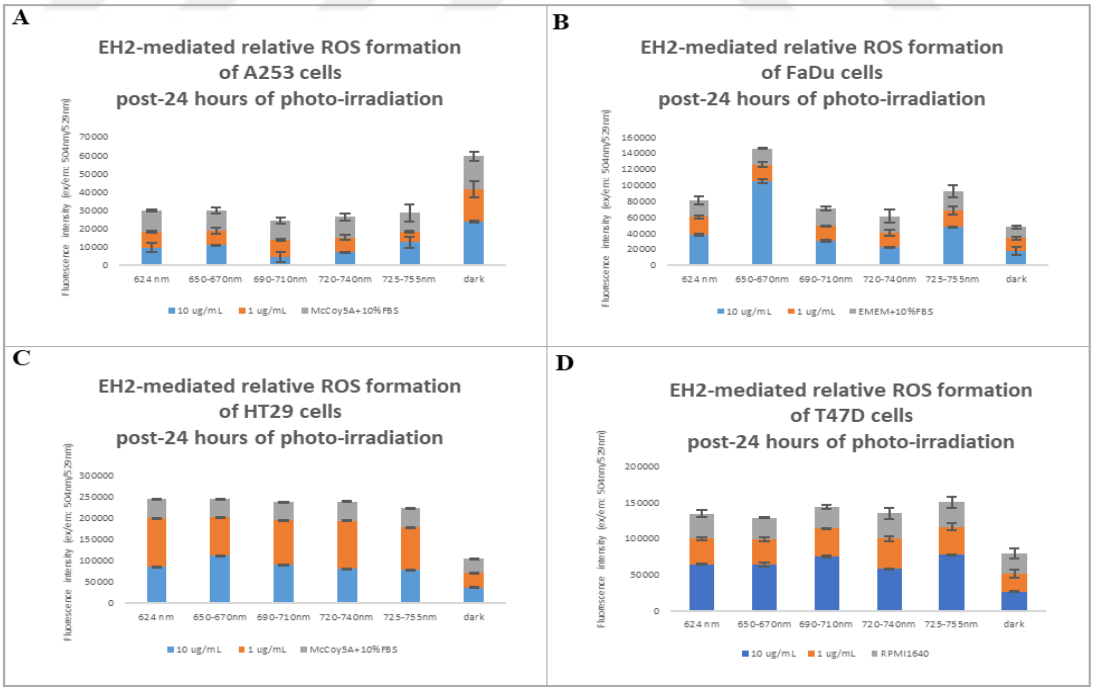



**Figure 52** Dose dependent post-24 hours photo-induced ROS formation of EH2 phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines.

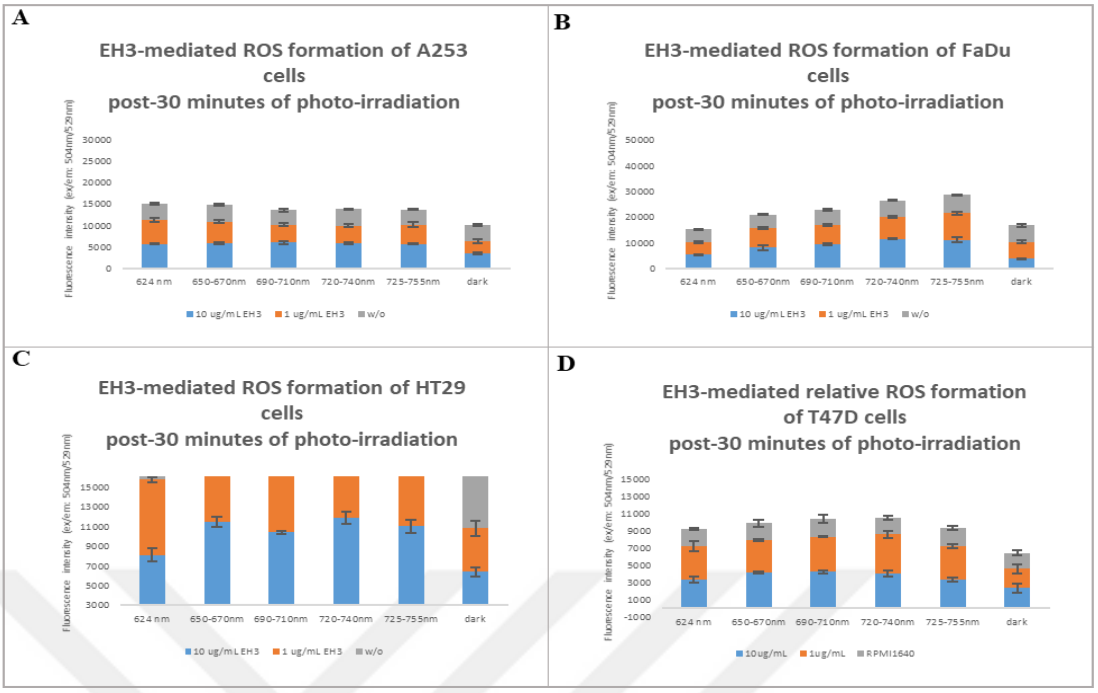



**Figure 53** Dose dependent post-30 minutes photo-induced ROS formation of EH3 phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines.

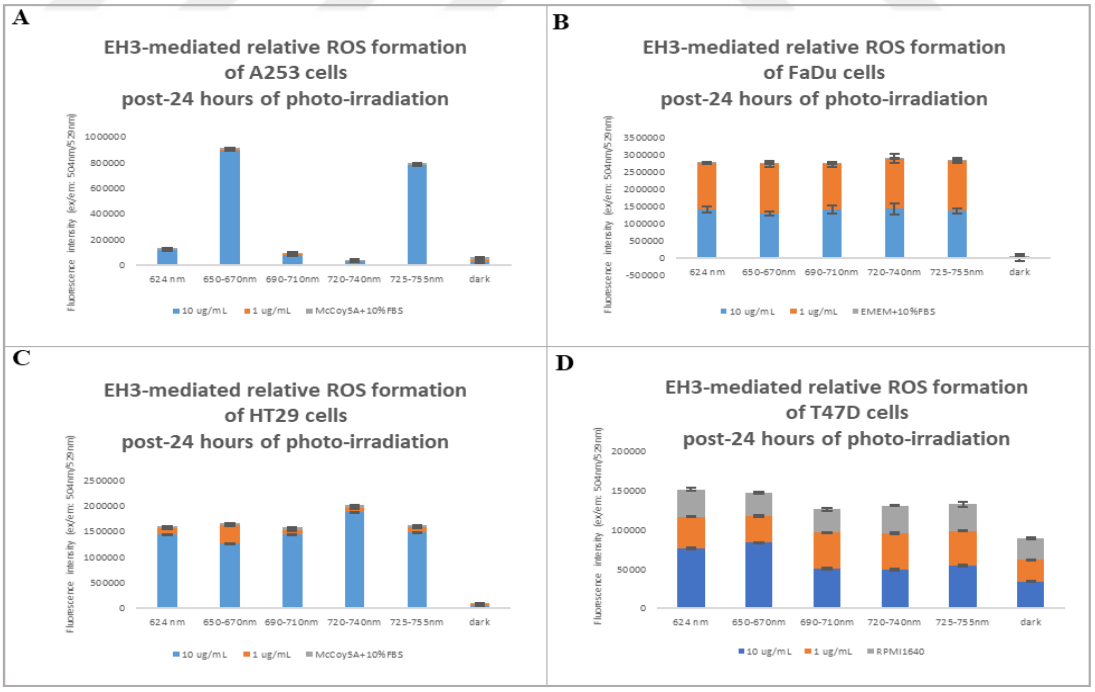



**Figure 54** Dose dependent post-24 hours photo-induced ROS formation of EH3 phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines.

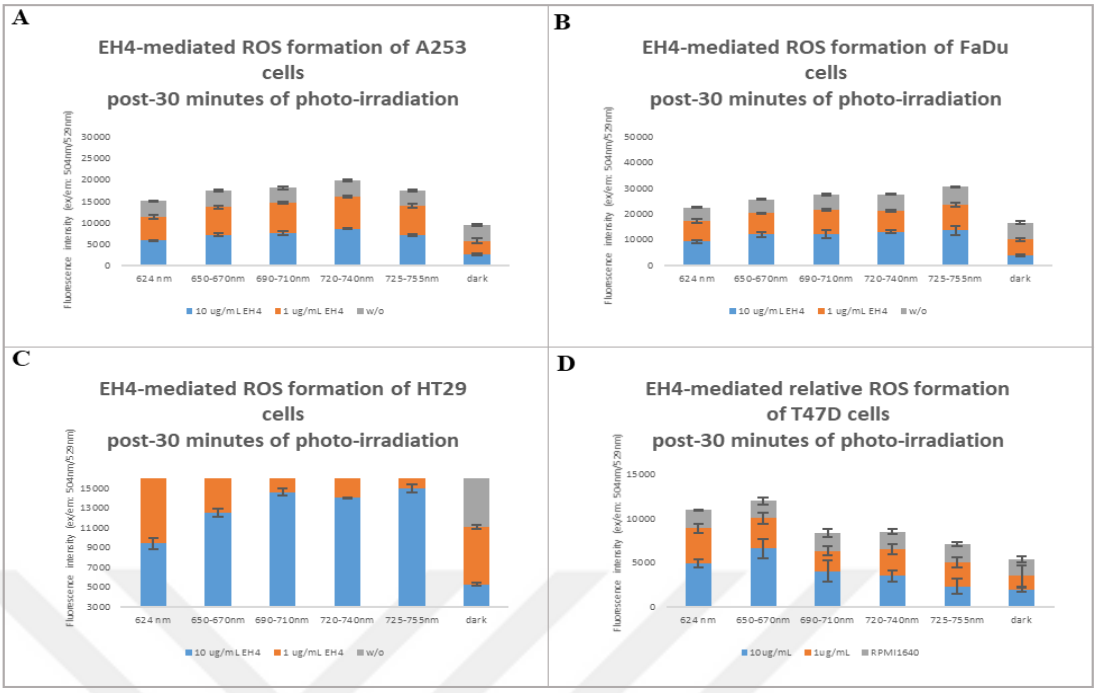



**Figure 55** Dose dependent post-30 minutes photo-induced ROS formation of EH4 phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines.

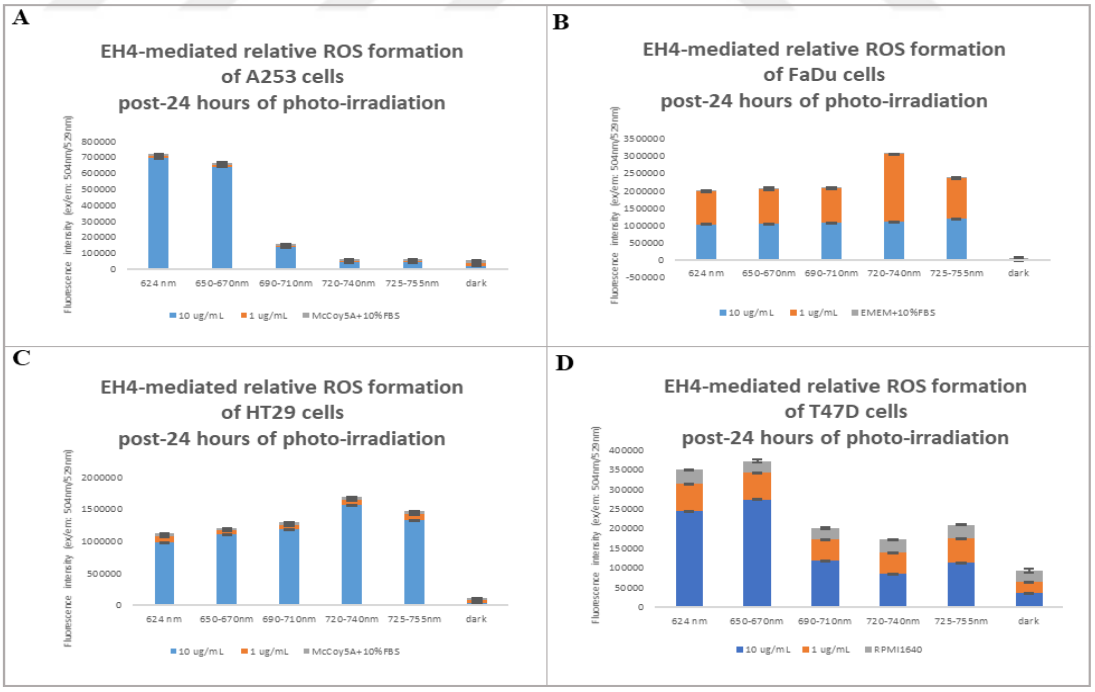



**Figure 56** Dose dependent post-24 hours photo-induced ROS formation of EH4 phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines.

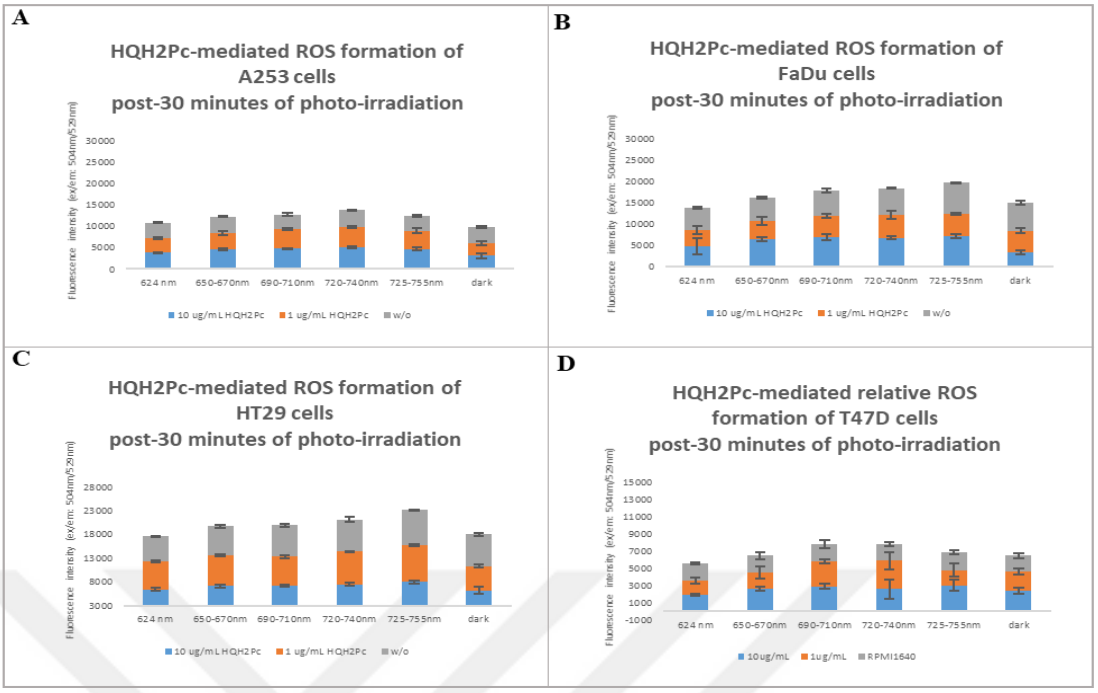



**Figure 57** Dose dependent post-30 minutes photo-induced ROS formation of $HQH_2Pc$ phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines.

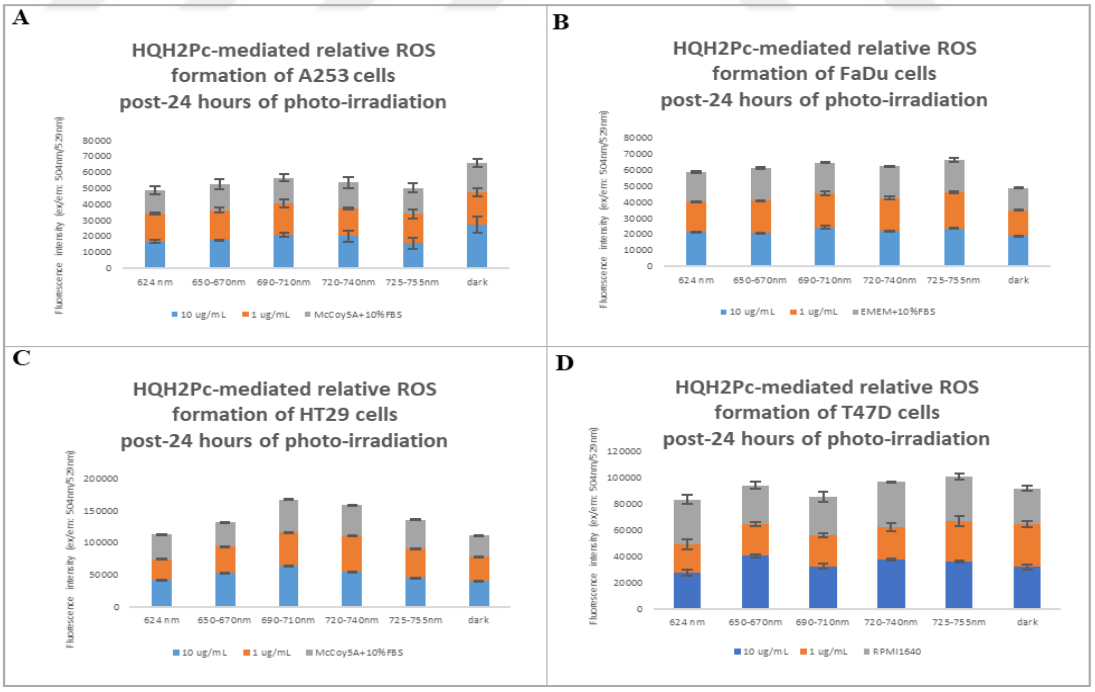



**Figure 58** Dose dependent post-24 hours photo-induced ROS formation of $HQH_2Pc$ phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines.

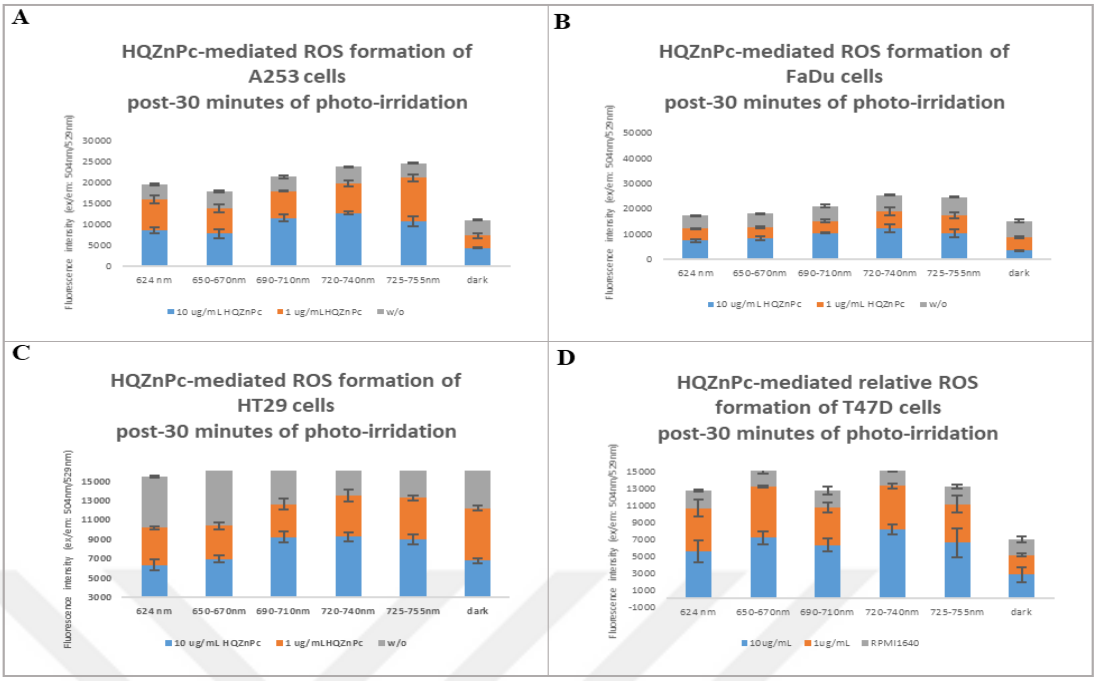



**Figure 59** Dose dependent post-30 minutes photo-induced ROS formation of HQZnPc phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines.

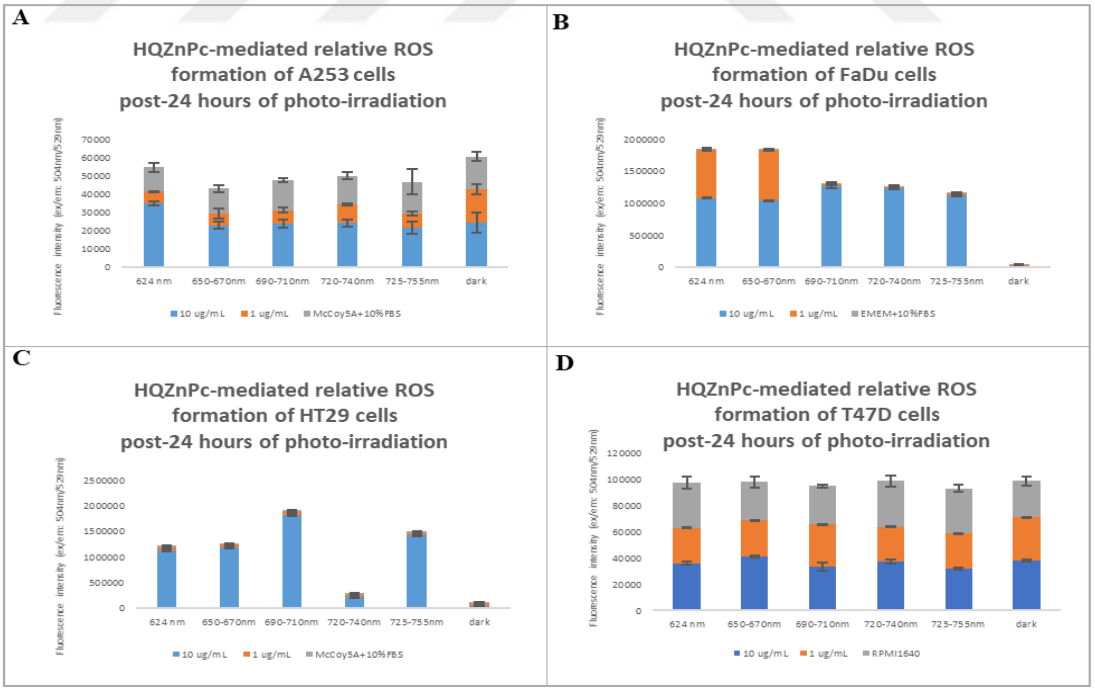



**Figure 60** Dose dependent post-24 hours photo-induced ROS formation of HQZnPc phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines.

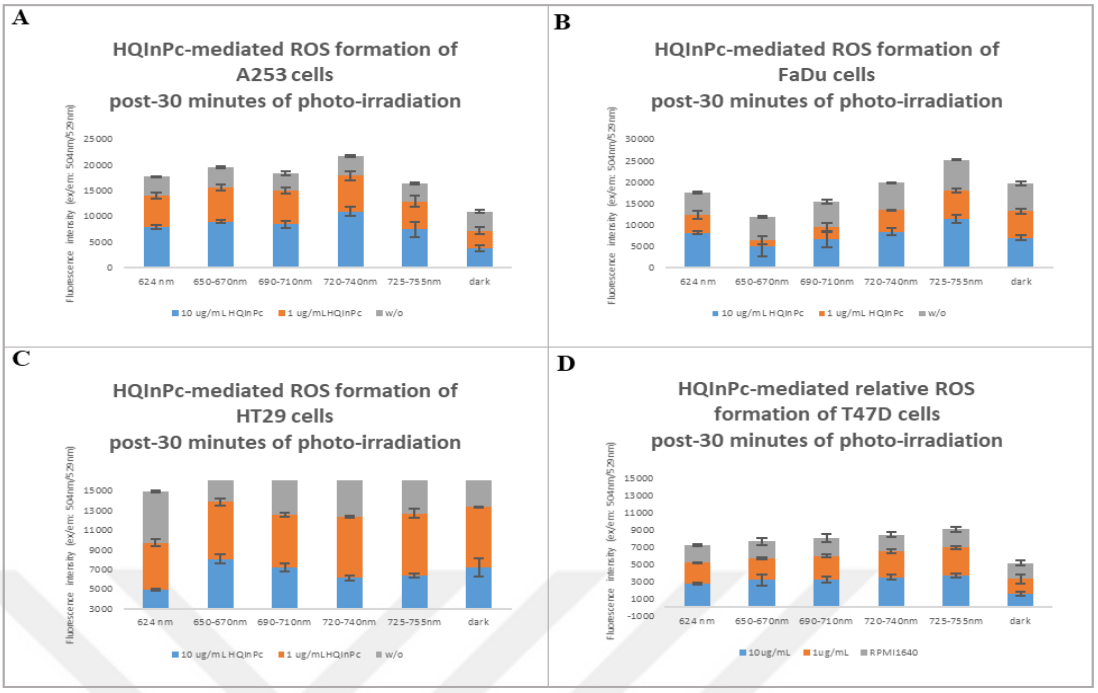



**Figure 61** Dose dependent post-30 minutes photo-induced ROS formation of HQInPc phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines.

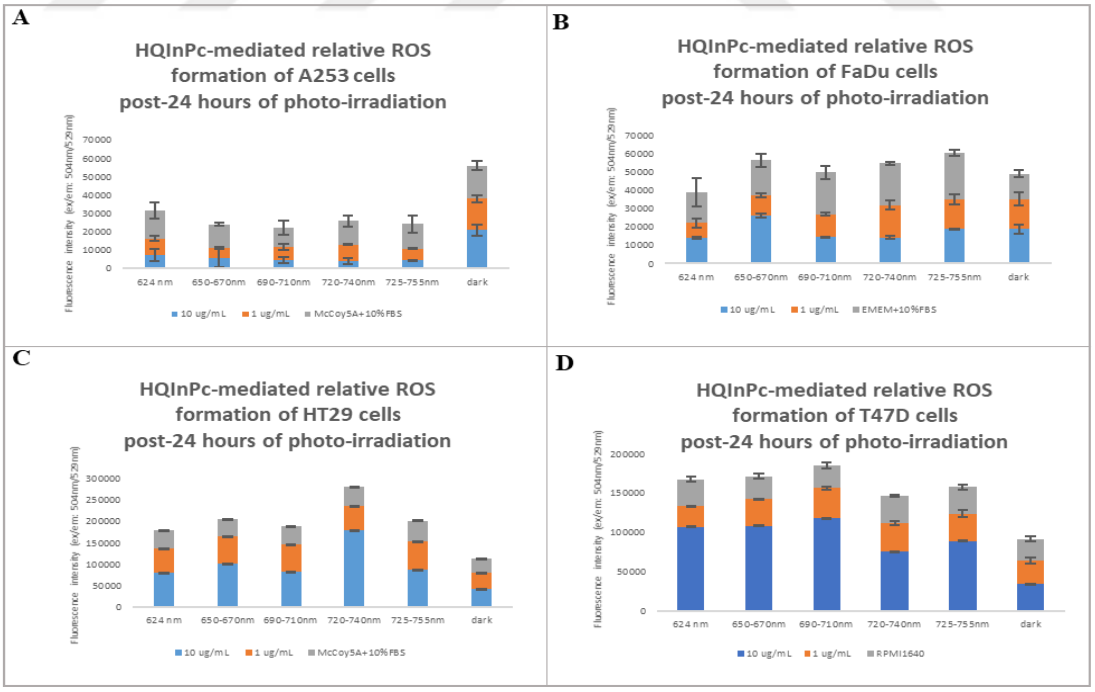



**Figure 62** Dose dependent post-24 hours photo-induced ROS formation of HQInPc phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines.

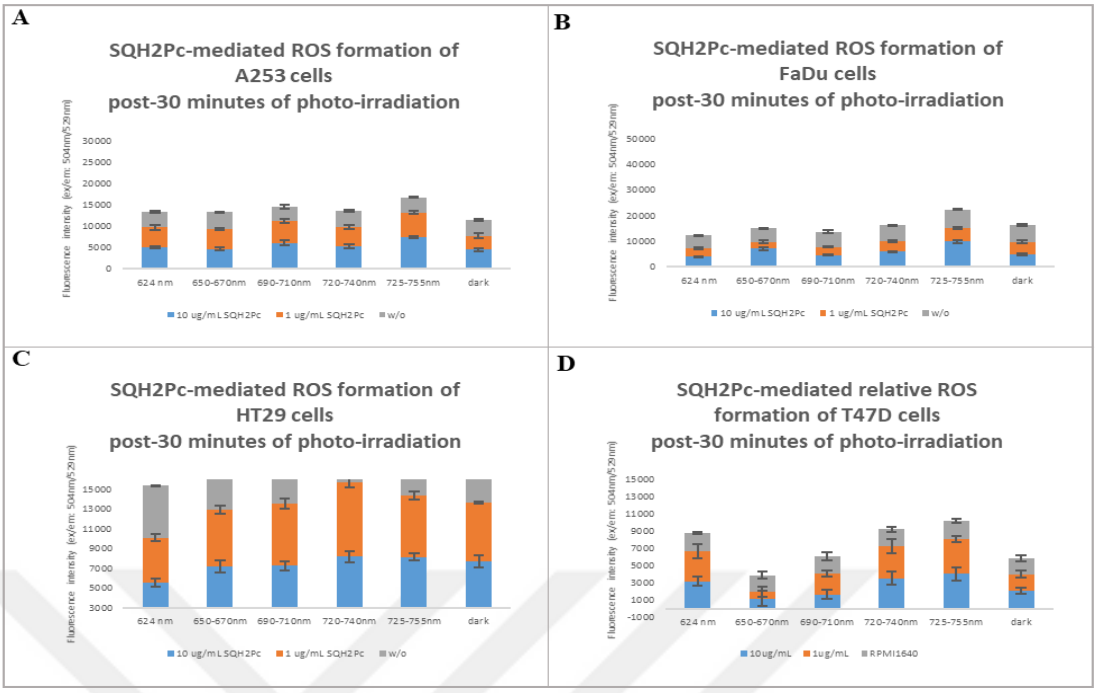



**Figure 63** Dose dependent post-30 minutes photo-induced ROS formation of $SQH_2Pc$ phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines.

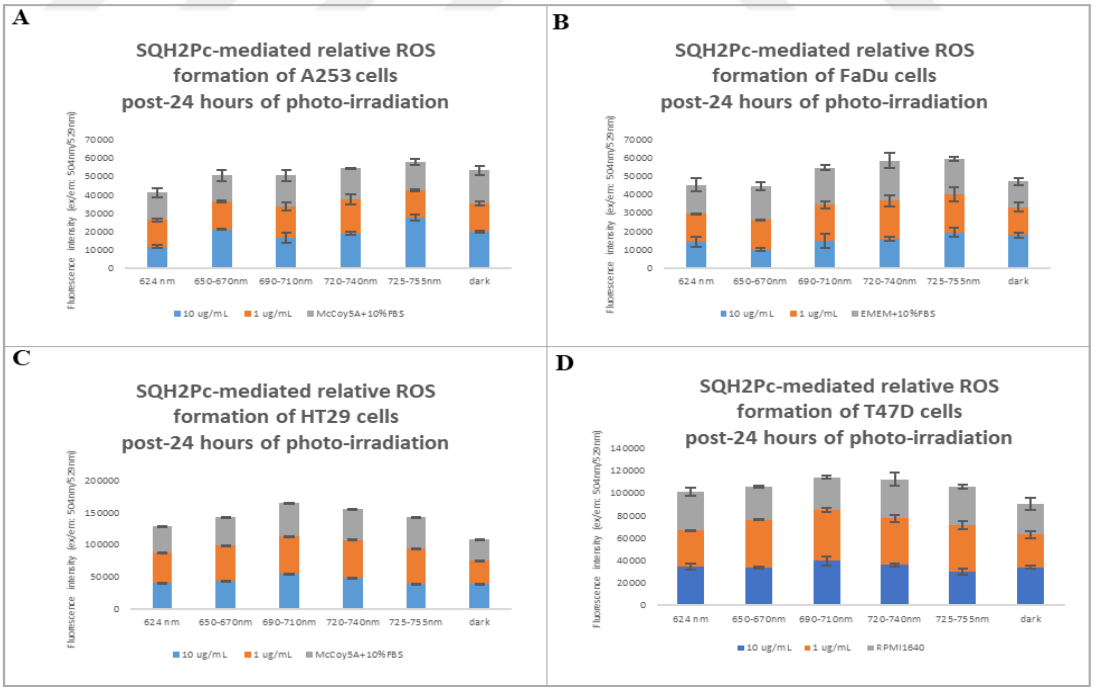



**Figure 64** Dose dependent post-24 hours photo-induced ROS formation of $SQH_2Pc$ phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines.

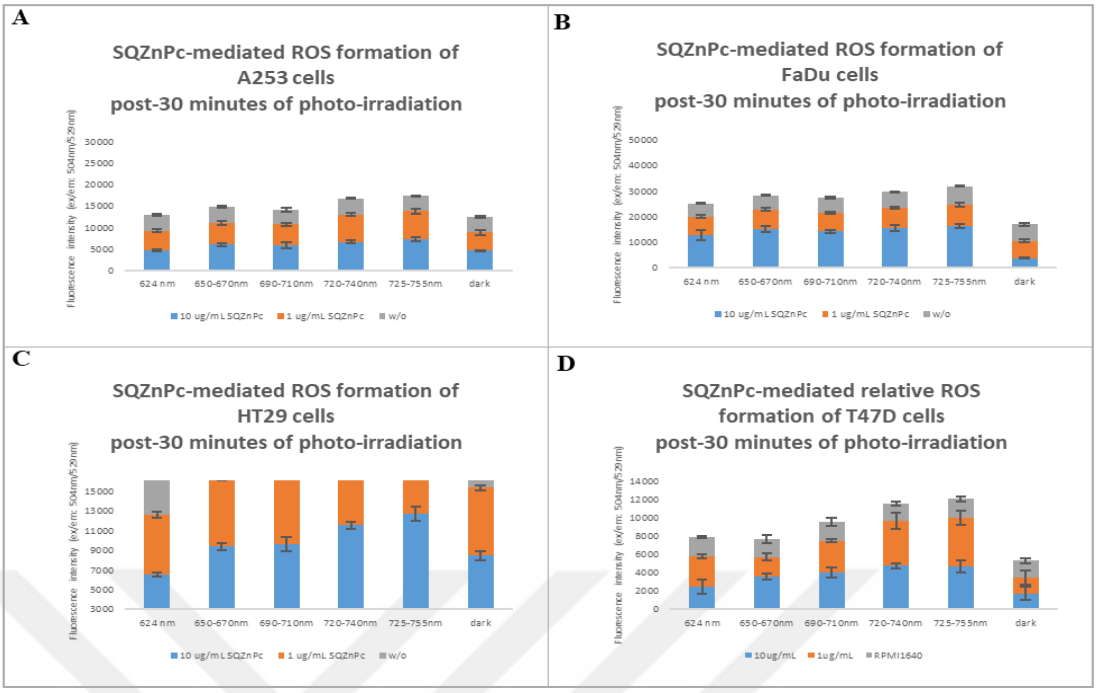



**Figure 65** Dose dependent post-30 minutes photo-induced ROS formation of SQZnPc phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines.

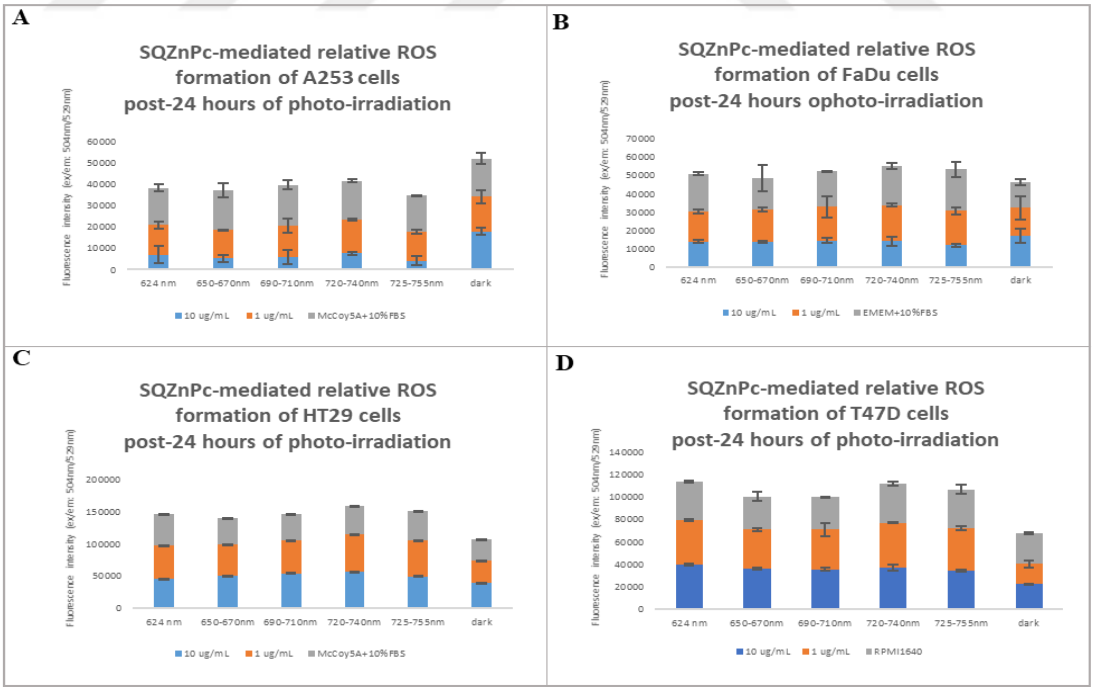



**Figure 66** Dose dependent post-24 hours photo-induced ROS formation of SQZnPc phthalocyanine on A. Salivary gland carcinoma, A253, B. Pharynx carcinoma, FaDu, C. Colorectal adenocarcinoma, HT29, D. Breast carcinoma, T47D cell lines.

## 4.8. Comparison of PDT-induced Cytotoxicity of Nine Phthalocyanine Molecules on Cancer Cell Lines

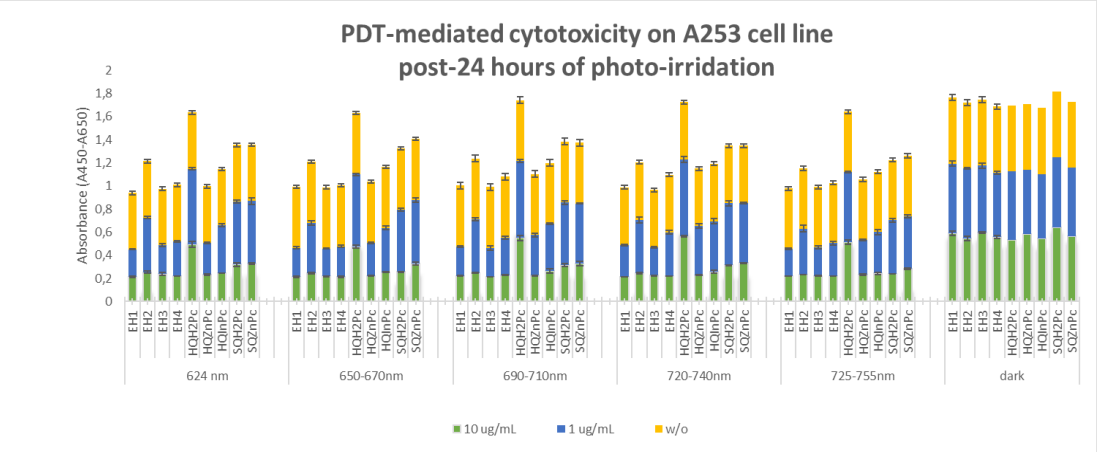



**Figure 67** PDT-mediated cytotoxicity of nine phthalocyanine molecules on A253 cell line.

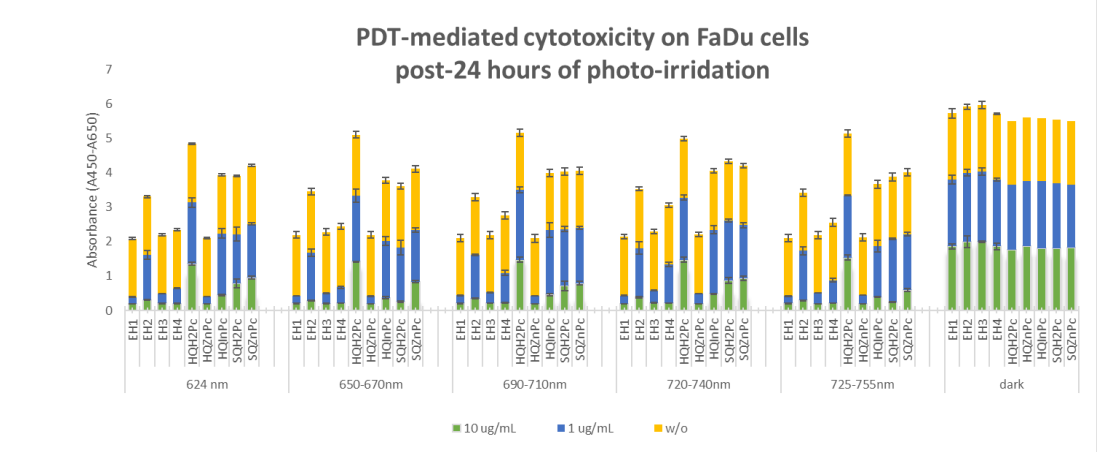



**Figure 68** PDT-mediated cytotoxicity of nine phthalocyanine molecules on FaDu cell line.

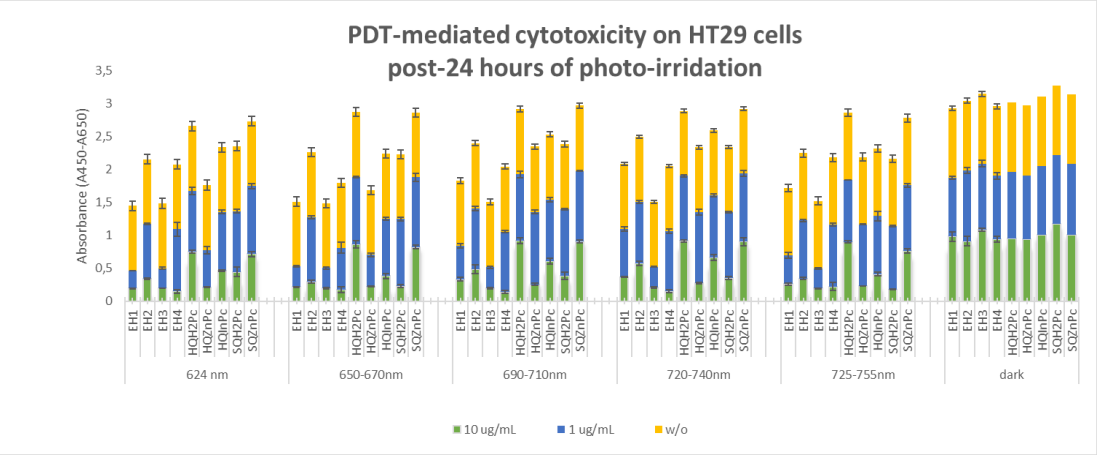



**Figure 69** PDT-mediated cytotoxicity of nine phthalocyanine molecules on HT29 cell line.

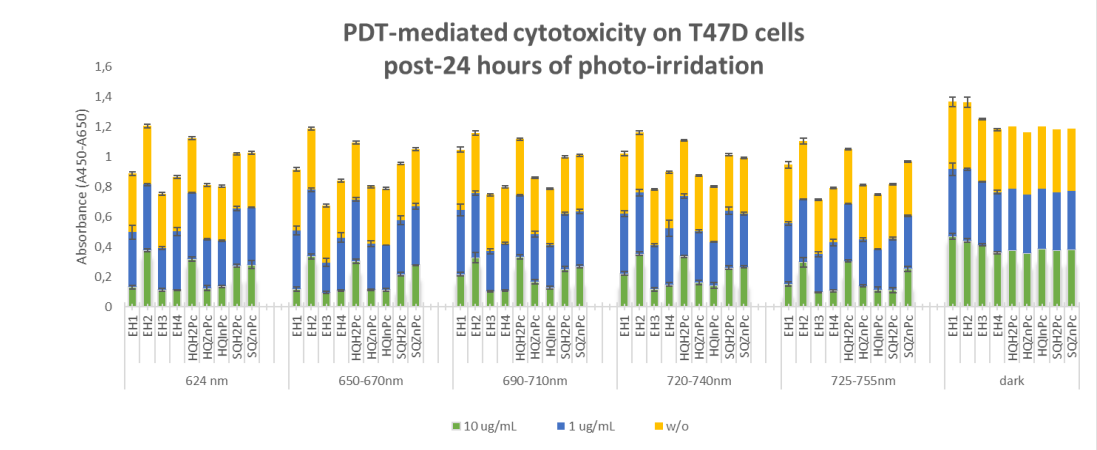



**Figure 70** PDT-mediated cytotoxicity of nine phthalocyanine molecules on T47D cell line.

Comparison of PDT-mediated cytotoxicity among nine phthalocyanine molecules revealed that the least cytotoxic response was obtained from the T47D cell line, and the highest cytotoxic response was obtained from FaDu cell line. When overall cytotoxicity after PDT was compared, from the highest to lowest cytotoxicity was orderly on FaDu, HT29, A253, T47D cell lines.

On the A253, FaDu, HT29 and T47D cell lines, EH1, EH3, EH4, and HQZnPc that was applied with a concentration of 10 µg/mL showed the highest cytotoxicity

followed by EH2, HQInPc, $SQH_2Pc$, and SQZnPc molecules. On T47D cell lines, different from the other cell lines, EH2 showed the lowest cytotoxicity. $HQH_2Pc$-mediated PDT did not show any cytotoxic response on the cell lines when it was applied with a concentration of 10 µg/ml.

EH1, EH3, EH4, and HQZnPc molecules when they were applied with a concentration of 1 µg/mL showed significant cytotoxicity on FaDu cell line, moderate cytotoxicity on A253 cell lines, whereas the other molecules did not show any cytotoxicity. EH1 and EH3 molecules when they were applied with a concentration of 1 µg/mL showed moderate cytotoxicity on HT29 cell line, whereas the other molecules did not show any cytotoxicity. On T47D cell line, the molecules did not show any cytotoxicity when they were applied with a concentration of 1 µg/ml. $HQH_2Pc$-mediated PDT did not show any cytotoxic response on the cell lines when it was applied with a concentration of 1 µg/ml.

## 4.9. Comparison of PDT-induced Immediate and Late ROS Production of Nine Phthalocyanine Molecules on Cancer Cell Lines

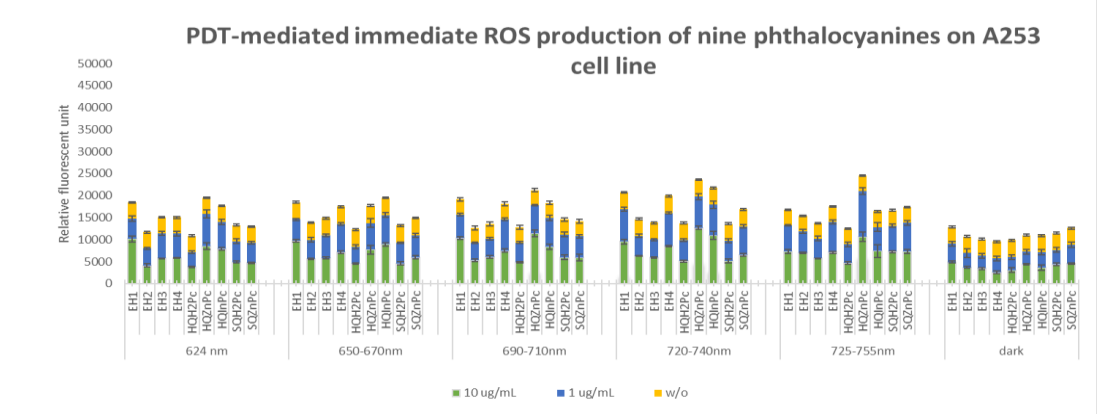



**Figure 71** PDT-mediated immediate ROS production of nine phthalocyanine molecules on A253 cell line

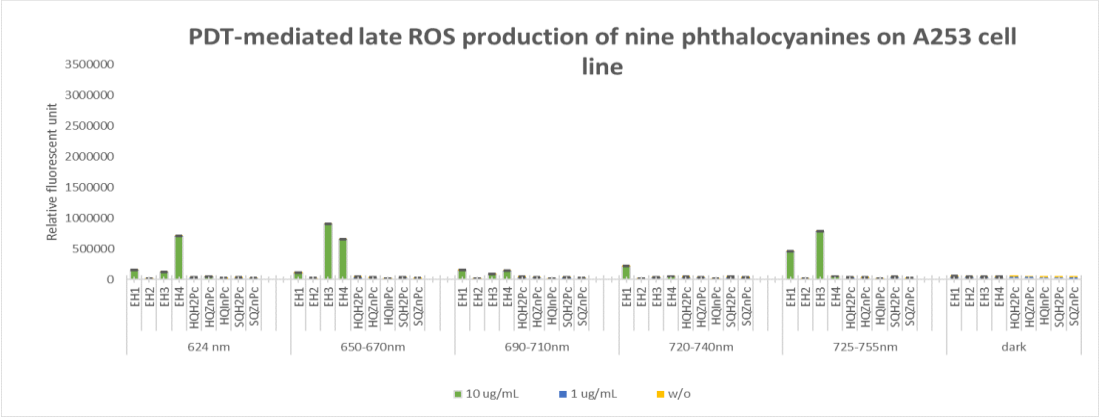



**Figure 72** PDT-mediated late ROS production of nine phthalocyanine molecules on A253 cell line

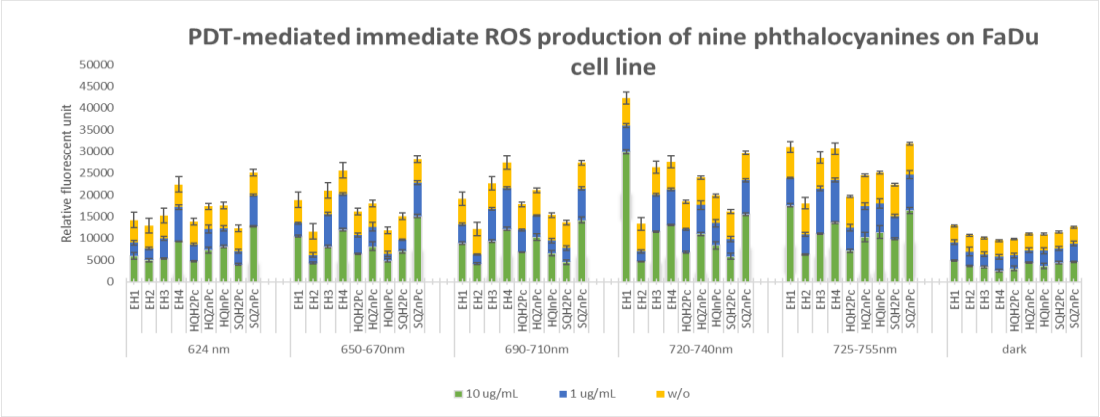



**Figure 73** PDT-mediated immediate ROS production of nine phthalocyanine molecules on FaDu cell line

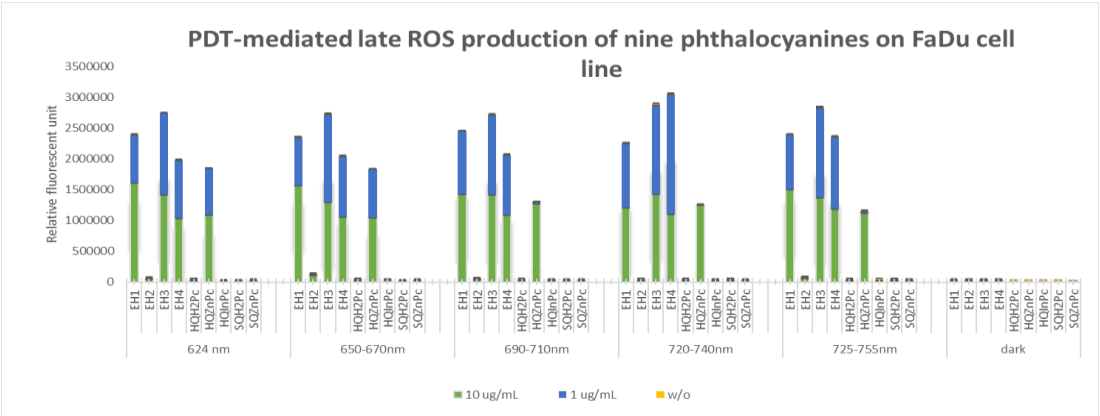



**Figure 74** PDT-mediated late ROS production of nine phthalocyanine molecules on FaDu cell line

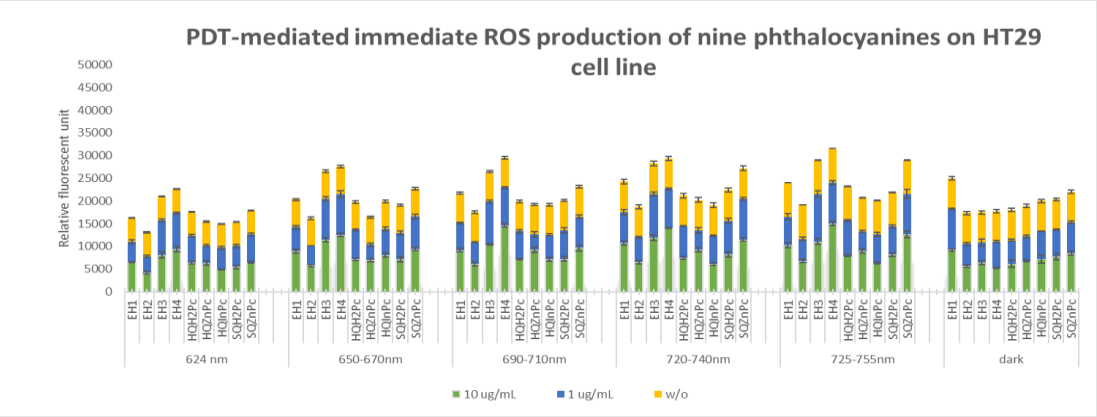



**Figure 75** PDT-mediated immediate ROS production of nine phthalocyanine molecules on HT29 cell line

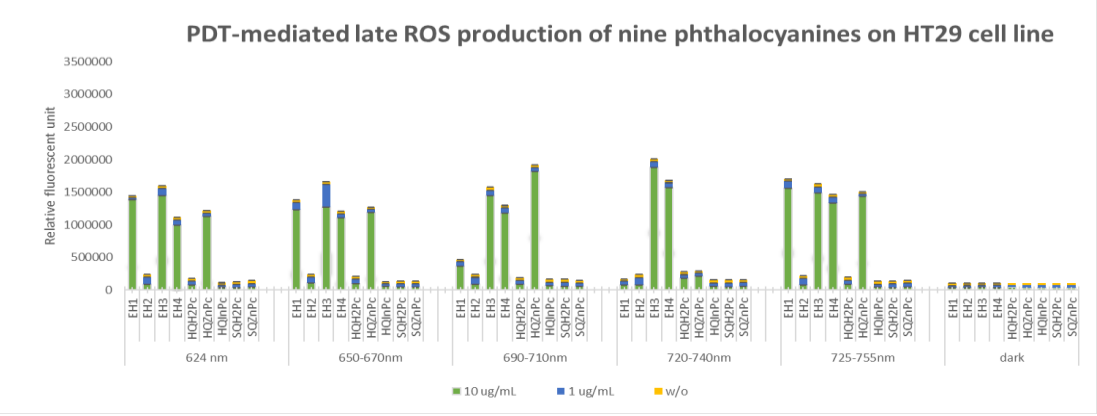



**Figure 76** PDT-mediated late ROS production of nine phthalocyanine molecules on HT29 cell line

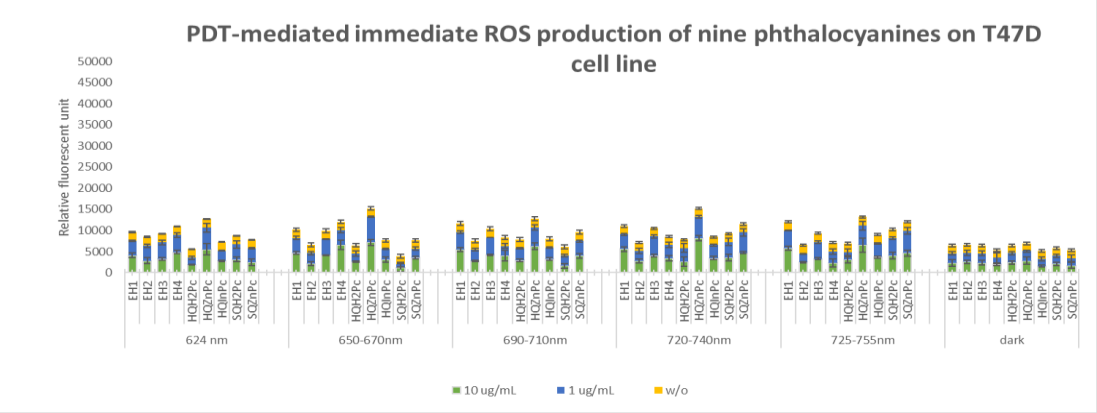



**Figure 77** PDT-mediated immediate ROS production of nine phthalocyanine molecules on T47D cell line

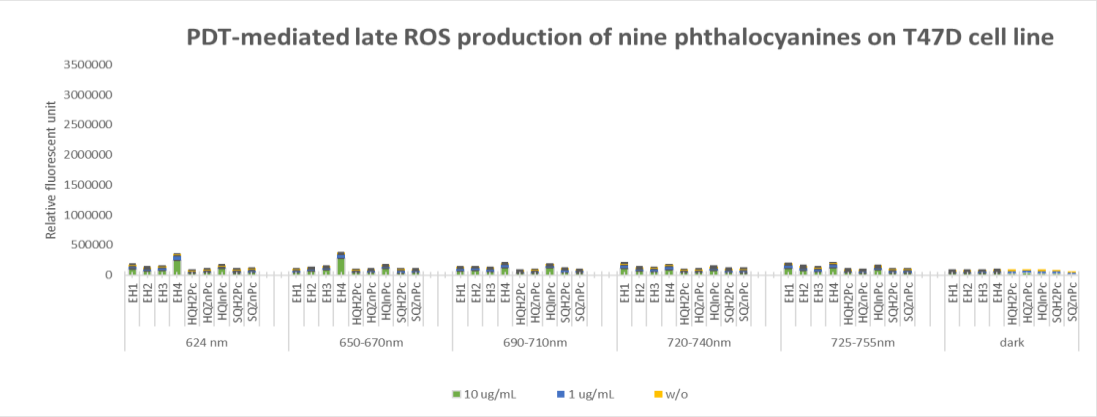



**Figure 78** PDT-mediated late ROS production of nine phthalocyanine molecules on T47D cell line

Comparison of PDT-mediated immediate ROS production among nine phthalocyanine molecules revealed that the least ROS production in terms of relative fluorescence unit was obtained from the T47D cell line, and the highest ROS production was obtained from FaDu cell line when it was applied with Pc concentration of 10 µg/ml. On the HT29 cell line, PDT of the molecules did not lead to immediate ROS production since yielding similar RFU values compared to dark control groups.

Comparison of PDT-mediated late ROS production among nine phthalocyanine molecules revealed that the least ROS production in terms of relative fluorescence unit was obtained from the T47D cell line, and the highest ROS production was obtained from FaDu cell line.

When overall ROS production after PDT was compared, from the highest to the lowest ROS production was orderly on FaDu, HT29, A253, T47D cell lines. ROS amount produced through all cancer cell lines in the dark was very low compared to the PDT-mediated ROS amount following 24 hours of light irradiation.

PDT-induced late ROS production of A253 cell lines varied across different wavelengths. Following 24 hours of PDT, the highest amount of ROS produced at 624 nm by EH4 molecule, at 650-670 nm by EH3 and EH4 molecules, and at 725-755 nm by EH2 and EH3 molecule when 10 μg / ml concentrations of the molecules were applied.

On the FaDu cell line, EH1, EH3, EH4, and HQZnPc molecules triggered the highest ROS production following 24 hours of PDT at all wavelengths that were applied. However, PDT with both concentration of HQZnPc molecule had a certain increase in ROS at 624 nm and 650-670 nm, while reducing ROS production at 690-710 nm, 720-740 nm, and 725-755nm when it was applied with a concentration of 1 μg/ml.

On the HT29 cell line, EH1, EH3, EH4, and HQZnPc molecules triggered the highest ROS production following 24 hours of PDT at all wavelengths that were applied.

On the T47D cell line, EH4 molecule triggered the highest ROS production following 24 hours of PDT when it was applied with a concentration of 10 μg/ml. However, among cell lines, the lowest ROS production was obtained from the T47D cell line.

## 4.10. Tables of Results

### 4.10.1. Tables of dark cytotoxicity

In this section, cytotoxic effects of the Pc molecules on the cell lines at dark conditions were revealed as percentage value relative to non-treated controls.

**Table 11** Relative percentage cell viability of cancer cell lines following 24 hours of EH1 phthalocyanine administrations at dark.

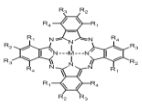
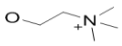
| EH1 | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|
|  | 100 µg/ml | 89 | 83 | 109 | 65 |
| | 10 µg/ml | 109 | 79 | 103 | 79 |
| Center metal Zn | 1 µg/ml | 102 | 78 | 107 | 91 |
| R2  | 0,1 µg/ml | 102 | 84 | 93 | 87 |

**Table 12** Relative percentage cell viability of cancer cell lines following 24 hours of EH2 phthalocyanine administrations at dark.

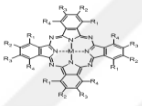
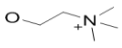
| EH2 | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|
|  | 100 µg/ml | 83 | 83 | 86 | 62 |
| | 10 µg/ml | 91 | 83 | 89 | 71 |
| Center metal Zn | 1 µg/ml | 98 | 75 | 85 | 70 |
| R1  | 0,1 µg/ml | 102 | 81 | 88 | 74 |

**Table 13** Relative percentage cell viability of cancer cell lines following 24 hours of EH3 phthalocyanine administrations at dark.

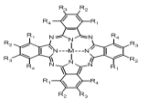
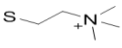
| EH3 | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|
|  | 100 µg/ml | 95 | 83 | 93 | 93 |
| | 10 µg/ml | 94 | 79 | 81 | 106 |
| Center metal Zn | 1 µg/ml | 98 | 82 | 87 | 103 |
| R1  | 0,1 µg/ml | 92 | 83 | 74 | 111 |

**Table 14** Relative percentage cell viability of cancer cell lines following 24 hours of EH4 phthalocyanine administrations at dark.

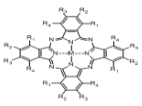
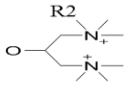
| EH4 | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|
|  | 100 µg/ml | 89 | 74 | 80 | 61 |
| | 10 µg/ml | 97 | 74 | 77 | 74 |
| Center metal Zn | 1 µg/ml | 96 | 72 | 76 | 71 |
|  | 0,1 µg/ml | 95 | 75 | 74 | 78 |

**Table 15** Relative percentage cell viability of cancer cell lines following 24 hours of $HQH_2Pc$ phthalocyanine administrations at dark.

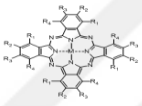
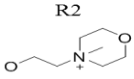
| HQH2Pc | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|
|  | 100 µg/ml | 69 | 83 | 83 | 53 |
| | 10 µg/ml | 80 | 81 | 96 | 72 |
| Center metal 2H | 1 µg/ml | 102 | 76 | 102 | 70 |
|  | 0,1 µg/ml | 98 | 79 | 98 | 80 |

**Table 16** Relative percentage cell viability of cancer cell lines following 24 hours of HQZnPc phthalocyanine administrations at dark.

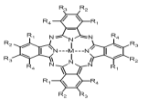
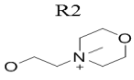
| HQZnPc | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|
|  | 100 µg/ml | 69 | 72 | 73 | 75 |
| | 10 µg/ml | 87 | 74 | 89 | 78 |
| Center metal Zn | 1 µg/ml | 90 | 74 | 81 | 89 |
|  | 0,1 µg/ml | 94 | 82 | 90 | 78 |

**Table 17** Relative percentage cell viability of cancer cell lines following 24 hours of HQInPc phthalocyanine administrations at dark.

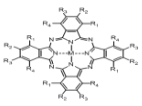
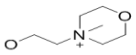
| HQInPc | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|
|  | 100 µg/ml | 90 | 88 | 83 | 112 |
| | 10 µg/ml | 95 | 91 | 82 | 119 |
| Center metal InCl | 1 µg/ml | 97 | 93 | 100 | 107 |
| R2  | 0,1 µg/ml | 96 | 92 | 121 | 98 |

**Table 18** Relative percentage cell viability of cancer cell lines following 24 hours of $SQH_2Pc$ phthalocyanine administrations at dark.

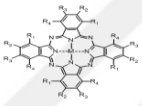
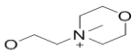
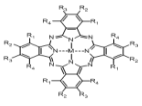
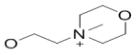
| SQH2Pc | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|
|  | 10 µg/ml<br>1 µg/ml | 100 | 65 | 93 | 75 |
| | 10 µg/ml<br>1 µg/ml | 98 | 71 | 71 | 80 |
| Center metal 2H | 10 µg/ml<br>1 µg/ml | 100 | 72 | 88 | 80 |
| R1  | 10 µg/ml<br>1 µg/ml | 97 | 75 | 84 | 89 |

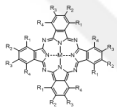
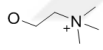
**Table 19** Relative percentage cell viability of cancer cell lines following 24 hours of SQZnPc phthalocyanine administrations at dark.

| SQZnPc | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|
|  | 100 µg/ml | 93 | 87 | 113 | 79 |
| | 10 µg/ml | 94 | 89 | 102 | 90 |
| Center metal Zn | 1 µg/ml | 102 | 91 | 107 | 90 |
| R1  | 0,1 µg/ml | 104 | 92 | 106 | 84 |

### 4.10.2. Comparative table of experiments with EH1 phthalocyanine

In this section, overall results obtained from EH1-mediated PDT with five different wavelengths were revealed as percentage value relative to non-treated controls.

**Table 20** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with EH1 phthalocyanine at 624 nm (light fluence of 2 joules/cm$^2$).

| EH1 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal Zn R2  | Cytotoxicity (WST-1) | 624 nm | 10 µg/ml | 37 | 10 | 18 | 30 |
| | | | 1 µg/ml | 42 | 11 | 26 | 81 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 25 | 7 | 28 | 22 |
| | | | 1 µg/ml | 44 | 5 | 70 | 64 |
| | ROS (30 minutes) | | 10 µg/ml | 269 | 157 | 98 | 220 |
| | | | 1 µg/ml | 123 | 81 | 67 | 185 |
| | ROS (24 hours) | | 10 µg/ml | 821 | 11492 | 4091 | 361 |
| | | | 1 µg/ml | 48 | 5624 | 112 | 164 |

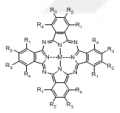
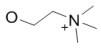
At wavelength of 624 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the EH1 molecule was measured in each cancer cell line but in different degrees. According to WST-1 assay, the most affected cell line was FaDu cell line where both high and low doses of EH1 molecule lead to appx. 90% cell death relative to the non-treated control. Other cell lines, HT29 and A253 cell lines were significantly affected by photodynamic therapy of both high and low doses of EH1 molecule, while the T47D cell line only affected from photodynamic therapy of high dose of EH1 molecule, and not affected from low lose.

Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general

trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

EH-1 induced immediate ROS production was seen by both high and low dose of the molecule on A253 and T47D cell line, only high dose on FaDu cell line, and there were not ROS production of HT29 cell line. When to look at the late ROS production following PDT, EH1-induced PDT triggered ROS production on each cell line, except A253 cell line when applied low dose of the molecule. The highest ROS production was measured from the FaDu cell line, followed by HT29, T47D and A253 cell lines, respectively.

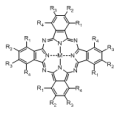
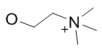
**Table 21** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with EH1 phthalocyanine at 650-670 nm (light fluence of 2 joules/cm$^2$).

| EH1 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal Zn R2  | Cytotoxicity (WST-1) | 650-670 nm | 10 µg/ml | 37 | 11 | 20 | 26 |
| | | | 1 µg/ml | 44 | 12 | 30 | 87 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 25 | 7 | 25 | 48 |
| | | | 1 µg/ml | 42 | 5 | 58 | 92 |
| | ROS (30 minutes) | | 10 µg/ml | 259 | 278 | 134 | 251 |
| | | | 1 µg/ml | 128 | 79 | 78 | 194 |
| | ROS (24 hours) | | 10 µg/ml | 533 | 11162 | 3641 | 154 |
| | | | 1 µg/ml | 52 | 5631 | 354 | 118 |

At wavelength of 650-670 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the EH1 molecule was measured in each cancer cell line except T47D cell line when it was applied with low dose of the molecule. According to the assays, the most affected cell line was FaDu cell line where both high and low doses of EH1 molecule lead to appx. 90% cell death relative to the non-treated control. Other cell lines, HT29 and A253 cell lines were significantly affected by photodynamic therapy of both high and low doses of EH1 molecule, while the T47D

cell line only affected from photodynamic therapy of high dose of EH1 molecule, and not affected from low lose. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay. EH-1 induced immediate ROS production was seen on each cell line in different degrees except FaDu and HT29 cell line when it was applied by low dose of the molecule. The highest amount of ROS was seen on the FaDu cell line caused by high dose of the molecule, it was followed by A253, T47D and HT29 cell lines, respectively. When to look at the late ROS production following PDT, EH1-induced PDT triggered ROS production on each cell line except A253 and T47D cell lines when were applied by low dose of the molecule. The highest ROS production was measured from the FaDu cell line, also HT29, A253, and T47D cell lines, respectively.

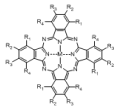
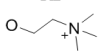
**Table 22** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with EH1 phthalocyanine at 690-710 nm (light fluence of 2 joules/cm$^2$).

| EH1 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal Zn R2  | Cytotoxicity (WST-1) | 690-710 nm | 10 µg/ml | 39 | 11 | 32 | 48 |
| | | | 1 µg/ml | 44 | 13 | 48 | 96 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 26 | 12 | 56 | 40 |
| | | | 1 µg/ml | 56 | 10 | 76 | 87 |
| | ROS (30 minutes) | | 10 µg/ml | 273 | 237 | 137 | 298 |
| | | | 1 µg/ml | 143 | 114 | 90 | 222 |
| | ROS (24 hours) | | 10 µg/ml | 787 | 10216 | 1083 | 222 |
| | | | 1 µg/ml | 34 | 7318 | 204 | 172 |

At wavelength of 690-710 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the EH1 molecule was measured in each cancer cell line except T47D cell line when it was applied by low dose of the molecule. According to both WST-1 and resazurin assays, the most affected cell line was FaDu cell line where both high and low doses of EH1 molecule lead to appx. 90% cell death relative to the

non-treated control. Other cell lines, HT29 and A253 cell lines were significantly affected by photodynamic therapy of both high and low doses of EH1 molecule, while the T47D cell line only affected from photodynamic therapy of high dose of EH1 molecule, and not affected from low lose. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay. EH-1 induced immediate ROS production was seen on each cell line when ere applied by high dose of the molecule. The highest amount of ROS was seen on the T47D cell line, it was followed by A253, FaDu, and HT29 cell lines, respectively. When to look at the late ROS production following PDT, EH1-induced PDT triggered ROS production on each cell line. The highest ROS production was measured from the FaDu cell line, also, HT29, A253 and T47D cell lines, respectively. The least ROS production was seen on the A253 cell line, even a low dose of EH1 did not trigger ROS on the A253 cell line.

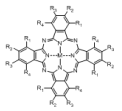
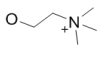
**Table 23** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with EH1 phthalocyanine at 720-740 nm (light fluence of 2 joules/cm$^2$).

| EH1 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal Zn R2  | Cytotoxicity (WST-1) | 720-740 nm | 10 µg/ml | 37 | 11 | 35 | 49 |
| | | | 1 µg/ml | 48 | 13 | 69 | 89 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 24 | 12 | 71 | 25 |
| | | | 1 µg/ml | 66 | 15 | 82 | 73 |
| | ROS (30 minutes) | | 10 µg/ml | 253 | 793 | 161 | 302 |
| | | | 1 µg/ml | 194 | 159 | 101 | 189 |
| | ROS (24 hours) | | 10 µg/ml | 1160 | 8640 | 241 | 396 |
| | | | 1 µg/ml | 32 | 7469 | 163 | 204 |

At wavelength of 720-740 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the EH1 molecule was measured in each cancer cell line but in different degrees. According to both WST-1 and resazurin assay, the most

affected cell line was FaDu cell line where both high and low doses of EH1 molecule lead to appx. 90% cell death relative to the non-treated control. Other cell lines, A253, HT29, and T47D cell lines were significantly affected by photodynamic therapy of only high dose of EH1 molecule, while they were not affected from PDT with low of the molecule. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay. EH-1 induced immediate ROS production was seen on each cell line in different degrees. The highest amount of ROS was seen on the FaDu cell line, it was followed by T47D, A253, and HT29 cell lines, respectively. Low dose of the molecule did not trigger HT29 cell line to produce ROS immediately. When to look at the late ROS production following PDT, EH1-induced PDT triggered ROS production on each cell line. The highest ROS production was measured from the FaDu cell line, also A253, T47D and HT29 cell lines, respectively. The least ROS production was seen on the A253 cell line, even a low dose of EH1 did not trigger ROS on the A253 cell line.

**Table 24** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with EH1 phthalocyanine at 725-755 nm (light fluence of 2 joules/cm$^2$).

| EH1 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal Zn R2  | Cytotoxicity (WST-1) | 725-755 nm | 10 µg/ml | 38 | 11 | 25 | 34 |
| | | | 1 µg/ml | 41 | 12 | 41 | 90 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 25 | 9 | 40 | 23 |
| | | | 1 µg/ml | 51 | 8 | 76 | 72 |
| | ROS (30 minutes) | | 10 µg/ml | 194 | 466 | 154 | 313 |
| | | | 1 µg/ml | 156 | 166 | 94 | 223 |
| | ROS (24 hours) | | 10 µg/ml | 2522 | 10718 | 4616 | 378 |
| | | | 1 µg/ml | 45 | 6441 | 332 | 205 |

At wavelength of 725-755 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the EH1 molecule was measured in each cancer cell line

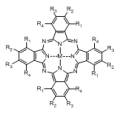
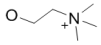
but in different degrees. According to WST-1 assay, the most affected cell line was FaDu cell line where both high and low doses of EH1 molecule lead to appx. 90% cell death relative to the non-treated control. Other cell lines, HT29 and A253 cell lines were significantly affected by photodynamic therapy of both high and low doses of EH1 molecule, while the T47D cell line only affected from photodynamic therapy of high dose of EH1 molecule, and not affected from low lose. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

EH-1 induced immediate ROS production was seen on each cell line in different degrees. The highest amount of ROS was seen on the FaDu cell line, it was followed by T47D, A253, and HT29 cell lines, respectively. When to look at the late ROS production following PDT, EH1-induced PDT triggered ROS production on each cell line. The highest ROS production was measured from the FaDu cell line, also T47D, HT29, and A253 cell lines, respectively. The least Ros production was seen on the A253 cell line, even a low dose of EH1 did not trigger ROS on the A253 cell line.

### 4.10.3. Comparative table of experiments with EH2 phthalocyanine

In this section, overall results obtained from EH2-mediated PDT with five different wavelengths were collected as percentage relative to non-treated control. Each data was revealed as % value.

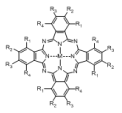
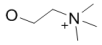
**Table 25** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with EH2 phthalocyanine at 624 nm (light fluence of 2 joules/cm$^2$).

| EH2 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal Zn R1  | Cytotoxicity (WST-1) | 624 nm | 10 µg/ml | 44 | 17 | 32 | 84 |
| | | | 1 µg/ml | 83 | 71 | 79 | 98 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 42 | 28 | 75 | 41 |
| | | | 1 µg/ml | 99 | 78 | 71 | 88 |
| | ROS (30 minutes) | | 10 µg/ml | 110 | 132 | 63 | 151 |
| | | | 1 µg/ml | 102 | 72 | 54 | 195 |
| | ROS (24 hours) | | 10 µg/ml | 52 | 270 | 253 | 235 |
| | | | 1 µg/ml | 49 | 163 | 341 | 129 |

At wavelength of 624 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the EH2 molecule was measured in each cancer cell line except T47D cell line for the high dose of EH2 molecule, and there was no effect was shown for the low dose of the molecule. According to WST-1 assay, the most affected cell line was FaDu cell line, followed by HT29, and A253 cell lines. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

Right after PDT, either high or low doses of EH2 molecule did not trigger ROS formation on the A253, FaDu, and HT29 cell lines. However, high dose of the molecule triggered immediate ROS formation on T47D cell line. Following PDT, late ROS formation was triggered with PDT of both doses of EH2 molecule on each cell line except A253 cell line. Compared to the results, it can be said that the cytotoxic responses of FaDu, HT29, and T47D cell lines were ROS dependent, however cytotoxic response of A253 cell line was not ROS-dependent.

**Table 26** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with EH2 phthalocyanine at 650-670 nm (light fluence of 2 joules/cm$^2$).

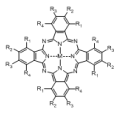
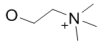
| EH2 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal Zn R1  | Cytotoxicity (WST-1) | 650-670 nm | 10 µg/ml | 43 | 15 | 28 | 75 |
| | | | 1 µg/ml | 76 | 76 | 92 | 99 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 37 | 18 | 70 | 36 |
| | | | 1 µg/ml | 98 | 82 | 75 | 82 |
| | ROS (30 minutes) | | 10 µg/ml | 149 | 115 | 86 | 116 |
| | | | 1 µg/ml | 115 | 49 | 65 | 133 |
| | ROS (24 hours) | | 10 µg/ml | 61 | 754 | 331 | 232 |
| | | | 1 µg/ml | 43 | 145 | 271 | 128 |

At wavelength of 650-670 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the EH2 molecule was measured on A253, FaDu, and HT29 cell lines for the high dose of EH2 molecule, and there was no effect was shown for the low dose of the molecule. According to WST-1 assay, the most affected cell line from EH2-mediated PDT was FaDu cell line, followed by HT29, and A253 cell lines.

Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

Right after PDT, high dose of EH2 molecule triggered ROS formation on the A253, FaDu, and both high and low dose triggered ROS formation onT47D cell lines. Following PDT, late ROS formation was triggered with PDT of both doses of EH2 molecule on each cell line except A253 cell line. Compared to the results, it can be said that the cytotoxic responses of FaDu, HT29, and T47D cell lines were ROS dependent, however cytotoxic response of A253 cell line was not ROS-dependent.

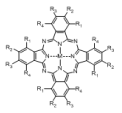
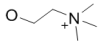
**Table 27** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with EH2 phthalocyanine at 690-710 nm (light fluence of 2 joules/cm$^2$).

| EH2 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal Zn R1  | Cytotoxicity (WST-1) | 690-710 nm | 10 µg/ml | 43 | 19 | 46 | 73 |
| | | | 1 µg/ml | 81 | 69 | 87 | 96 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 46 | 31 | 78 | 37 |
| | | | 1 µg/ml | 89 | 88 | 76 | 86 |
| | ROS (30 minutes) | | 10 µg/ml | 140 | 110 | 91 | 149 |
| | | | 1 µg/ml | 104 | 56 | 72 | 146 |
| | ROS (24 hours) | | 10 µg/ml | 25 | 219 | 268 | 274 |
| | | | 1 µg/ml | 52 | 135 | 311 | 141 |

At wavelength of 690-710 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the EH2 molecule was measured in each cancer cell line except T47D cell line for the high dose of EH2 molecule, and there was no effect was shown for the low dose of the molecule. According to WST-1 assay, the most affected cell line was FaDu cell line, followed by HT29, and A253 cell lines. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

Right after PDT, high doses of EH2 molecule triggered immediate ROS formation on the A253, FaDu, and both dose of the molecule triggered ROS formation on T47D cell lines. However, neither low nor high dose of the molecule did not trigger immediate ROS formation on HT29 cell line. Following PDT, late ROS formation was triggered with PDT of both doses of EH2 molecule on each cell line except A253 cell line. Compared to the results, it can be said that the cytotoxic responses of FaDu, HT29, and T47D cell lines were ROS dependent, however cytotoxic response of A253 cell line was not ROS-dependent.

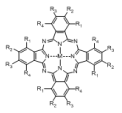
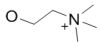
**Table 28** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with EH2 phthalocyanine at 720-740nm (light fluence of 2 joules/cm$^2$).

| EH2 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal Zn R1  | Cytotoxicity (WST-1) | 720-740 nm | 10 µg/ml | 43 | 21 | 54 | 79 |
| | | | 1 µg/ml | 81 | 78 | 88 | 91 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 48 | 44 | 82 | 50 |
| | | | 1 µg/ml | 101 | 86 | 76 | 86 |
| | ROS (30 minutes) | | 10 µg/ml | 167 | 123 | 98 | 152 |
| | | | 1 µg/ml | 120 | 62 | 82 | 129 |
| | ROS (24 hours) | | 10 µg/ml | 40 | 161 | 238 | 214 |
| | | | 1 µg/ml | 44 | 128 | 337 | 151 |

At wavelength of 720-740 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the EH2 molecule was measured in each cancer cell line except T47D cell line for the high dose of EH2 molecule, and there was no effect was shown for the low dose of the molecule. According to WST-1 assay, the most affected cell line was FaDu cell line, followed by HT29, and A253 cell lines. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

Right after PDT, high doses of EH2 molecule triggered immediate ROS formation on the FaDu cell line, and both dose of the molecule triggered ROS formation on A253 and T47D cell lines. However, neither low nor high dose of the molecule did not trigger immediate ROS formation on HT29 cell line. Following PDT, late ROS formation was triggered with PDT of both doses of EH2 molecule on each cell line except A253 cell line. Compared to the results, it can be said that the cytotoxic responses of FaDu, HT29, and T47D cell lines were ROS dependent, however cytotoxic response of A253 cell line was not ROS-dependent.

**Table 29** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with EH2 phthalocyanine at 725-755 nm (light fluence of 2 joules/cm$^2$).

| EH2 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal Zn R1  | Cytotoxicity (WST-1) | 725-755 nm | 10 µg/ml | 41 | 16 | 32 | 66 |
| | | | 1 µg/ml | 70 | 79 | 83 | 93 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 36 | 25 | 77 | 31 |
| | | | 1 µg/ml | 98 | 82 | 78 | 82 |
| | ROS (30 minutes) | | 10 µg/ml | 187 | 168 | 101 | 135 |
| | | | 1 µg/ml | 129 | 121 | 74 | 105 |
| | ROS (24 hours) | | 10 µg/ml | 69 | 344 | 237 | 281 |
| | | | 1 µg/ml | 32 | 148 | 294 | 140 |

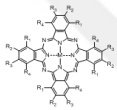
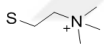
At wavelength of 725-755 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the EH2 molecule was measured in each cancer cell line for the high dose of EH2 molecule, and there was no effect was shown for the low dose of the molecule. According to WST-1 assay, the most affected cell line was FaDu cell line, followed by HT29, A253, and T47D cell lines. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

Right after PDT, either high or low doses of EH2 molecule did not trigger ROS formation on the cell lines. Following PDT, low dose of EH2 did not lead to late ROS formation, while high dose of EH2 induced late ROS production on each cell line in different degrees. The highest amount of ROS was seen on the T47D cell line, it was followed by FaDu, HT29, and A253 cell lines, respectively. Compared to the results, it can be said that the cytotoxic responses of each cell line were ROS dependent.

### 4.10.4. Comparative table of experiments with EH3 phthalocyanine

In this section, overall results obtained from EH3-mediated PDT with five different wavelengths were collected as percentage relative to non-treated control. Each data was revealed as % value.

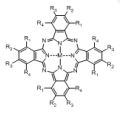
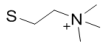
**Table 30** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with EH3 phthalocyanine at 624 nm (light fluence of 2 joules/cm$^2$).

| EH3 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal Zn R1  | Cytotoxicity (WST-1) | 624 nm | 10 µg/ml | 41 | 11 | 19 | 28 |
| | | | 1 µg/ml | 44 | 16 | 29 | 66 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 22 | 8 | 24 | 30 |
| | | | 1 µg/ml | 57 | 11 | 64 | 84 |
| | ROS (30 minutes) | | 10 µg/ml | 151 | 141 | 121 | 178 |
| | | | 1 µg/ml | 151 | 124 | 114 | 207 |
| | ROS (24 hours) | | 10 µg/ml | 644 | 10073 | 4276 | 277 |
| | | | 1 µg/ml | 33 | 9579 | 343 | 147 |

At wavelength of 624 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the EH3 molecule was measured in each cancer cell line but in different degrees. According to both WST-1 and resazurin assays, the most affected cell line was FaDu cell line where both high and low doses of EH1 molecule lead to appx. 90% cell death relative to the non-treated control. Other cell lines, HT29 and A253 cell lines were significantly affected by photodynamic therapy of both high and low doses of EH1 molecule, while the T47D cell line only affected from photodynamic therapy with high dose of EH3 molecule. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

PDT-induced immediate ROS production was seen moderately on each cell line for both low and high doses of the molecule. Following PDT, both low and high doses EH3 molecule lead to late ROS formation, except that low dose of the molecule did not trigger late ROS formation on A253 cell line. The highest amount of ROS was seen on the FaDu cell line, it was followed by HT29, A253, and T74D cell lines, respectively. It can be said that PDT-mediated cytotoxic response on the cell lines was ROS dependent.

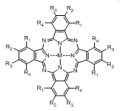
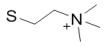
**Table 31** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with EH3 phthalocyanine at 650-670 nm (light fluence of 2 joules/cm$^2$).

| EH3 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal Zn R1  | Cytotoxicity (WST-1) | 650-670 nm | 10 µg/ml | 38 | 11 | 18 | 23 |
| | | | 1 µg/ml | 42 | 16 | 29 | 48 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 17 | 7 | 25 | 25 |
| | | | 1 µg/ml | 56 | 10 | 53 | 81 |
| | ROS (30 minutes) | | 10 µg/ml | 155 | 215 | 171 | 225 |
| | | | 1 µg/ml | 136 | 199 | 135 | 204 |
| | ROS (24 hours) | | 10 µg/ml | 4991 | 9281 | 3760 | 305 |
| | | | 1 µg/ml | 31 | 10245 | 1040 | 122 |

At wavelength of 650-670 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the EH3 molecule was measured in each cancer cell line but in different degrees. According to both WST-1 and resazurin assays, the most affected cell line was FaDu cell line where both high and low doses of EH1 molecule lead to appx. 90% cell death relative to the non-treated control. Other cell lines, HT29 and A253 cell lines were significantly affected by photodynamic therapy of both high and low doses of EH1 molecule, while the T47D cell line only affected from photodynamic therapy with high dose of EH3 molecule. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

PDT-induced immediate ROS production was seen moderately on each cell line for both low and high doses of the molecule. Following PDT, both low and high doses EH3 molecule lead to late ROS formation, except that low dose of the molecule did not trigger late ROS formation on A253 cell line. The highest amount of ROS was seen on the FaDu cell line, it was followed by HT29, A253, and T74D cell lines, respectively. It can be said that PDT-mediated cytotoxic response on the cell lines was ROS dependent.

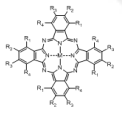
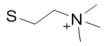
**Table 32** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with EH3 phthalocyanine at 690-710 nm (light fluence of 2 joules/cm$^2$).

| EH3 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal Zn R1  | Cytotoxicity (WST-1) | 690-710 nm | 10 µg/ml | 37 | 12 | 19 | 25 |
| | | | 1 µg/ml | 44 | 17 | 30 | 64 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 21 | 8 | 30 | 45 |
| | | | 1 µg/ml | 59 | 13 | 72 | 82 |
| | ROS (30 minutes) | | 10 µg/ml | 160 | 249 | 155 | 227 |
| | | | 1 µg/ml | 110 | 196 | 141 | 222 |
| | ROS (24 hours) | | 10 µg/ml | 429 | 10091 | 4282 | 184 |
| | | | 1 µg/ml | 32 | 9339 | 254 | 167 |

At wavelength of 690-710 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the EH3 molecule was measured in each cancer cell line but in different degrees. According to both WST-1 and resazurin assays, the most affected cell line was FaDu cell line where both high and low doses of EH1 molecule lead to appx. 90% cell death relative to the non-treated control. Other cell lines, HT29 and A253 cell lines were significantly affected by photodynamic therapy of both high and low doses of EH1 molecule, while the T47D cell line only affected from photodynamic therapy with high dose of EH3 molecule. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

PDT-induced immediate ROS production was seen moderately on each cell line for both low and high doses of the molecule. Following PDT, both low and high doses EH3 molecule lead to late ROS formation, except that low dose of the molecule did not trigger late ROS formation on A253 cell line. The highest amount of ROS was seen on the FaDu cell line, it was followed by HT29, A253, and T74D cell lines, respectively. It can be said that PDT-mediated cytotoxic response on the cell lines was ROS dependent.

**Table 33** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with EH3 phthalocyanine at 720-740nm (light fluence of 2 joules/cm$^2$).

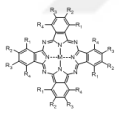
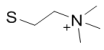
| EH3 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal Zn R1  | Cytotoxicity (WST-1) | 720-740 nm | 10 µg/ml | 39 | 12 | 20 | 28 |
| | | | 1 µg/ml | 43 | 20 | 30 | 71 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 28 | 9 | 34 | 54 |
| | | | 1 µg/ml | 60 | 16 | 73 | 85 |
| | ROS (30 minutes) | | 10 µg/ml | 156 | 305 | 177 | 218 |
| | | | 1 µg/ml | 108 | 227 | 145 | 245 |
| | ROS (24 hours) | | 10 µg/ml | 153 | 10202 | 5559 | 180 |
| | | | 1 µg/ml | 25 | 10442 | 275 | 168 |

At wavelength of 720-740 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the EH3 molecule was measured in each cancer cell line except T47D cell line when applied by low dose of the molecule. According to both WST-1 and resazurin assays, the most affected cell line was FaDu cell line where both high and low doses of EH1 molecule lead to appx. 90% cell death relative to the non-treated control. Other cell lines, HT29 and A253 cell lines were significantly affected by photodynamic therapy of both high and low doses of EH1 molecule, while the T47D cell line only affected from photodynamic therapy with high dose of EH3 molecule. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends

of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

PDT-induced immediate ROS production was seen moderately on each cell line for both low and high doses of the molecule. Following PDT, both low and high doses EH3 molecule lead to late ROS formation, except that low dose of the molecule did not trigger late ROS formation on A253 cell line. The highest amount of ROS was seen on the FaDu cell line, it was followed by HT29, and T74D cell lines, respectively. It can be said that PDT-mediated cytotoxic response on the cell lines was ROS dependent.

**Table 34** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with EH3 phthalocyanine at 725-755 nm (light fluence of 2 joules/cm$^2$).

| EH3 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal Zn R1  | Cytotoxicity (WST-1) | 725-755 nm | 10 µg/ml | 39 | 11 | 18 | 23 |
| | | | 1 µg/ml | 43 | 17 | 29 | 61 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 18 | 7 | 29 | 42 |
| | | | 1 µg/ml | 60 | 12 | 70 | 84 |
| | ROS (30 minutes) | | 10 µg/ml | 151 | 293 | 165 | 177 |
| | | | 1 µg/ml | 120 | 275 | 156 | 212 |
| | ROS (24 hours) | | 10 µg/ml | 4344 | 9815 | 4424 | 198 |
| | | | 1 µg/ml | 25 | 10421 | 270 | 160 |

At wavelength of 725-755 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the EH3 molecule was measured in each cancer cell line but in different degrees.

According to WST-1 assay, the most affected cell line was FaDu cell line where both high and low doses of EH1 molecule lead to appx. 90% cell death relative to the

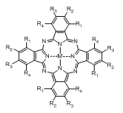
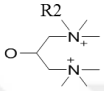
non-treated control. Other cell lines, HT29 and A253 cell lines were significantly affected by photodynamic therapy of both high and low doses of EH1 molecule, while the T47D cell line only affected from photodynamic therapy of high dose of EH3 molecule, and moderately affected from low lose. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

PDT-induced immediate ROS production was seen moderately on each cell line, however, there were significant ROS amount was measured triggered by PDT lately for each cell line except on A253 and HT29 cell line when treated with the low dose of the molecule. It can be said that PDT-mediated cytotoxic response on the cell lines was ROS dependent.

### 4.10.5. Comparative table of experiments with EH4 phthalocyanine

In this section, overall results obtained from EH4-mediated PDT with five different wavelengths were collected as percentage relative to non-treated control. Each data was revealed as % value.

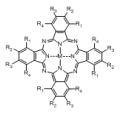
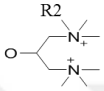
**Table 35** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with EH4 phthalocyanine at 624 nm (light fluence of 2 joules/cm$^2$).

| EH4 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal Zn  R2 | Cytotoxicity (WST-1) | 624 nm | 10 µg/ml | 38 | 11 | 14 | 27 |
| | | | 1 µg/ml | 53 | 24 | 89 | 94 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 14 | 4 | 12 | 15 |
| | | | 1 µg/ml | 33 | 4 | 63 | 50 |
| | ROS (30 minutes) | | 10 µg/ml | 153 | 245 | 140 | 264 |
| | | | 1 µg/ml | 148 | 211 | 119 | 216 |
| | ROS (24 hours) | | 10 µg/ml | 3865 | 7379 | 2932 | 889 |
| | | | 1 µg/ml | 64 | 6788 | 263 | 254 |

At wavelength of 624 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by EH4 molecule was measured in each cancer cell line except HT29 and T47D cell lines when it was applied by low dose of the molecule. According to WST-1 assay, the most affected cell line was FaDu cell line where both high and low doses of EH1 molecule lead to significant cell death relative to the non-treated control. Other cell lines, HT29 A253, and T47D cell lines were significantly affected by photodynamic therapy of high EH1 molecule, while the low dose of EH4 molecule did not trigger significant cell death. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

EH4 induced immediate ROS production was moderately seen on each cell line. The highest amount of ROS was seen on the T47D cell line, it was followed by FaDu, A253, and HT29 cell lines, respectively. The EH4-induced PDT triggered late ROS production on each cell line. The higher ROS production was measured from the FaDu cell line, followed by A253, HT29, and T47D cell lines, respectively. The least ROS production was seen on the A253 cell line, even the low dose of EH4 did not trigger ROS on the A253 cell line.

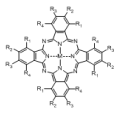
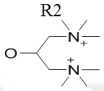
**Table 36** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with EH4 phthalocyanine at 650-670 nm (light fluence of 2 joules/cm$^2$).

| EH4 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal Zn | Cytotoxicity (WST-1) | 650-670 nm | 10 µg/ml | 37 | 12 | 17 | 26 |
| | | | 1 µg/ml | 46 | 25 | 60 | 85 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 11 | 4 | 12 | 14 |
| | | | 1 µg/ml | 28 | 4 | 73 | 45 |
| | ROS (30 minutes) | | 10 µg/ml | 190 | 317 | 188 | 356 |
| | | | 1 µg/ml | 170 | 219 | 134 | 187 |
|  | ROS (24 hours) | | 10 µg/ml | 3570 | 7537 | 3290 | 993 |
| | | | 1 µg/ml | 72 | 7103 | 177 | 248 |

At wavelength of 650-670 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by EH4 molecule was measured in each cancer cell line except HT29 and T47D cell lines when it was applied by low dose of the molecule. According to WST-1 assay, the most affected cell line was FaDu cell line where both high and low doses of EH1 molecule lead to significant cell death relative to the non-treated control. Other cell lines, HT29 A253, and T47D cell lines were significantly affected by photodynamic therapy with high dose of EH4 molecule, while the low dose of EH4 molecule did not trigger significant cell death. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

EH4-induced immediate ROS production was moderately seen on each cell line. The highest amount of ROS was seen on the T47D cell line, it was followed by FaDu, A253, and HT29 cell lines, respectively. The EH4-induced PDT triggered late ROS production on each cell line. The higher ROS production was measured from the FaDu cell line, followed by A253, HT29, and T47D cell lines, respectively. The least ROS production was seen on the A253 cell line, even the low dose of EH4 did not trigger ROS on the A253 cell line.

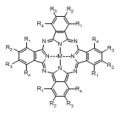
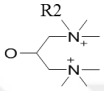
**Table 37** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with EH4 phthalocyanine at 690-710 nm (light fluence of 2 joules/cm$^2$).

| EH4 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal Zn  R2 | Cytotoxicity (WST-1) | 690-710 nm | 10 µg/ml | 40 | 12 | 13 | 26 |
| | | | 1 µg/ml | 57 | 47 | 87 | 75 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 16 | 5 | 17 | 34 |
| | | | 1 µg/ml | 54 | 8 | 76 | 71 |
| | ROS (30 minutes) | | 10 µg/ml | 199 | 324 | 218 | 218 |
| | | | 1 µg/ml | 191 | 249 | 125 | 123 |
| | ROS (24 hours) | | 10 µg/ml | 771 | 7730 | 3508 | 427 |
| | | | 1 µg/ml | 24 | 7064 | 216 | 199 |

At wavelength of 690-710 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by EH4 molecule was measured in each cancer cell line except HT29 and T47D cell lines when it was applied by low dose of the molecule. According to WST-1 assay, the most affected cell line was FaDu and HT29 cell line where high dose of EH1 molecule lead to significant cell death relative to the non-treated control. Other cell lines, A253, and T47D cell lines were significantly affected by photodynamic therapy of high EH4 molecule. PDT with low dose of the molecule did not lead to significant cellular death according to WST-1 assay. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

EH4 induced immediate ROS production was moderately seen on each cell line. The highest amount of ROS was seen on the FaDu cell line, it was followed by HT29, T47D, and A253 cell lines, respectively. The EH4-induced PDT triggered late ROS production on each cell line. The higher ROS production was measured from the FaDu cell line, followed by HT29, A253 and T47D cell lines, respectively. The least ROS production was seen on the A253 cell line, even the low dose of EH4 did not trigger ROS on the A253 cell line.

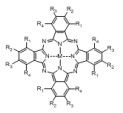
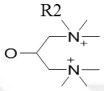
**Table 38** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with EH4 phthalocyanine at 720-740 nm (light fluence of 2 joules/cm$^2$).

| EH4 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal Zn | Cytotoxicity (WST-1) | 720-740 nm | 10 µg/ml | 38 | 12 | 15 | 36 |
| | | | 1 µg/ml | 66 | 61 | 86 | 90 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 25 | 6 | 39 | 51 |
| | | | 1 µg/ml | 68 | 22 | 76 | 78 |
| | ROS (30 minutes) | | 10 µg/ml | 227 | 346 | 209 | 188 |
| | | | 1 µg/ml | 199 | 218 | 129 | 167 |
|  | ROS (24 hours) | | 10 µg/ml | 237 | 7820 | 4660 | 309 |
| | | | 1 µg/ml | 37 | 14053 | 223 | 191 |

At wavelength of 720-740 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by EH4 molecule was measured in each cancer cell line except HT29 and T47D cell lines when it was applied by low dose of the molecule. According to WST-1 assay, the most affected cell line was FaDu cell line where both high and low doses of EH1 molecule lead to significant cell death relative to the non-treated control. Other cell lines, HT29 A253, and T47D cell lines were significantly affected by photodynamic therapy of high EH1 molecule, while the low dose of EH4 molecule did not trigger significant cell death. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

EH4 induced immediate ROS production was moderately seen on each cell line. The highest amount of ROS was seen on the T47D cell line, it was followed by FaDu, A253, and HT29 cell lines, respectively. The EH4-induced PDT triggered late ROS production on each cell line. The higher ROS production was measured from the FaDu cell line, followed by A253, HT29, and T47D cell lines, respectively. The least ROS production was seen on the A253 cell line, even the low dose of EH4 did not trigger ROS on the A253 cell line.

**Table 39** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with EH4 phthalocyanine at 725-755 nm (light fluence of 2 joules/cm$^2$).

| EH4 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal Zn | Cytotoxicity (WST-1) | 725-755 nm | 10 µg/ml | 38 | 12 | 21 | 26 |
| | | | 1 µg/ml | 50 | 36 | 89 | 78 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 19 | 5 | 20 | 36 |
| | | | 1 µg/ml | 52 | 8 | 73 | 77 |
| | ROS (30 minutes) | | 10 µg/ml | 188 | 362 | 224 | 126 |
| | | | 1 µg/ml | 183 | 261 | 136 | 145 |
|  | ROS (24 hours) | | 10 µg/ml | 245 | 8447 | 3945 | 413 |
| | | | 1 µg/ml | 28 | 8436 | 268 | 221 |

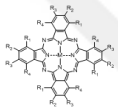
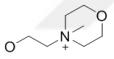
At wavelength of 725-755 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by EH4 molecule was measured in each cancer cell line, but in different degrees According to WST-1 assay, the most affected cell line was FaDu cell line where both high and low doses of EH1 molecule lead to significant cell death relative to the non-treated control. Other cell lines, HT29 A253, and T47D cell lines were significantly affected by photodynamic therapy of both high EH1 molecule, while the low dose of EH4 molecule did not trigger significant cell death. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

EH4 induced immediate ROS production was moderately seen on each cell line. The highest amount of ROS was seen on the FaDu cell line, it was followed by HT29, A253, and T47D cell lines, respectively. The EH4-induced PDT triggered late ROS production on each cell line. The highest ROS production was measured from the FaDu cell line, also T47D, HT29, and A253 cell lines, respectively. The least ROS production was seen on the A253 cell line, even the low dose of EH1 did not trigger ROS on the A253 cell line.

### 4.10.6. Comparative table of experiments with $HQH_2Pc$ phthalocyanine

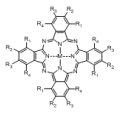
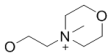
In this section, overall results obtained from $HQH_2Pc$-mediated PDT with five different wavelengths were collected as percentage relative to non-treated control. Each data was revealed as % value.

**Table 40** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with $HQH_2Pc$ phthalocyanine at 624 nm (light fluence of 2 joules/$cm^2$).

| HQH2Pc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal 2H R2  | Cytotoxicity (WST-1) | 624 nm | 10 µg/ml | 87 | 74 | 71 | 77 |
| | | | 1 µg/ml | 114 | 97 | 87 | 106 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 81 | 94 | 91 | 102 |
| | | | 1 µg/ml | 105 | 94 | 101 | 108 |
| | ROS (30 minutes) | | 10 µg/ml | 101 | 125 | 96 | 103 |
| | | | 1 µg/ml | 90 | 103 | 89 | 87 |
| | ROS (24 hours) | | 10 µg/ml | 93 | 152 | 237 | 100 |
| | | | 1 µg/ml | 97 | 136 | 172 | 78 |

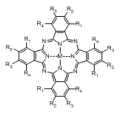
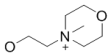
At wavelength of 624 nm with 2 joules/$cm^2$ light fluence, photo-induced cellular damage caused by the $HQH_2Pc$ molecule was not measured in the cancer cell line. Both WST-1 and resazurin assays had supported each other. Neither immediate nor late PDT-induced ROS was not formed. $HQH_2Pc$ molecule is not suitable for PDT.

**Table 41** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with $HQH_2Pc$ phthalocyanine at 650-670 nm (light fluence of 2 joules/$cm^2$).

| HQH2Pc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  | Cytotoxicity (WST-1) | 650-670 nm | 10 µg/ml | 83 | 77 | 82 | 73 |
| | | | 1 µg/ml | 109 | 104 | 97 | 99 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 79 | 93 | 94 | 91 |
| | | | 1 µg/ml | 106 | 95 | 100 | 113 |
| Center metal 2H | ROS (30 minutes) | | 10 µg/ml | 121 | 171 | 107 | 141 |
| | | | 1 µg/ml | 100 | 114 | 97 | 103 |
| R2 | ROS (24 hours) | | 10 µg/ml | 97 | 148 | 301 | 146 |
|  | | | 1 µg/ml | 104 | 147 | 189 | 88 |

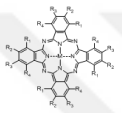
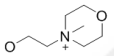
At wavelength of 650-670 nm with 2 joules/$cm^2$ light fluence, photo-induced cellular damage caused by the $HQH_2Pc$ molecule was not measured in the cancer cell line. Both WST-1 and resazurin assays had supported each other. Neither immediate nor late PDT-induced ROS was not formed. $HQH_2Pc$ molecule is not suitable for PDT.

**Table 42** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with $HQH_2Pc$ phthalocyanine at 690-710 nm (light fluence of 2 joules/$cm^2$).

| HQH2Pc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  | Cytotoxicity (WST-1) | 690-710 nm | 10 µg/ml | 96 | 80 | 87 | 80 |
| | | | 1 µg/ml | 116 | 110 | 95 | 98 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 87 | 94 | 93 | 92 |
| | | | 1 µg/ml | 107 | 96 | 97 | 112 |
| Center metal 2H | ROS (30 minutes) | | 10 µg/ml | 129 | 182 | 107 | 156 |
| | | | 1 µg/ml | 119 | 134 | 92 | 157 |
| R2 | ROS (24 hours) | | 10 µg/ml | 117 | 174 | 246 | 117 |
|  | | | 1 µg/ml | 108 | 151 | 188 | 87 |

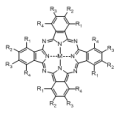
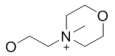
At wavelength of 690-710 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the $HQH_2Pc$ molecule was not measured in the cancer cell line. Both WST-1 and resazurin assays had supported each other. Neither immediate nor late PDT-induced ROS was not formed. $HQH_2Pc$ molecule is not suitable for PDT.

**Table 43** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with $HQH_2Pc$ phthalocyanine at 720-740 nm (light fluence of 2 joules/cm$^2$).

| HQH2Pc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal 2H R2  | Cytotoxicity (WST-1) | 720-740 nm | 10 µg/ml | 99 | 80 | 87 | 81 |
| | | | 1 µg/ml | 116 | 98 | 93 | 96 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 88 | 96 | 96 | 94 |
| | | | 1 µg/ml | 104 | 93 | 101 | 111 |
| | ROS (30 minutes) | | 10 µg/ml | 136 | 180 | 112 | 139 |
| | | | 1 µg/ml | 127 | 140 | 104 | 178 |
| | ROS (24 hours) | | 10 µg/ml | 112 | 158 | 530 | 135 |
| | | | 1 µg/ml | 96 | 148 | 172 | 90 |

At wavelength of 720-740 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the $HQH_2Pc$ molecule was not measured in the cancer cell line. Both WST-1 and resazurin assays had supported each other. Neither immediate nor late PDT-induced ROS was not formed. $HQH_2Pc$ molecule is not suitable for PDT.

**Table 44** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with $HQH_2Pc$ phthalocyanine at 725-755 nm (light fluence of 2 joules/$cm^2$).

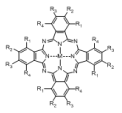
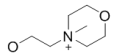
| HQH2Pc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  | Cytotoxicity (WST-1) | 725-755 nm | 10 µg/ml | 90 | 83 | 85 | 74 |
| | | | 1 µg/ml | 106 | 98 | 89 | 91 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 85 | 93 | 90 | 99 |
| | | | 1 µg/ml | 103 | 98 | 94 | 113 |
| Center metal 2H | ROS (30 minutes) | | 10 µg/ml | 124 | 189 | 119 | 163 |
| | | | 1 µg/ml | 113 | 141 | 116 | 96 |
| R2  | ROS (24 hours) | | 10 µg/ml | 86 | 170 | 258 | 131 |
| | | | 1 µg/ml | 103 | 161 | 195 | 111 |

At wavelength of 725-755 nm with 2 joules/$cm^2$ light fluence, photo-induced cellular damage caused by the $HQH_2Pc$ molecule was not measured in the cancer cell line. Both WST-1 and resazurin assays had supported each other. Neither immediate nor late PDT-induced ROS was not formed. $HQH_2Pc$ molecule is not suitable for PDT.

### 4.10.7. Comparative table of experiments with HQZnPc phthalocyanine

In this section, overall results obtained from HQZnPc-mediated PDT with five different wavelengths were collected as percentage relative to non-treated control. Each data was revealed as % value.

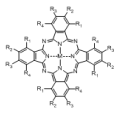
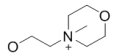
**Table 45** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with HQZnPc phthalocyanine at 624 nm (light fluence of 2 joules/cm$^2$).

| HQZnPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  | Cytotoxicity (WST-1) | 624 nm | 10 µg/ml | 41 | 10 | 20 | 30 |
| | | | 1 µg/ml | 48 | 12 | 54 | 78 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 26 | 5 | 14 | 54 |
| | | | 1 µg/ml | 58 | 7 | 78 | 93 |
| Center metal Zn | ROS (30 minutes) | | 10 µg/ml | 225 | 193 | 95 | 299 |
| | | | 1 µg/ml | 194 | 128 | 58 | 275 |
| R2  | ROS (24 hours) | | 10 µg/ml | 197 | 7788 | 3355 | 131 |
| | | | 1 µg/ml | 34 | 5413 | 153 | 98 |

At wavelength of 624 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the HQZnPc molecule was measured in each cancer cell line but different degrees. According to WST-1 assay, the most affected cell line was FaDu cell line where both high and low doses of EH1 molecule lead to appx. 90% cell death relative to the non-treated control. Other cell lines, A253, HT29, and T47D cell lines were significantly affected by photodynamic therapy of high HQZnPc molecule, and not affected by low lose. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

HQZnPc induced immediate ROS production was seen on each cell lines except HT29 cell line. The highest amount of ROS was seen on the T47D cell line, it was followed by A253, and FaDu cell lines, respectively. PDT mediated late ROS production was significantly seen on FaDu when applied by both high and low doses of the molecule, on HT29 when applied only high dose of the molecule. Only high dose of the molecule triggered late ROS production on A253 and T47D cell lines, moderately. However, low dose of the molecule did not lead to ROS production on A253 and T47D cell lines.

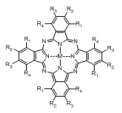
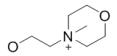
**Table 46** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with HQZnPc phthalocyanine at 650-670 nm (light fluence of 2 joules/cm$^2$).

| HQZnPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  | Cytotoxicity (WST-1) | 650-670 nm | 10 µg/ml | 39 | 11 | 21 | 27 |
| | | | 1 µg/ml | 50 | 12 | 45 | 74 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 28 | 5 | 14 | 49 |
| | | | 1 µg/ml | 55 | 8 | 75 | 89 |
| Center metal Zn | ROS (30 minutes) | | 10 µg/ml | 204 | 217 | 104 | 387 |
| | | | 1 µg/ml | 161 | 119 | 51 | 327 |
| R2  | ROS (24 hours) | | 10 µg/ml | 128 | 7465 | 3524 | 150 |
| | | | 1 µg/ml | 37 | 5646 | 152 | 99 |

At wavelength of 650-670 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the HQZnPc molecule was measured in each cancer cell line but different degrees. According to WST-1 assay, the most affected cell line was FaDu cell line where both high and low doses of EH1 molecule lead to appx. 90% cell death relative to the non-treated control. Other cell lines, A253, HT29, and T47D cell lines were significantly affected by photodynamic therapy of high HQZnPc molecule, and not affected by low lose. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

HQZnPc induced immediate ROS production was seen on each cell lines except HT29 cell line. The highest amount of ROS was seen on the T47D cell line, it was followed by FaDu, and A253 cell lines, respectively. PDT mediated late ROS production was significantly seen on FaDu when applied by both high and low doses of the molecule, on HT29 when applied only high dose of the molecule. Only high dose of the molecule triggered late ROS production on A253 and T47D cell lines, moderately. However, low dose of the molecule did not lead to ROS production on A253 and T47D cell lines.

**Table 47** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with HQZnPc phthalocyanine at 690-710 nm (light fluence of 2 joules/cm$^2$).

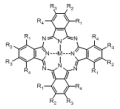
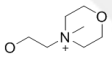
| HQZnPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  | Cytotoxicity (WST-1) | 690-710 nm | 10 µg/ml | 39 | 10 | 24 | 40 |
| | | | 1 µg/ml | 62 | 13 | 104 | 76 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 30 | 8 | 31 | 79 |
| | | | 1 µg/ml | 79 | 18 | 77 | 98 |
| Center metal Zn | ROS (30 minutes) | | 10 µg/ml | 304 | 274 | 138 | 338 |
| | | | 1 µg/ml | 169 | 128 | 51 | 241 |
| R2  | ROS (24 hours) | | 10 µg/ml | 132 | 9102 | 5381 | 121 |
| | | | 1 µg/ml | 42 | 211 | 181 | 118 |

At wavelength of 690-710 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the HQZnPc molecule was measured in each cancer cell line but different degrees. According to WST-1 assay, the most affected cell line was FaDu cell line where both high and low doses of EH1 molecule lead to appx. 90% cell death relative to the non-treated control. Other cell lines, A253, HT29, and T47D cell lines were significantly affected by photodynamic therapy of high HQZnPc molecule, and not affected by low lose. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

HQZnPc induced immediate ROS production was seen on each cell lines except HT29 cell line when it was applied by low dose of the molecule. The highest amount of ROS was seen on the T47D cell line, it was followed by A253, and FaDu cell lines, respectively. PDT mediated late ROS production was significantly seen on FaDu and HT29 when applied by both high dose of the molecule. Low dose of the molecule triggered late ROS production on FaDu and HT29 cell lines, moderately. Only high dose of the molecule triggered late ROS production on A253 and T47D cell lines,

moderately. However, low dose of the molecule did not lead to ROS production on A253 and T47D cell lines.

**Table 48** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with HQZnPc phthalocyanine at 720-740 nm (light fluence of 2 joules/cm$^2$).

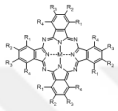
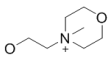
| HQZnPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal Zn R2  | Cytotoxicity (WST-1) | 720-740 nm | 10 µg/ml | 40 | 11 | 26 | 38 |
| | | | 1 µg/ml | 74 | 16 | 102 | 82 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 30 | 9 | 48 | 81 |
| | | | 1 µg/ml | 88 | 36 | 81 | 103 |
| | ROS (30 minutes) | | 10 µg/ml | 336 | 292 | 138 | 441 |
| | | | 1 µg/ml | 188 | 177 | 64 | 275 |
| | ROS (24 hours) | | 10 µg/ml | 135 | 8905 | 628 | 136 |
| | | | 1 µg/ml | 57 | 84 | 139 | 96 |

At wavelength of 720-740 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the HQZnPc molecule was measured in each cancer cell line but different degrees. According to WST-1 assay, the most affected cell line was FaDu cell line where both high and low doses of EH1 molecule lead to appx. 90% cell death relative to the non-treated control. Other cell lines, A253, HT29, and T47D cell lines were significantly affected by photodynamic therapy of high HQZnPc molecule, and not affected by low lose. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

HQZnPc induced immediate ROS production was seen on each cell lines except HT29 cell line when it was applied by low dose of the molecule. The highest amount of ROS was seen on the T47D cell line, it was followed by A253, and FaDu cell lines, respectively. PDT mediated late ROS production was significantly seen on FaDu when

applied by high dose of the molecule, also it moderately triggered ROS production on HT29, T47D and A253 cell lines. However, low dose of the molecule did not lead to ROS production on the cell lines.

**Table 49** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with HQZnPc phthalocyanine at 725-755 nm (light fluence of 2 joules/cm$^2$).

| HQZnPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal Zn R2  | Cytotoxicity (WST-1) | 725-755 nm | 10 µg/ml | 40 | 10 | 22 | 34 |
| | | | 1 µg/ml | 53 | 14 | 88 | 74 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 32 | 6 | 25 | 71 |
| | | | 1 µg/ml | 76 | 18 | 75 | 101 |
| | ROS (30 minutes) | | 10 µg/ml | 283 | 272 | 134 | 353 |
| | | | 1 µg/ml | 274 | 189 | 64 | 247 |
| | ROS (24 hours) | | 10 µg/ml | 122 | 8035 | 4245 | 115 |
| | | | 1 µg/ml | 43 | 213 | 130 | 99 |

At wavelength of 725-755 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the HQZnPc molecule was measured in each cancer cell line but different degrees. According to WST-1 assay, the most affected cell line was FaDu cell line where both high and low doses of EH1 molecule lead to appx. 90% cell death relative to the non-treated control. Other cell lines, A253, HT29, and T47D cell lines were significantly affected by photodynamic therapy of high HQZnPc molecule, and not affected by low lose. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

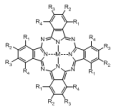
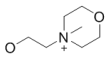
HQZnPc induced immediate ROS production was seen on each cell line in different degrees. The highest amount of ROS was seen on the T47D cell line, it was followed by A253, FaDu, and HT29 cell lines, respectively. At the light of proper

wavelengths, PDT mediated late ROS production was significantly seen FaDu, T47D, and HT29 cell lines when treated with the high dose of the molecule, and there was no significant ROS measured right after PDT with the low dose of HQZnPc molecule. However, the light with higher energy induced ROS production on FaDu, HT29 and T47D cell lines even low HQZnPc dose.

### 4.10.8. Comparative table of experiments with HQInPc phthalocyanine

In this section, overall results obtained from HQInPc-mediated PDT with five different wavelengths were collected as percentage relative to non-treated control. Each data was revealed as % value.

**Table 50** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with HQInPc phthalocyanine at 624 nm (light fluence of 2 joules/cm$^2$).

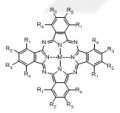
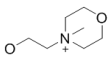
| HQInPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal InCl R2  | Cytotoxicity (WST-1) | 624 nm | 10 µg/ml | 43 | 24 | 44 | 33 |
| | | | 1 µg/ml | 72 | 98 | 84 | 73 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 47 | 37 | 79 | 24 |
| | | | 1 µg/ml | 90 | 83 | 84 | 76 |
| | ROS (30 minutes) | | 10 µg/ml | 208 | 216 | 73 | 150 |
| | | | 1 µg/ml | 163 | 111 | 71 | 130 |
| | ROS (24 hours) | | 10 µg/ml | 40 | 101 | 123 | 389 |
| | | | 1 µg/ml | 51 | 59 | 98 | 94 |

At wavelength of 624 nm with 2 joules/cm$^2$ light fluence, photo -induced cellular damage caused by the high dose HQInPc molecule was measured in each cancer cell line, but not by the low dose of the molecule. According to WST-1 assay, the most affected cell line was FaDu, followed by T47D, HT29, and A253 cell lines. Considering 10 percent difference is acceptable, there were some differences above

this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

HQInPc induced immediate ROS production was seen on each cell line except the HT29 cell line. The highest amount of ROS was seen on the FaDu cell line, it was followed by A253 and T47D cell lines, respectively. HQInPc-induced PDT triggered late ROS production on only T47D cell line when it was applied by high dose of the molecule. There was no significant late ROS formation triggered following PDT on the cell lines.

**Table 51** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with HQInPc phthalocyanine at 650-670 nm (light fluence of 2 joules/cm$^2$).

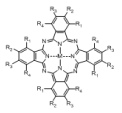
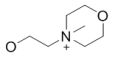
| HQInPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal InCl R2  | Cytotoxicity (WST-1) | 650-670 nm | 10 µg/ml | 44 | 20 | 36 | 28 |
| | | | 1 µg/ml | 67 | 89 | 82 | 71 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 48 | 23 | 72 | 21 |
| | | | 1 µg/ml | 89 | 57 | 85 | 69 |
| | ROS (30 minutes) | | 10 µg/ml | 237 | 131 | 120 | 170 |
| | | | 1 µg/ml | 176 | 39 | 86 | 135 |
| | ROS (24 hours) | | 10 µg/ml | 30 | 188 | 157 | 393 |
| | | | 1 µg/ml | 32 | 79 | 122 | 122 |

At wavelength of 650-670 nm with 2 joules/cm$^2$ light fluence, photo -induced cellular damage caused by the high dose HQInPc molecule was measured in each cancer cell line, but not by the low dose of the molecule. According to WST-1 assay, the most affected cell line was FaDu, followed by T47D, HT29, and A253 cell lines. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends

of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

HQInPc induced immediate ROS production was seen on each cell lines except FaDu and HT29 cell lines when it was applied by low dose of the molecule. The highest amount of ROS was seen on the A253, it was followed by T74D, FaDu and HT29 cell lines, respectively. HQInPc-induced PDT triggered late ROS production on T47D cell line, when it was applied by high dose of the molecule. It was followed by FaDu, and HT29 cell lines. There was no late ROS formation triggered following PDT on A253 cell line when it was applied by both doses, and FaDu when it was applied low dose of the molecule.

**Table 52** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with HQInPc phthalocyanine at 690-710 nm (light fluence of 2 joules/cm$^2$).

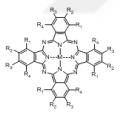
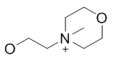
| HQInPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal InCl R2  | Cytotoxicity (WST-1) | 690-710 nm | 10 µg/ml | 46 | 25 | 57 | 31 |
| | | | 1 µg/ml | 71 | 102 | 88 | 68 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 57 | 42 | 82 | 30 |
| | | | 1 µg/ml | 95 | 75 | 83 | 77 |
| | ROS (30 minutes) | | 10 µg/ml | 222 | 173 | 107 | 174 |
| | | | 1 µg/ml | 173 | 77 | 80 | 148 |
| | ROS (24 hours) | | 10 µg/ml | 26 | 104 | 191 | 428 |
| | | | 1 µg/ml | 38 | 89 | 154 | 139 |

At wavelength of 690-710 nm with 2 joules/cm$^2$ light fluence, photo -induced cellular damage caused by the high dose HQInPc molecule was measured in each cancer cell line, but not by the low dose of the molecule. According to WST-1 assay, the most affected cell line was FaDu, followed by T47D, HT29, and A253 cell lines. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends

of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

HQInPc induced immediate ROS production was seen on each cell line except FaDu and HT29 cell lines when it was applied by low dose of the molecule. The highest amount of ROS was seen on A253 cell line, it was followed by T47D and FaDu cell lines, respectively. HQInPc-induced PDT triggered late ROS production on T47D and HT29 cell lines when it was applied by both low and high dose of the molecule. There was no significant late ROS formation triggered following PDT on A253 and FaDu cell lines.

**Table 53** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with HQInPc phthalocyanine at 720-740 nm (light fluence of 2 joules/cm$^2$).

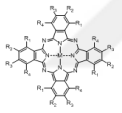
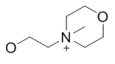
| HQInPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  | Cytotoxicity (WST-1) | 720-740 nm | 10 µg/ml | 45 | 26 | 62 | 34 |
| | | | 1 µg/ml | 76 | 101 | 90 | 69 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 60 | 55 | 77 | 41 |
| | | | 1 µg/ml | 96 | 80 | 96 | 84 |
| Center metal InCl | ROS (30 minutes) | | 10 µg/ml | 289 | 222 | 92 | 188 |
| | | | 1 µg/ml | 186 | 135 | 92 | 163 |
| R2  | ROS (24 hours) | | 10 µg/ml | 22 | 103 | 162 | 274 |
| | | | 1 µg/ml | 50 | 127 | 169 | 131 |

At wavelength of 624 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the high dose HQInPc molecule was measured in each cancer cell line except HT29 cell line. Low dose of the molecule did not lead to cytotoxic response on any cell lines. According to WST-1 assay, the most affected cell line was FaDu, followed by T47D, and A253 cell lines. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin

assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

HQInPc-induced immediate ROS production was seen on each cell line except the HT29 cell line. The highest amount of ROS was seen on the A253 cell line, it was followed by FaDu and T47D cell lines, respectively. HQInPc-induced PDT triggered late ROS production on T47D and HT29 cell line when it was applied by high dose of the molecule. There was no significant late ROS formation triggered following PDT on A253 and FaDu cell line.

**Table 54** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with HQInPc phthalocyanine at 725-755 nm (light fluence of 2 joules/cm$^2$).

| HQInPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal InCl R2  | Cytotoxicity (WST-1) | 725-755 nm | 10 µg/ml | 42 | 21 | 39 | 28 |
| | | | 1 µg/ml | 63 | 81 | 83 | 65 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 54 | 32 | 77 | 30 |
| | | | 1 µg/ml | 90 | 57 | 73 | 76 |
| | ROS (30 minutes) | | 10 µg/ml | 196 | 303 | 95 | 197 |
| | | | 1 µg/ml | 145 | 175 | 95 | 178 |
| | ROS (24 hours) | | 10 µg/ml | 25 | 136 | 136 | 323 |
| | | | 1 µg/ml | 35 | 115 | 137 | 125 |

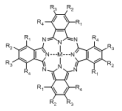
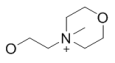
At wavelength of 624 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the high dose HQInPc molecule was measured in each cancer cell line, but not by the low dose of the molecule. According to WST-1 assay, the most affected cell line was FaDu, followed by T47D, HT29, and A253 cell lines. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

HQInPc-induced immediate ROS production was seen on each cell line except the HT29 cell line. The highest amount of ROS was seen on the FaDu cell line, it was followed by T47D and A253 cell lines, respectively. HQInPc-induced PDT triggered late ROS production on the only T47D cell line. The high dose of HQInPc molecule triggered a significant amount of ROS, while the low dose of the molecule leads to only moderately ROS formation on the T47D cell line. There was no significant amount of ROS formed lately following PDT on A253, FaDu, and HT29 cell line.

### 4.10.9. Comparative table of experiments with $SQH_2Pc$ phthalocyanine

In this section, overall results obtained from $SQH_2Pc$-mediated PDT with five different wavelengths were collected as percentage relative to non-treated control. Each data was revealed as % value.

**Table 55** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with $SQH_2Pc$ phthalocyanine at 624 nm (light fluence of 2 joules/$cm^2$).

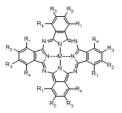
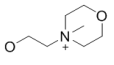
| SQH2Pc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  | Cytotoxicity (WST-1) | 624 nm | 10 µg/ml | 56 | 43 | 42 | 66 |
| | | | 1 µg/ml | 95 | 77 | 87 | 91 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 67 | 84 | 86 | 87 |
| | | | 1 µg/ml | 108 | 94 | 88 | 101 |
| Center metal 2H | ROS (30 minutes) | | 10 µg/ml | 130 | 107 | 83 | 171 |
| | | | 1 µg/ml | 125 | 80 | 68 | 190 |
| R1  | ROS (24 hours) | | 10 µg/ml | 65 | 101 | 121 | 126 |
| | | | 1 µg/ml | 81 | 112 | 138 | 116 |

At wavelength of 624 nm with 2 joules/$cm^2$ light fluence, photo-induced cellular damage caused by the $SQH_2Pc$ molecule was measured in each cancer cell line moderately only when were applied by high dose of the molecule. Considering 10

percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

Right after PDT, high doses of $SQH_2Pc$ molecule triggered immediate ROS formation on the T47D and A253 cell lines when applied both dose of the molecule. However, neither low nor high dose of the molecule did not trigger immediate ROS formation on FaDu and HT29 cell lines. Following PDT, late ROS formation was triggered with PDT of both doses of $SQH_2Pc$ molecule on A253 and T47D cell lines. PDT with neither dose of $SQH_2Pc$ molecule did not triggered late ROS formation on A253 and FaDu cell line.

**Table 56** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with $SQH_2Pc$ phthalocyanine at 650-670 nm (light fluence of 2 joules/$cm^2$).

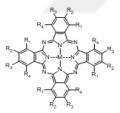
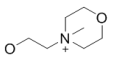
| SQH2Pc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  | Cytotoxicity (WST-1) | 650-670 nm | 10 µg/ml | 45 | 14 | 22 | 52 |
| | | | 1 µg/ml | 95 | 85 | 96 | 86 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 27 | 72 | 82 | 77 |
| | | | 1 µg/ml | 105 | 92 | 88 | 95 |
| Center metal 2H | ROS (30 minutes) | | 10 µg/ml | 121 | 186 | 107 | 64 |
| | | | 1 µg/ml | 124 | 70 | 87 | 40 |
| R1  | ROS (24 hours) | | 10 µg/ml | 117 | 71 | 130 | 120 |
| | | | 1 µg/ml | 86 | 116 | 162 | 156 |

At wavelength of 650-670 nm with 2 joules/$cm^2$ light fluence, photo-induced cellular damage caused by the $SQH_2Pc$ molecule was measured in each cancer cell line only when were applied by high dose of the molecule. The most cytotoxic response was taken from FaDu cell line, followed by HT29, A253 and T47D cell lines, respectively. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according

to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

Right after PDT, high doses of $SQH_2Pc$ molecule triggered immediate ROS formation on A253 and FaDu cell lines when applied both dose of the molecule. However, neither low nor high dose of the molecule did not trigger immediate ROS formation on HT29 and T47D cell lines. Following PDT, late ROS formation was moderately triggered with PDT of both doses of $SQH_2Pc$ molecule on HT29 and T47D cell lines, high dose of the molecule on A253 cell line. PDT with neither dose of $SQH_2Pc$ molecule did not triggered late ROS formation on FaDu cell line.

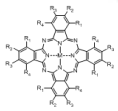
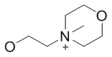
**Table 57** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with $SQH_2Pc$ phthalocyanine at 690-710 nm (light fluence of 2 joules/$cm^2$).

| SQH2Pc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal 2H R1  | Cytotoxicity (WST-1) | 690-710 nm | 10 µg/ml | 55 | 39 | 36 | 60 |
| | | | 1 µg/ml | 95 | 90 | 96 | 89 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 59 | 76 | 86 | 89 |
| | | | 1 µg/ml | 104 | 92 | 86 | 92 |
| | ROS (30 minutes) | | 10 µg/ml | 159 | 120 | 108 | 89 |
| | | | 1 µg/ml | 136 | 86 | 94 | 130 |
| | ROS (24 hours) | | 10 µg/ml | 92 | 106 | 161 | 144 |
| | | | 1 µg/ml | 95 | 140 | 176 | 165 |

At wavelength of 690-710 nm with 2 joules/$cm^2$ light fluence, photo-induced cellular damage caused by the $SQH_2Pc$ molecule was measured in each cancer cell line moderately only when were applied by high dose of the molecule. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

Right after PDT, high doses of $SQH_2Pc$ molecule triggered immediate ROS formation on the FaDu and A253 cell lines when applied both dose of the molecule. However, neither low nor high dose of the molecule did not trigger immediate ROS formation on HT29 and T47D cell lines. Following PDT, late ROS formation was triggered with PDT of both doses of $SQH_2Pc$ molecule on HT29 and T47D cell lines. PDT with neither dose of $SQH_2Pc$ molecule did not triggered late ROS formation on A253 and FaDu cell line.

**Table 58** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with $SQH_2Pc$ phthalocyanine at 720-740 nm (light fluence of 2 joules/$cm^2$).

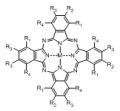
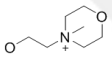
| SQH2Pc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal 2H R1  | Cytotoxicity (WST-1) | 720-740 nm | 10 µg/ml | 42 | 47 | 33 | 63 |
| | | | 1 µg/ml | 81 | 95 | 94 | 91 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 58 | 73 | 84 | 88 |
| | | | 1 µg/ml | 100 | 89 | 82 | 97 |
| | ROS (30 minutes) | | 10 µg/ml | 136 | 156 | 122 | 190 |
| | | | 1 µg/ml | 123 | 105 | 112 | 202 |
| | ROS (24 hours) | | 10 µg/ml | 105 | 113 | 142 | 130 |
| | | | 1 µg/ml | 103 | 150 | 177 | 151 |

At wavelength of 720-740 nm with 2 joules/$cm^2$ light fluence, photo-induced cellular damage caused by the $SQH_2Pc$ molecule was measured in each cancer cell line moderately only when were applied by high dose of the molecule. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

Right after PDT, both doses of $SQH_2Pc$ molecule moderately triggered immediate ROS formation on each cell lines. Following PDT, late ROS formation was triggered with PDT of both doses of $SQH_2Pc$ molecule on HT29 and T47D cell lines. PDT with

neither dose of $SQH_2Pc$ molecule did not triggered late ROS formation on A253 and FaDu cell line.

**Table 59** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with $SQH_2Pc$ phthalocyanine at 725-755 nm (light fluence of 2 joules/$cm^2$).

| SQH2Pc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  | Cytotoxicity (WST-1) | 725-755 nm | 10 µg/ml | 56 | 13 | 17 | 27 |
| | | | 1 µg/ml | 95 | 100 | 91 | 82 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 23 | 37 | 73 | 67 |
| | | | 1 µg/ml | 98 | 84 | 77 | 93 |
| Center metal 2H | ROS (30 minutes) | | 10 µg/ml | 192 | 262 | 122 | 217 |
| | | | 1 µg/ml | 156 | 139 | 93 | 222 |
| R1  | ROS (24 hours) | | 10 µg/ml | 154 | 139 | 116 | 109 |
| | | | 1 µg/ml | 82 | 150 | 164 | 150 |

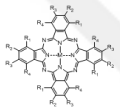
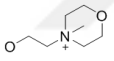
At wavelength of 725-755 nm with 2 joules/$cm^2$ light fluence, photo-induced cellular damage caused by the high dose $SQH_2Pc$ molecule was measured in each cancer cell line but in different degrees. The low dose of the molecule did not have any cytotoxic effects on the cell lines. $SQH_2Pc$ molecule was most effective on FaDu and HT29 cell lines, followed by T47D and A253 cell lines, respectively. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

Right after PDT, both doses of $SQH_2Pc$ molecule moderately triggered immediate ROS formation on each cell lines except HT29 cell line when it was applied by low dose of the molecule. Following PDT, late ROS formation was triggered with PDT of both doses of $SQH_2Pc$ molecule on FaDu, HT29, and T47D cell line. $SQH_2Pc$ molecule only lead to late ROS formation when it was applied by high dose.

### 4.10.10. Comparative table of experiments with SQZnPc phthalocyanine

In this section, overall results obtained from SQZnPc-mediated PDT with five different wavelengths were collected as percentage relative to non-treated control. Each data was revealed as % value.

**Table 60** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with SQZnPc phthalocyanine at 624 nm (light fluence of 2 joules/cm$^2$).

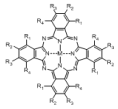
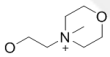
| SQZnPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal Zn R1  | Cytotoxicity (WST-1) | 624 nm | 10 µg/ml | 57 | 52 | 68 | 68 |
| | | | 1 µg/ml | 95 | 84 | 98 | 91 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 80 | 79 | 83 | 81 |
| | | | 1 µg/ml | 107 | 93 | 86 | 92 |
| | ROS (30 minutes) | | 10 µg/ml | 124 | 338 | 97 | 131 |
| | | | 1 µg/ml | 121 | 191 | 91 | 180 |
| | ROS (24 hours) | | 10 µg/ml | 39 | 99 | 135 | 144 |
| | | | 1 µg/ml | 77 | 118 | 156 | 143 |

At wavelength of 624 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the high dose of the SQZnPc molecule was moderately measured in A253 and FaDu cell lines, but not on HT29 and T47D cell lines. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

SQZnPc-induced immediate ROS production was seen on each cell line in different degrees. The highest amount of ROS was seen on the FaDu cell line, it was followed by T47D, A253, and HT29 cell lines, respectively. Following PDT, late ROS formation was triggered with PDT of both doses of SQZnPc molecule on HT29, And

T47D cell line. SQZnPc molecule did not lead to late ROS formation on A253 and FaDu cell lines.

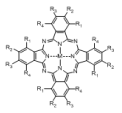
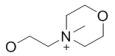
**Table 61** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with SQZnPc phthalocyanine at 650-670 nm (light fluence of 2 joules/cm$^2$).

| SQZnPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal Zn R1  | Cytotoxicity (WST-1) | 650-670 nm | 10 µg/ml | 57 | 45 | 78 | 67 |
| | | | 1 µg/ml | 96 | 81 | 100 | 95 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 72 | 63 | 82 | 82 |
| | | | 1 µg/ml | 108 | 89 | 84 | 95 |
| | ROS (30 minutes) | | 10 µg/ml | 158 | 401 | 140 | 193 |
| | | | 1 µg/ml | 133 | 204 | 108 | 114 |
| | ROS (24 hours) | | 10 µg/ml | 29 | 99 | 147 | 131 |
| | | | 1 µg/ml | 74 | 125 | 150 | 126 |

At wavelength of 650-670 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the high dose of the SQZnPc molecule was moderately measured in A253 and FaDu cell lines, but not on HT29 and T47D cell lines. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

SQZnPc-induced immediate ROS production was seen on each cell line in different degrees. The highest amount of ROS was seen on the FaDu cell line, it was followed by T47D, A253, and HT29 cell lines, respectively. Following PDT, late ROS formation was triggered with PDT of both doses of SQZnPc molecule on HT29, And T47D cell line. SQZnPc molecule did not lead to late ROS formation on A253 and FaDu cell lines.

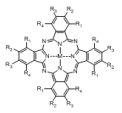
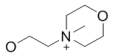
**Table 62** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with SQZnPc phthalocyanine at 690-710 nm (light fluence of 2 joules/cm$^2$).

| SQZnPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  Center metal Zn R1  | Cytotoxicity (WST-1) | 690-710 nm | 10 µg/ml | 57 | 43 | 86 | 65 |
| | | | 1 µg/ml | 91 | 87 | 101 | 88 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 78 | 70 | 81 | 84 |
| | | | 1 µg/ml | 100 | 91 | 80 | 95 |
| | ROS (30 minutes) | | 10 µg/ml | 157 | 376 | 144 | 216 |
| | | | 1 µg/ml | 128 | 193 | 104 | 187 |
| | ROS (24 hours) | | 10 µg/ml | 34 | 103 | 160 | 128 |
| | | | 1 µg/ml | 82 | 132 | 152 | 129 |

At wavelength of 670-710 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the high dose of the SQZnPc molecule was moderately measured in A253 and FaDu cell lines, but not on HT29 and T47D cell lines. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

SQZnPc-induced immediate ROS production was seen on each cell line in different degrees. The highest amount of ROS was seen on the FaDu cell line, it was followed by T47D, A253, and HT29 cell lines, respectively. Following PDT, late ROS formation was triggered with PDT of both doses of SQZnPc molecule on HT29, And T47D cell line. SQZnPc molecule did not lead to late ROS formation on A253 and FaDu cell lines.

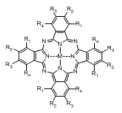
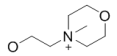
**Table 63** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with SQZnPc phthalocyanine at 720-740 nm (light fluence of 2 joules/cm$^2$).

| SQZnPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  | Cytotoxicity (WST-1) | 720-740 nm | 10 µg/ml | 58 | 50 | 85 | 64 |
| | | | 1 µg/ml | 91 | 84 | 98 | 85 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 86 | 81 | 79 | 84 |
| | | | 1 µg/ml | 101 | 91 | 78 | 95 |
| Center metal Zn | ROS (30 minutes) | | 10 µg/ml | 174 | 411 | 172 | 256 |
| | | | 1 µg/ml | 171 | 208 | 134 | 261 |
| R1  | ROS (24 hours) | | 10 µg/ml | 42 | 102 | 166 | 134 |
| | | | 1 µg/ml | 88 | 141 | 175 | 145 |

At wavelength of 720-740 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the high dose of the SQZnPc molecule was moderately measured in A253 and FaDu cell lines, but not on HT29 and T47D cell lines. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

SQZnPc-induced immediate ROS production was seen on each cell line in different degrees. The highest amount of ROS was seen on the FaDu cell line, it was followed by T47D, A253, and HT29 cell lines, respectively. Following PDT, late ROS formation was triggered with PDT of both doses of SQZnPc molecule on HT29, And T47D cell line. SQZnPc molecule did not lead to late ROS formation on A253 and FaDu cell lines.

**Table 64** Relative percentage cell viability and ROS formation of cancer cell lines after photodynamic therapy with SQZnPc phthalocyanine at 725-755 nm (light fluence of 2 joules/cm$^2$).

| SQZnPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
|  | Cytotoxicity (WST-1) | 725-755 nm | 10 µg/ml | 49 | 32 | 73 | 61 |
| | | | 1 µg/ml | 80 | 88 | 94 | 85 |
| | Cytotoxicity (Resazurin) | | 10 µg/ml | 72 | 69 | 76 | 82 |
| | | | 1 µg/ml | 101 | 87 | 73 | 93 |
| Center metal Zn | ROS (30 minutes) | | 10 µg/ml | 194 | 435 | 190 | 249 |
| | | | 1 µg/ml | 173 | 219 | 132 | 287 |
| R1  | ROS (24 hours) | | 10 µg/ml | 22 | 85 | 147 | 126 |
| | | | 1 µg/ml | 77 | 135 | 168 | 136 |

At wavelength of 725-755 nm with 2 joules/cm$^2$ light fluence, photo-induced cellular damage caused by the high dose of the SQZnPc molecule was moderately measured in A253 and FaDu cell lines, but not on HT29 and T47D cell lines. Considering 10 percent difference is acceptable, there were some differences above this limit between WST-1 and resazurin assay. However, according to general trends of viability depend on the Pc concentration, the resazurin assay results mostly supported the WST-1 assay.

SQZnPc-induced immediate ROS production was seen on each cell line in different degrees. The highest amount of ROS was seen on the FaDu cell line, it was followed by T47D, A253, and HT29 cell lines, respectively. Following PDT, late ROS formation was triggered with PDT of both doses of SQZnPc molecule on HT29, And T47D cell line. SQZnPc molecule did not lead to late ROS formation on A253 and FaDu cell lines.

## 4.11. Statistical Analysis

For each assay, data were represented as mean ± SD of 3 independent experimental repeats. Statistical analysis for comparison of the data relative to the non-treated control was done using the two-way ANOVA. The statistic studies of cytotoxic response to the molecule was carried out based on WST-1 assay results.

**Table 65** Significance of EH1-mediated PDT (at 624 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| EH1 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R2<br>  | Cytotoxicity | 624 nm | 10 µg/ml | *** | *** | ** | ** |
| | | | 1 µg/ml | *** | *** | ** | ns |
| | ROS (30 minutes) | | 10 µg/ml | ** | * | ns | ** |
| | | | 1 µg/ml | ns | ** | * | ns |
| | ROS (24 hours) | | 10 µg/ml | * | ** | ns | ** |
| | | | 1 µg/ml | ** | *** | ** | ns |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 66** Significance of EH1-mediated PDT (at 650-670 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| EH1 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R2<br>  | Cytotoxicity | 650-670 nm | 10 µg/ml | *** | *** | ** | ** |
| | | | 1 µg/ml | ** | *** | ** | * |
| | ROS (30 minutes) | | 10 µg/ml | *** | * | * | ** |
| | | | 1 µg/ml | ns | ns | * | * |
| | ROS (24 hours) | | 10 µg/ml | ns | *** | ns | ** |
| | | | 1 µg/ml | * | *** | ** | * |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 67** Significance of EH1-mediated PDT (at 690-710 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| EH1 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R2<br>  | Cytotoxicity | 690-710 nm | 10 µg/ml | *** | *** | ** | ** |
| | | | 1 µg/ml | *** | *** | * | ns |
| | ROS (30 minutes) | | 10 µg/ml | *** | ns | * | ** |
| | | | 1 µg/ml | * | * | * | ns |
| | ROS (24 hours) | | 10 µg/ml | ** | *** | * | ** |
| | | | 1 µg/ml | ns | *** | ns | ns |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 68** Significance of EH1-mediated PDT (at 720-740 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| EH1 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R2<br>  | Cytotoxicity | 720-740 nm | 10 µg/ml | *** | *** | ** | ** |
| | | | 1 µg/ml | *** | *** | * | ** |
| | ROS (30 minutes) | | 10 µg/ml | ** | ** | * | ** |
| | | | 1 µg/ml | * | ns | ns | ** |
| | ROS (24 hours) | | 10 µg/ml | ** | ** | * | ** |
| | | | 1 µg/ml | ns | * | * | ** |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 69** Significance of EH1-mediated PDT (at 725-755 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| EH1 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R2<br>  | Cytotoxicity | 725-755 nm | 10 µg/ml | *** | *** | ** | *** |
| | | | 1 µg/ml | *** | *** | * | ns |
| | ROS (30 minutes) | | 10 µg/ml | ** | ** | ** | *** |
| | | | 1 µg/ml | ** | ns | ns | ns |
| | ROS (24 hours) | | 10 µg/ml | * | ** | ns | *** |
| | | | 1 µg/ml | * | *** | * | ns |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 70** Significance of EH2-mediated PDT (at 624 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| EH2 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R1<br>  | Cytotoxicity | 624 nm | 10 µg/ml | *** | *** | ** | * |
| | | | 1 µg/ml | * | ns | ns | ns |
| | ROS (30 minutes) | | 10 µg/ml | ns | ns | ** | * |
| | | | 1 µg/ml | ns | ** | ** | ns |
| | ROS (24 hours) | | 10 µg/ml | * | ** | ns | * |
| | | | 1 µg/ml | ns | *** | * | ns |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 71** Significance of EH2-mediated PDT (at 650-670 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| EH2 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R1<br>  | Cytotoxicity | 650-670 nm | 10 µg/ml | *** | *** | ** | ** |
| | | | 1 µg/ml | * | ns | ns | ns |
| | ROS (30 minutes) | | 10 µg/ml | * | ns | ns | ** |
| | | | 1 µg/ml | ns | * | ns | ns |
| | ROS (24 hours) | | 10 µg/ml | ** | ** | ns | ** |
| | | | 1 µg/ml | * | ** | ns | ns |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 72** Significance of EH2-mediated PDT (at 690-710 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| EH2 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R1<br>  | Cytotoxicity | 690-710 nm | 10 µg/ml | *** | *** | ** | * |
| | | | 1 µg/ml | * | ** | ns | ns |
| | ROS (30 minutes) | | 10 µg/ml | * | * | ns | * |
| | | | 1 µg/ml | ns | * | * | ns |
| | ROS (24 hours) | | 10 µg/ml | * | ** | ns | * |
| | | | 1 µg/ml | ns | * | * | ns |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 73** Significance of EH2-mediated PDT (at 720-740 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| EH2 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R1<br>  | Cytotoxicity | 720-740 nm | 10 µg/ml | *** | *** | ** | * |
| | | | 1 µg/ml | * | ns | ns | *** |
| | ROS (30 minutes) | | 10 µg/ml | ** | ns | ns | * |
| | | | 1 µg/ml | * | ns | ns | *** |
| | ROS (24 hours) | | 10 µg/ml | ** | * | ns | * |
| | | | 1 µg/ml | * | ** | ns | *** |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 74** Significance of EH2-mediated PDT (at 725-755 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| EH2 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R1<br>  | Cytotoxicity | 725-755 nm | 10 µg/ml | *** | *** | ** | * |
| | | | 1 µg/ml | * | * | ns | *** |
| | ROS (30 minutes) | | 10 µg/ml | ns | ns | ns | * |
| | | | 1 µg/ml | ns | ns | * | *** |
| | ROS (24 hours) | | 10 µg/ml | * | ** | ns | * |
| | | | 1 µg/ml | * | *** | ns | *** |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 75** Significance of EH3-mediated PDT (at 624 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| EH3 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R1<br>  | Cytotoxicity | 624 nm | 10 µg/ml | ** | *** | ** | *** |
| | | | 1 µg/ml | ** | *** | ** | * |
| | ROS (30 minutes) | | 10 µg/ml | ** | * | ns | *** |
| | | | 1 µg/ml | * | ns | * | * |
| | ROS (24 hours) | | 10 µg/ml | ns | ** | ** | *** |
| | | | 1 µg/ml | ns | ** | * | * |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 76** Significance of EH3-mediated PDT (at 650-670 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| EH3 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R1<br>  | Cytotoxicity | 650-670 nm | 10 µg/ml | *** | *** | ** | ** |
| | | | 1 µg/ml | *** | *** | * | ** |
| | ROS (30 minutes) | | 10 µg/ml | ** | * | ** | ** |
| | | | 1 µg/ml | ** | ns | * | ** |
| | ROS (24 hours) | | 10 µg/ml | ns | *** | *** | ** |
| | | | 1 µg/ml | ns | ** | ns | ** |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 77** Significance of EH3-mediated PDT (at 690-710 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| EH3 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R1<br>  | Cytotoxicity | 690-710 nm | 10 µg/ml | *** | *** | ** | * |
| | | | 1 µg/ml | ** | *** | * | *** |
| | ROS (30 minutes) | | 10 µg/ml | * | ** | ** | * |
| | | | 1 µg/ml | * | ns | ** | *** |
| | ROS (24 hours) | | 10 µg/ml | ** | ** | ** | * |
| | | | 1 µg/ml | ** | ns | ** | *** |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 78** Significance of EH3-mediated PDT (at 720-740 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| EH3 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R1<br>  | Cytotoxicity | 740-740 nm | 10 µg/ml | *** | *** | ** | * |
| | | | 1 µg/ml | *** | *** | ** | * |
| | ROS (30 minutes) | | 10 µg/ml | ** | ** | ** | * |
| | | | 1 µg/ml | ns | ns | ** | * |
| | ROS (24 hours) | | 10 µg/ml | * | ns | * | * |
| | | | 1 µg/ml | ns | ** | ns | * |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 79** Significance of EH3-mediated PDT (at 725-755 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| EH3 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R1<br>  | Cytotoxicity | 725-755 nm | 10 µg/ml | *** | *** | ** | ** |
| | | | 1 µg/ml | ** | *** | ** | * |
| | ROS (30 minutes) | | 10 µg/ml | ** | ns | ** | ** |
| | | | 1 µg/ml | * | ** | * | * |
| | ROS (24 hours) | | 10 µg/ml | *** | * | *** | ** |
| | | | 1 µg/ml | * | ** | ns | * |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 80** Significance of EH4-mediated PDT (at 624 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| EH4 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R2<br>  | Cytotoxicity | 624 nm | 10 µg/ml | *** | *** | ** | ** |
| | | | 1 µg/ml | *** | *** | ns | ns |
| | ROS (30 minutes) | | 10 µg/ml | ** | * | * | ** |
| | | | 1 µg/ml | * | ns | * | ns |
| | ROS (24 hours) | | 10 µg/ml | ns | *** | *** | ** |
| | | | 1 µg/ml | *** | *** | *** | ns |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 81** Significance of EH4-mediated PDT (at 650-670 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| EH4 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R2<br>  | Cytotoxicity | 650-670 nm | 10 µg/ml | *** | *** | ** | *** |
| | | | 1 µg/ml | ** | *** | * | ns |
| | ROS (30 minutes) | | 10 µg/ml | ** | * | ** | *** |
| | | | 1 µg/ml | * | * | * | ns |
| | ROS (24 hours) | | 10 µg/ml | * | *** | ** | *** |
| | | | 1 µg/ml | *** | *** | ** | ns |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 82** Significance of EH4-mediated PDT (at 690-710 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| EH4 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R2<br>  | Cytotoxicity | 690-710 nm | 10 µg/ml | *** | *** | ** | ** |
| | | | 1 µg/ml | ** | ** | ns | ** |
| | ROS (30 minutes) | | 10 µg/ml | ** | * | *** | ** |
| | | | 1 µg/ml | ** | * | * | ** |
| | ROS (24 hours) | | 10 µg/ml | ns | *** | ** | ** |
| | | | 1 µg/ml | *** | ** | * | ** |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 83** Significance of EH4-mediated PDT (at 720-740 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| EH4 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R2<br>  | Cytotoxicity | 720-740 nm | 10 µg/ml | *** | *** | ** | ** |
| | | | 1 µg/ml | ** | ** | ns | * |
| | ROS (30 minutes) | | 10 µg/ml | *** | * | *** | ** |
| | | | 1 µg/ml | *** | * | ** | * |
| | ROS (24 hours) | | 10 µg/ml | * | *** | ns | ** |
| | | | 1 µg/ml | ns | ** | * | * |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 84** Significance of EH4-mediated PDT (at 725-755 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| EH4 | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R2<br>  | Cytotoxicity | 725-755 nm | 10 µg/ml | *** | *** | ns | *** |
| | | | 1 µg/ml | ** | ** | ns | ** |
| | ROS (30 minutes) | | 10 µg/ml | ** | * | ** | *** |
| | | | 1 µg/ml | * | ns | * | ** |
| | ROS (24 hours) | | 10 µg/ml | ** | *** | * | *** |
| | | | 1 µg/ml | ns | *** | ns | ** |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 85** Significance of $HQH_2Pc$-mediated PDT (at 624 nm with light fluence 2 joules/$cm^2$), two-way ANOVA results.

| HQH2Pc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal 2H<br>R2  | Cytotoxicity | 624 nm | 10 µg/ml | ns | ** | ns | ** |
| | | | 1 µg/ml | * | ns | ns | ** |
| | ROS (30 minutes) | | 10 µg/ml | ns | ns | ns | ** |
| | | | 1 µg/ml | ns | ns | ns | ** |
| | ROS (24 hours) | | 10 µg/ml | ns | ** | ns | ** |
| | | | 1 µg/ml | *** | *** | ** | ** |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 86** Significance of $HQH_2Pc$-mediated PDT (at 650-670 nm with light fluence 2 joules/$cm^2$), two-way ANOVA results.

| HQH2Pc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal 2H<br>R2  | Cytotoxicity | 650-670 nm | 10 µg/ml | * | ** | ns | ** |
| | | | 1 µg/ml | ns | ns | ns | ** |
| | ROS (30 minutes) | | 10 µg/ml | * | ns | ns | ** |
| | | | 1 µg/ml | ns | ns | ns | ** |
| | ROS (24 hours) | | 10 µg/ml | ns | ** | ns | ** |
| | | | 1 µg/ml | ns | ** | ns | ** |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 87** Significance of $HQH_2Pc$-mediated PDT (at 690-710 nm with light fluence 2 joules/$cm^2$), two-way ANOVA results.

| HQH2Pc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal 2H<br>R2  | Cytotoxicity | 690-710 nm | 10 µg/ml | ns | * | ns | *** |
| | | | 1 µg/ml | * | ns | ns | ** |
| | ROS (30 minutes) | | 10 µg/ml | * | ns | ns | *** |
| | | | 1 µg/ml | ns | ns | ns | ** |
| | ROS (24 hours) | | 10 µg/ml | ** | ** | * | *** |
| | | | 1 µg/ml | ns | *** | ns | ** |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 88** Significance of $HQH_2Pc$-mediated PDT (at 720-740 nm with light fluence 2 joules/$cm^2$), two-way ANOVA results.

| HQH2Pc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal 2H<br>R2  | Cytotoxicity | 720-740 nm | 10 µg/ml | ns | * | ns | ** |
| | | | 1 µg/ml | ns | ns | ns | *** |
| | ROS (30 minutes) | | 10 µg/ml | ** | ns | ns | ** |
| | | | 1 µg/ml | ns | ns | ns | *** |
| | ROS (24 hours) | | 10 µg/ml | ** | *** | ** | ** |
| | | | 1 µg/ml | ** | *** | *** | *** |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 89** Significance of $HQH_2Pc$-mediated PDT (at 725-755 nm with light fluence 2 joules/$cm^2$), two-way ANOVA results.

| HQH2Pc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal 2H<br>R2  | Cytotoxicity | 725-755 nm | 10 µg/ml | ns | * | ns | * |
| | | | 1 µg/ml | * | ns | ns | *** |
| | ROS (30 minutes) | | 10 µg/ml | * | ns | * | * |
| | | | 1 µg/ml | ns | ns | * | *** |
| | ROS (24 hours) | | 10 µg/ml | *** | ** | *** | * |
| | | | 1 µg/ml | ** | *** | ** | *** |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 90** Significance of HQZnPc-mediated PDT (at 624 nm with light fluence 2 joules/$cm^2$), two-way ANOVA results.

| HQZnPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R2  | Cytotoxicity | 624 nm | 10 µg/ml | *** | *** | * | ns |
| | | | 1 µg/ml | ** | ns | ns | * |
| | ROS (30 minutes) | | 10 µg/ml | * | ns | ns | ns |
| | | | 1 µg/ml | * | * | ** | * |
| | ROS (24 hours) | | 10 µg/ml | ** | ns | *** | ns |
| | | | 1 µg/ml | *** | ** | ** | * |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 91** Significance of HQZnPc-mediated PDT (at 650-670 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| HQZnPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R2<br>  | Cytotoxicity | 650-670 nm | 10 µg/ml | *** | *** | ** | * |
| | | | 1 µg/ml | ** | ns | ns | ns |
| | ROS (30 minutes) | | 10 µg/ml | * | * | ns | * |
| | | | 1 µg/ml | ns | * | ns | ns |
| | ROS (24 hours) | | 10 µg/ml | ** | ns | ** | * |
| | | | 1 µg/ml | ns | ** | ns | ns |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 92** Significance of HQZnPc-mediated PDT (at 690-710 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| HQZnPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R2<br>  | Cytotoxicity | 690-710 nm | 10 µg/ml | ** | *** | ** | * |
| | | | 1 µg/ml | *** | ns | ns | ns |
| | ROS (30 minutes) | | 10 µg/ml | ** | ** | * | * |
| | | | 1 µg/ml | *** | ns | * | ns |
| | ROS (24 hours) | | 10 µg/ml | ns | * | ns | * |
| | | | 1 µg/ml | *** | ** | ** | ns |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 93** Significance of HQZnPc-mediated PDT (at 720-740 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| HQZnPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R2<br>  | Cytotoxicity | 720-740 nm | 10 µg/ml | ** | *** | * | ** |
| | | | 1 µg/ml | * | ns | ns | ns |
| | ROS (30 minutes) | | 10 µg/ml | *** | * | ** | ** |
| | | | 1 µg/ml | ns | ns | ns | ns |
| | ROS (24 hours) | | 10 µg/ml | ns | * | ns | ** |
| | | | 1 µg/ml | * | *** | * | ns |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 94** Significance of HQZnPc-mediated PDT (at 725-755 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| HQZnPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R2<br>  | Cytotoxicity | 725-755 nm | 10 µg/ml | *** | *** | ** | * |
| | | | 1 µg/ml | * | ns | ns | ns |
| | ROS (30 minutes) | | 10 µg/ml | ** | * | * | * |
| | | | 1 µg/ml | ** | ns | * | ns |
| | ROS (24 hours) | | 10 µg/ml | ** | * | * | * |
| | | | 1 µg/ml | *** | ** | *** | ns |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 95** Significance of HQInPc-mediated PDT (at 624 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| HQInPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal InCl<br>R2<br>  | Cytotoxicity | 624 nm | 10 µg/ml | *** | *** | ** | ** |
| | | | 1 µg/ml | *** | *** | * | *** |
| | ROS (30 minutes) | | 10 µg/ml | *** | ns | ** | ** |
| | | | 1 µg/ml | * | ns | ** | *** |
| | ROS (24 hours) | | 10 µg/ml | ns | * | ns | ** |
| | | | 1 µg/ml | *** | ** | *** | *** |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 96** Significance of HQZnPc-mediated PDT (at 650-670 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| HQZnPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R2<br>  | Cytotoxicity | 650-670 nm | 10 µg/ml | *** | *** | ** | * |
| | | | 1 µg/ml | ** | ns | ns | ns |
| | ROS (30 minutes) | | 10 µg/ml | * | * | ns | * |
| | | | 1 µg/ml | ns | * | ns | ns |
| | ROS (24 hours) | | 10 µg/ml | ** | ** | ** | * |
| | | | 1 µg/ml | ns | ** | ns | ns |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 97** Significance of HQZnPc-mediated PDT (at 690-710 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| HQInPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal InCl<br>R2<br>  | Cytotoxicity | 690-710 nm | 10 µg/ml | *** | *** | ** | *** |
| | | | 1 µg/ml | ** | *** | ns | ** |
| | ROS (30 minutes) | | 10 µg/ml | ** | ns | ns | *** |
| | | | 1 µg/ml | * | * | ns | ** |
| | ROS (24 hours) | | 10 µg/ml | ns | *** | * | *** |
| | | | 1 µg/ml | ** | ** | ** | ** |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 98** Significance of HQZnPc-mediated PDT (at 720-740 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| HQInPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal InCl<br>R2<br>  | Cytotoxicity | 720-740 nm | 10 µg/ml | *** | *** | ** | ** |
| | | | 1 µg/ml | * | *** | ns | ** |
| | ROS (30 minutes) | | 10 µg/ml | ** | ns | ns | ** |
| | | | 1 µg/ml | * | ns | ns | ** |
| | ROS (24 hours) | | 10 µg/ml | * | * | * | ** |
| | | | 1 µg/ml | * | ** | ** | ** |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 99** Significance of HQZnPc-mediated PDT (at 725-755 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| HQInPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal InCl<br>R2<br>  | Cytotoxicity | 725-755 nm | 10 µg/ml | *** | *** | ** | *** |
| | | | 1 µg/ml | *** | *** | ns | *** |
| | ROS (30 minutes) | | 10 µg/ml | * | * | * | *** |
| | | | 1 µg/ml | ns | ns | ns | *** |
| | ROS (24 hours) | | 10 µg/ml | ** | ** | ** | *** |
| | | | 1 µg/ml | ns | ** | * | *** |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 100** Significance of $SQH_2Pc$-mediated PDT (at 624 nm with light fluence 2 joules/$cm^2$), two-way ANOVA results.

| SQH2Pc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal 2H<br>R1  | Cytotoxicity | 624 nm | 10 µg/ml | ** | ** | *** | ** |
| | | | 1 µg/ml | ns | ns | ns | * |
| | ROS (30 minutes) | | 10 µg/ml | * | * | ** | ** |
| | | | 1 µg/ml | ns | ** | * | * |
| | ROS (24 hours) | | 10 µg/ml | ns | * | ns | ** |
| | | | 1 µg/ml | *** | ** | ** | * |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 101** Significance of $SQH_2Pc$-mediated PDT (at 650-670 nm with light fluence 2 joules/$cm^2$), two-way ANOVA results.

| SQH2Pc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal 2H<br>R1  | Cytotoxicity | 650-670 nm | 10 µg/ml | *** | *** | ** | ** |
| | | | 1 µg/ml | ns | ns | ns | ns |
| | ROS (30 minutes) | | 10 µg/ml | ns | ns | ns | ** |
| | | | 1 µg/ml | * | * | * | ns |
| | ROS (24 hours) | | 10 µg/ml | ns | ** | ns | ** |
| | | | 1 µg/ml | ** | ** | *** | ns |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 102** Significance of $SQH_2Pc$-mediated PDT (at 670-710 nm with light fluence 2 joules/$cm^2$), two-way ANOVA results.

| SQH2Pc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal 2H<br>R1  | Cytotoxicity | 690-710 nm | 10 µg/ml | ** | ** | ** | ** |
| | | | 1 µg/ml | ns | * | ns | * |
| | ROS (30 minutes) | | 10 µg/ml | * | * | ns | ** |
| | | | 1 µg/ml | * | * | ns | * |
| | ROS (24 hours) | | 10 µg/ml | * | ** | * | ** |
| | | | 1 µg/ml | ** | ** | * | * |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 103** Significance of $SQH_2Pc$-mediated PDT (at 720-740 nm with light fluence 2 joules/$cm^2$), two-way ANOVA results.

| SQH2Pc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal 2H<br>R1  | Cytotoxicity | 720-740 nm | 10 µg/ml | *** | ** | ** | ** |
| | | | 1 µg/ml | ns | * | ns | ** |
| | ROS (30 minutes) | | 10 µg/ml | ns | ns | ns | ** |
| | | | 1 µg/ml | ns | ns | ns | ** |
| | ROS (24 hours) | | 10 µg/ml | ** | *** | ** | ** |
| | | | 1 µg/ml | ns | ** | ns | ** |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 104** Significance of $SQH_2Pc$-mediated PDT (at 725-755 nm with light fluence 2 joules/$cm^2$), two-way ANOVA results.

| SQH2Pc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal 2H<br>R1  | Cytotoxicity | 725-755 nm | 10 µg/ml | *** | *** | ** | ** |
| | | | 1 µg/ml | * | ns | ns | *** |
| | ROS (30 minutes) | | 10 µg/ml | ** | ns | * | ** |
| | | | 1 µg/ml | ** | * | ns | *** |
| | ROS (24 hours) | | 10 µg/ml | *** | ** | ** | ** |
| | | | 1 µg/ml | ns | ns | ns | *** |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 105** Significance of SQZnPc-mediated PDT (at 624 nm with light fluence 2 joules/$cm^2$), two-way ANOVA results.

| SQZnPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R1  | Cytotoxicity | 624 nm | 10 µg/ml | *** | *** | ns | * |
| | | | 1 µg/ml | ns | * | ns | ** |
| | ROS (30 minutes) | | 10 µg/ml | * | * | ns | * |
| | | | 1 µg/ml | ns | ns | ns | ** |
| | ROS (24 hours) | | 10 µg/ml | ** | ** | ** | * |
| | | | 1 µg/ml | ns | *** | * | ** |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 106** Significance of SQZnPc-mediated PDT (at 650-670 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| SQZnPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R1<br>  | Cytotoxicity | 650-670 nm | 10 µg/ml | ** | *** | * | ** |
| | | | 1 µg/ml | ns | * | ns | * |
| | ROS (30 minutes) | | 10 µg/ml | ** | ** | * | ** |
| | | | 1 µg/ml | ns | ns | * | * |
| | ROS (24 hours) | | 10 µg/ml | *** | ** | ** | ** |
| | | | 1 µg/ml | * | ns | ns | * |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 107** Significance of SQZnPc-mediated PDT (at 690-710 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| SQZnPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R1<br>  | Cytotoxicity | 690-710 nm | 10 µg/ml | ** | ** | ns | ** |
| | | | 1 µg/ml | * | * | ns | ** |
| | ROS (30 minutes) | | 10 µg/ml | * | ** | *** | ** |
| | | | 1 µg/ml | ns | ns | ns | ** |
| | ROS (24 hours) | | 10 µg/ml | * | * | * | ** |
| | | | 1 µg/ml | ns | ** | ns | ** |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 108** Significance of SQZnPc-mediated PDT (at 720-740 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| SQZnPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R1<br>  | Cytotoxicity | 720-740 nm | 10 µg/ml | *** | *** | ns | * |
| | | | 1 µg/ml | * | * | ns | ** |
| | ROS (30 minutes) | | 10 µg/ml | ** | ** | *** | * |
| | | | 1 µg/ml | * | ns | * | ** |
| | ROS (24 hours) | | 10 µg/ml | *** | *** | *** | * |
| | | | 1 µg/ml | ** | ** | ** | ** |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

**Table 109** Significance of SQZnPc-mediated PDT (at 725-755 nm with light fluence 2 joules/cm$^2$), two-way ANOVA results.

| SQZnPc | Assay | Wavelength | Concentration | A253 | FaDu | HT29 | T47D |
|---|---|---|---|---|---|---|---|
| Center metal Zn<br>R1<br>  | Cytotoxicity | 725-755 nm | 10 µg/ml | *** | ** | ns | * |
| | | | 1 µg/ml | * | * | * | ** |
| | ROS (30 minutes) | | 10 µg/ml | * | ** | ** | * |
| | | | 1 µg/ml | * | ns | ** | ** |
| | ROS (24 hours) | | 10 µg/ml | ** | ns | * | * |
| | | | 1 µg/ml | *** | ** | ** | ** |

p-value is less than 0.05, marked with one star (*), p-value is less than 0.01, marked with two stars (**), p-value is less than 0.001, marked with three stars (***), ns for not significant.

# 5. DISCUSSION

Photodynamic therapy is two-step cancer treatment alternatives to other treatments with its low side effects. Photosensitizer, light and molecular oxygen are the components of PDT which have an impact on the efficiency of the treatment. In this thesis study, in vitro characterization of nine specifically synthesized cationic phthalocyanine molecules has been studied.

In the light of the aim, several questions were studied in order to understand "in vitro PDT effects" of the molecules. Firstly, cellular uptake capacities of the molecules were studied. Furthermore, biocompatibility and appropriate concentrations were also calculated where the molecules did not have cytotoxic effects on the cell lines under dark conditions, and proper light fluence for PDT was determined.

After all these experiments, PDT-mediated cytotoxicity and ROS formation were evaluated at the determined concentration and light fluency.

To see the biocompatibility of the EH1, EH2, EH3, EH4, $HQH_2Pc$, HQZnPc, HQInPc, and $SQH_2Pc$, and SQZnPc Cationic Tetra Substituted Symmetrical Phthalocyanine molecules, cytotoxicity assay was performed on the L929 line as considering prescription of International Organization for Standardization. Effect of different doses of cytotoxicity in the dark environment (10 µg /mL and 1 µg /mL) were determined and then was used as working doses for the following studies. As a result of the light studies performed in the visible spectrum, the molecules were considered as biocompatible at the determined doses.

Cytotoxicity studies were performed by WST-1 assay in four different concentrations to determine the concentration range without toxic effects on A253,

FaDu, and HT29 and T47D cell lines in the dark environment. Phthalocyanines at a dose of 1 μg/mL and 10 μg/mL with a viability of 70% and above were determined as the non-toxic range.

Light-irradiated cytotoxicity studies were performed at two concentrations, 1 μg/mL and 10 μg/mL determined as non-toxic by dark cytotoxicity study. This study was carried out by WST-1 assay and verified by resazurin assay.

Although the molecules have absorbance spectrums at wavelengths with higher energies such as 300 nm, these wavelengths are not sufficient for depth penetration into the tissues. The other absorbance range (600-800 nm) of these molecules is suitable for PDT applications. For each molecule, their specific wavelengths in their absorbance spectrums which is higher than 600 nm were considered. However, at the other wavelengths (624-755 nm) that were studied, cellular response was obtained like their specific wavelengths. The reason for this situation could be that the wavelength intervals were close to the specific ones. Also, the absorbance spectrums could be affected from the ingredients of culture media since the molecules has different behavior in different substance such as DMSO and water.

Taking into account the absorption spectrum, at a wavelength of 650-670 nm with 2 joules/$cm^2$ of light fluence, EH1-mediated photodynamic therapy was the most effective on the FaDu cell line (with a p-value is smaller than 0.001 according to ANOVA analysis) where high and low doses of EH1 molecule lead to appx. 90 percent of cell death followed by HT29, A253, and T47D cell lines. EH1 induced immediate ROS production was seen the highest on FaDu cell line (with a p-value is smaller than 0.05 according to ANOVA analysis) caused by the high dose of the molecule with approx. 300% ROS production relative to non-treated control. It was followed by A253, T47D and HT29 cell lines, respectively. Further, EH1-induced PDT triggered late ROS production on FaDu cell line with approx. 10000% ROS production (with a p-value is smaller than 0.001 according to ANOVA analysis) relative to control,

followed by HT29, A253, and T47D cell lines, respectively. When gathering cytotoxicity and ROS results, it can be said that PDT-induced cytotoxicity of EH1 molecule was mostly dependent on ROS production.

Taking into account the absorption spectrum, at the wavelength of 650-670 nm with 2 joules/$cm^2$ light fluence, EH2-mediated photodynamic therapy was the most effective on FaDu cell line (with a p-value is smaller than 0.001 according to ANOVA analysis), followed by HT29, A253. EH2 induced immediate ROS production was seen only on A253 cell line (with a p-value is smaller than 0.05 according to ANOVA analysis) caused by the high dose of the molecule with approx. 150% ROS production relative to the non-treated control. Further, EH2-induced PDT triggered late ROS production on FaDu cell line (with a p-value is smaller than 0.01 according to ANOVA analysis) with approx. 750% ROS production relative to control, followed by HT29, and T47D cell lines, respectively. In contrast to the immediate ROS production of A253 cell line, there was no ROS measured lately. When gathering cytotoxicity and ROS results, it can be said that PDT-induced cytotoxicity of EH2 molecule was mostly dependent on ROS production, however, it can be triggered by immediate or late ROS production.

Taking into account the absorption spectrum, at the wavelength of 650-670 nm with 2 joules/$cm^2$ light fluence, EH3-mediated photodynamic therapy was the most effective on FaDu cell line (with a p-value is smaller than 0.001 according to ANOVA analysis) where both high and low doses of EH3 molecule lead to appx. 90% cell death relative to the non-treated control. It was followed by A253, HT29, and T47D cell lines. EH3 induced immediate ROS production was seen the highest on T47D cell line (with a p-value is smaller than 0.01 according to ANOVA analysis) caused by the high dose of the molecule with approx. 250% ROS production relative to the non-treated control. It was followed by FaDu, HT29, and A253 cell lines, respectively. Further, EH3-induced PDT triggered late ROS production on FaDu cell line (with a p-value is smaller than 0.001 according to ANOVA analysis) with approx. 10000% ROS production relative to control, followed by A253, HT29, and T47D cell lines,

respectively. When the cytotoxicity and ROS results were analyzed, it can be said that PDT-induced cytotoxicity of EH3 molecule was mostly dependent on ROS production.

Taking into account the absorption spectrum, at the wavelength of 650-670 nm with 2 joules/$cm^2$ light fluence, EH4-mediated photodynamic therapy was the most effective on FaDu cell line (with a p-value is smaller than 0.001 according to ANOVA analysis), followed by HT29, A253, and T47D cell lines. EH4 induced immediate ROS production was seen the highest on T47D cell line (with a p-value is smaller than 0.001 according to ANOVA analysis) caused by high dose of the molecule with approx. 350% ROS production relative to the non-treated control. It was followed by FaDu, A253, and HT29 cell lines, respectively. Further, EH4-induced PDT triggered late ROS production on FaDu cell line (with a p-value is smaller than 0.001 according to ANOVA analysis) with approx. 7500% ROS production relative to control, followed by A253, HT29, and T47D cell lines, respectively. When gathering cytotoxicity and ROS results, it can be said that PDT-induced cytotoxicity of EH4 molecule was mostly dependent on ROS production.

Taking into account the absorption spectrum, at the wavelength of 650-670 nm with 2 joules/$cm^2$ light fluence, $HQH_2Pc$-mediated photodynamic therapy did not trigger cellular damage on any cell lines. Further, neither immediate nor late PDT-induced ROS was not formed. $HQH_2Pc$ molecule is not suitable for PDT.

Taking into account the absorption spectrum, at the wavelength of 650-670 nm with 2 joules/$cm^2$ light fluence, HQZnPc-mediated photodynamic therapy was the most effective on FaDu cell line (with a p-value is smaller than 0.001 according to ANOVA analysis) where both high and low doses of HQZnPc molecule lead to appx. 90% cell death relative to the non-treated control. It was followed by A253, HT29, and T47D cell lines, respectively. HQZnPc induced immediate ROS production was seen the highest on T47D cell line (with a p-value is smaller than 0.05 according to ANOVA

analysis) caused by the high dose of the molecule with approx. 350% ROS production relative to the non-treated control. It was followed by FaDu, A253, and HT29 cell lines, respectively. Further, HQZnPc-induced PDT triggered late ROS production on FaDu cell line (with a p-value is smaller than 0.01 according to ANOVA analysis) with approx. 7500% ROS production relative to control, followed by HT29, T47D, and A253 cell lines, respectively. When gathering cytotoxicity and ROS results, it can be said that PDT-induced cytotoxicity of HQZnPc molecule was mostly dependent on ROS production.

Taking into account the absorption spectrum, at the wavelength of 650-670 nm with 2 joules/$cm^2$ light fluence, HQInPc-mediated photodynamic therapy was the most effective on FaDu cell line (with a p-value is smaller than 0.001 according to ANOVA analysis), followed by T47D, HT29, and A253 cell lines, respectively. HQInPc induced immediate ROS production was seen the highest on A253 cell line (with a p-value is smaller than 0.01 according to ANOVA analysis) caused by the high dose of the molecule with approx. 250% ROS production relative to the non-treated control. It was followed by T47D, FaDu, and HT29 cell lines, respectively. Further, HQInPc-induced PDT triggered late ROS production on T47D cell line (with a p-value is smaller than 0.01 according to ANOVA analysis) with approx. 400% ROS production relative to control, followed by FaDu, and HT29 cell lines, respectively. When gathering cytotoxicity and ROS results, it can be said that PDT-induced cytotoxicity of HQInPc molecule was mostly dependent on ROS production, however, it can be triggered by immediate or late ROS production.

Taking into account the absorption spectrum, at the wavelength of 690-710 nm with 2 joules/$cm^2$ light fluence, $SQH_2Pc$-mediated photodynamic therapy was the moderately effective only on FaDu and HT29 cell lines (with a p-value is smaller than 0.01 according to ANOVA analysis) when were applied with the high dose of the molecule. $SQH_2Pc$ induced immediate ROS production was seen the highest on A253 cell line (with a p-value is smaller than 0.05 according to ANOVA analysis) caused by the high dose of the molecule with approx. 150% ROS production relative to the non-

treated control. It was followed by FaDu cell line. Further, $SQH_2Pc$-induced PDT triggered late ROS production on HT29 cell line (with a p-value is smaller than 0.05 according to ANOVA analysis) with approx. 150% ROS production relative to control, followed by T47D cell line. When gathering cytotoxicity and ROS results, it can be said that PDT-induced cytotoxicity of $SQH_2Pc$ molecule was mostly dependent on ROS production, however, it can be triggered by immediate or late ROS production.

Taking into account the absorption spectrum, at the wavelength of 690-710 nm with 2 joules/$cm^2$ light fluence, SQZnPc-mediated photodynamic therapy was the moderately effective only on A253 and FaDu cell lines line (with a p-value is smaller than 0.01 according to ANOVA analysis). SQZnPc induced immediate ROS production was seen the highest on FaDu cell line line (with a p-value is smaller than 0.01 according to ANOVA analysis) caused by the high dose of the molecule with approx. 350% ROS production relative to the non-treated control. It was followed by T47D, A253, and HT29 cell lines, respectively. Further, SQZnPc-induced PDT triggered late ROS production on HT29 cell line line (with a p-value is smaller than 0.05 according to ANOVA analysis) with approx. 150% ROS production relative to control, followed by T47D cell line. When gathering cytotoxicity and ROS results, it can be said that PDT-induced cytotoxicity of SQZnPc molecule was mostly dependent on ROS production, however, it can be triggered by immediate or late ROS production.

When all the data that were presented among the phthalocyanine molecules were collected, the most cytotoxic response was obtained from EH1-induced PDT, followed by EH3, HQZnPc, EH4, SQZnPc, EH2, HQInPc, and SQH2Pc.

Two different methods (WST-1 and Resazurin methods) were used in cell viability studies and the results were compared. In most of the cases in the study, although the resazurin assay results were similar to the WST-1 assay, in some cases, there were some differences. Even though the experiments were repeated to eliminate the differences, the same differences were experienced. The results obtained by WST-

1 experiments have been considered more reliable and reproducible than those obtained by resazurin experiments. Since, for resazurin assay, the evaluation is based on fluorescence intensity whereas for WST-1 assay, it is based on absorbance, and each experiment was carried out using clear surface plates that are not suitable for fluorescence readings and can scatter taken fluoresces. For this reason, the results of WST-1 experiments were taken as a basis in cell viability / proliferation assays in this thesis study.

When the relationship between cytotoxicity and ROS production after Pc-mediated PDT was considered, it can be said that for each cancer cell line, the cytotoxic response was mostly depending on ROS formation following PDT.

It is known that the molecules can generate enough energy to form singlet oxygen. However, the PDT-mediated singlet oxygen formation on cells could not be studied because of its very short half-life. Therefore, in this study, we could only show PDT mediated responses of type-1 reaction (electron/photon is transferred from Pc to $O_2$) that triggers cellular damage through ROS. Further, at different times, PDT-mediated ROS amounts were measured. We may say that given the findings, PDT begins to trigger ROS formation right after the treatment. However, the most significant ROS formation was obtained later on the treatment. It could be better to take kinetic models of ROS formation to make more precious comments on when the highest amount of ROS produced after treatment.

In some cases, ROS production did not lead to cell death. This situation could be due to that each cell line requires a different amount of ROS to trigger cell death, and although there was ROS production, the requirement of the cell to trigger death could not be meet.

Further, the cytotoxic response against Pc-based PDT and cellular uptake capacities of Pc molecules was evaluated together. Flow cytometry assay at Fluorochrome (Ex-Max 650 nm/Em-Max 785 nm) to see cellular uptake of these nine phthalocyanine molecules on the A253, FaDu, HT29, and T47D cell lines due to their fluorescence was performed. Consequently, a higher capacity of binding is found at SQ group phthalocyanines, $SQH_2Pc$ and SQZnPc, further EH4 and EH2 showed relatively agreeable binding capacities on each cell lines relative to control. In contradiction to cellular uptake results, EH1, EH3, and HQZnPc molecules have higher light-dependent toxicity compared to the molecules that have higher binding capacities. This contradiction could be explained by the higher energy transfer capacities of these molecules.

In conclusion, at wavelength range between 624-755 nm with 2 joules/$cm^2$ light fluence, each cell line used in this thesis responded to Pc-based PDT, even to varying degrees. The highest cytotoxicity was mostly measured in the FaDu cell line. Between the phthalocyanine molecules, the most cytotoxic response was obtained from EH1-induced PDT, followed by EH3, HQZnPc, EH4, SQZnPc, EH2, HQInPc, and SQH2Pc. PDT-induced cytotoxicity of the molecules was mostly ROS-dependent. Each molecule also had cellular uptake capacities. Since their high binding capacities, fluorescence features, and non-cytotoxic effect at low dose of the EH2, SQH2Pc, and SQZnPc molecules, they can be used as a diagnostic reagent for solid tumors of cancer. According to the in vitro experimental results, Pc derivatives used in this thesis can be considered as strong candidates that can be used as photosensitizing agents for further studies due to their binding capacities, biocompatibility and high level of cancer cytotoxicity.

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# 7. RESUME

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|---|---|---|---|
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| | **Foreign Language Examination Grade** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| KPDS | ÜDS | IELTS | TOEFL IBT | TOEFL PBT | TOEFL CBT | FCE | CAE | CPE | YÖK DİL |
| | | | | | | | | | |

KPDS: Kamu Personeli Yabancı Dil Sınavı; ÜDS: ÜniversiteleraRası Kurul Yabancı Dil Sınavı; IELT S: International English Language T esting System; TOEFL IBT: Test of English as a Foreign Language-Internet-Based T est TOEFL PBT: T est of English as a Foreign Language-Paper-Based T est; TOEFL CBT: T est of English as a Foreign Language-Computer-Based T est; FCE: First Certificate in English;

CAE: Certificate in Advanced English; CPE: Certificate of Proficiency in English

| | **Quantitative** | **Equally Weighted** | **Verbal** |
|---|---|---|---|
| **ALES Note** | | | |

**Computer Skills**

| **Program** | |
|---|---|
| Gen5 | Image J |
| VMD | Office Package |
| NAMD | Novo Express |