INCREASING PHOTODYNAMIC THERAPY EFFICACY BY THE NATURAL COMPOUND CURCUMIN

by

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ACADEMIC ETHICS AND INTEGRITY STATEMENT

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ABSTRACT

INCREASING PHOTODYNAMIC THERAPY EFFICACY BY THE NATURAL COMPOUND CURCUMIN

Photodynamic therapy (PDT) is a cancer treatment in which an injected or applied photosensitizing agent is activated by light of a specific wavelength which causes a sequence of photochemical and photobiological processes resulting in irreversible selective damage to the target tissue. PDT is a promising antitumor treatment method for its high selectivity, non-invasiveness and minimal side effects. However, due to the resistance of some cancer cell lines to PDT, it exhibits results with low efficiency. Therefore, there is an urgent need to overcome this resistance to increase the PDT's efficiency, making PDT more widely applicable. Thus, this M.Sc. study introduces the usage of Curcumin, which is a non-toxic natural compound that has antitumor characteristics, with 5-ALA-PDT to increase PDT's efficacy. First, 5-ALA mediated PDT and Curcumin antitumor characteristics were evaluated on two cell lines, PC-3 and Caco-2. Then, the determined PDT doses were applied to the cell lines together with two different Curcumin concentrations. Illumination was performed using 635-nm diode laser after 6-hr incubation of 5-ALA and Curcumin. The outcomes of this study prove the success of the proposed combination on the highly resistive colon cancer (Caco-2 cell line) with 62.4% decrease in cell viability.

Keywords: Photodynamic Therapy, 5-ALA, Curcumin, PC-3, Caco-2.

ÖZET

DOĞAL BİLEŞİK ZERDEÇAL İLE FOTODİNAMİK TERAPİ ETKİNLİĞİNİN ARTIRILMASI

Fotodinamik terapi (FDT), hedef dokuya enjekte edilmiş veya uygulanmış bir ışığa duyarlılaştırıcı ajanın, belirli bir dalga boyunda ışıkla aktive edilmesi sonucu geri dönüşü olmayan seçici bir hasara yol açan bir fotokimyasal ve fotobiyolojik işlem dizisine neden olan bir kanser tedavisidir. FDT, yüksek seçiciliği, invazif olmaması ve yan etkilerinin minimal olması sebebiyle umut verici bir antitümör tedavi yöntemidir. Bununla birlikte, bazı kanser hücre hatlarının FDT'ye direnci nedeniyle, düşük verimli sonuçlar vermektedir. Bu nedenle, FDT'nin verimliliğini artırmak ve daha yaygın şekilde uygulanmasını sağlamak için bu direncin üstesinden gelmeye acil bir ihtiyaç vardır. Bu sebeple, bu Yüksek Lisans çalışması, FDT'nin etkinliğini arttırmak için antitümör özelliklerine sahip toksik olmayan bir doğal bileşik olan Zerdeçal'ın, 5-ALA-FDT ile kullanımını ortaya koymaktadır. İlk önce, 5-ALA aracılı FDT ve Zerdeçal'ın antitümör özellikleri, PC-3 ve Caco-2 olmak üzere iki hücre hatlarında değerlendirildi. Daha sonra, belirlenen FDT dozları, iki farklı Zerdeçal konsantrasyonuyla birlikte hücre hatlarına uygulandı. ışıklandırma, 6-saatlik 5-ALA ve Zerdeçal inkübasyonundan sonra 635-nm diyot lazer kullanılarak yapıldı. Bu çalışmanın sonuçları, önerilen kombinasyonun yüksek dirençli kolon kanseri (Caco-2 hücre hattı) üzerindeki başarısını kanıtlamaktadır ve hücre canlılığında % 62.4 azalma göstermiştir.

Anahtar Sözcükler: Fotodinamik Terapi, 5-ALA, Zerdeçal, PC-3, Caco-2.

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LIST OF SYMBOLS

J	Joule
W	Watt
J/cm^2	Energy Density
W/cm^2	Power Density
CO_2	Carbon dioxide
OD	Optical density
A	Absorption
M	Molar
$^1O_2$	Singlet state Oxygen
Fe^{2+}	Ferrous ion

LIST OF ABBREVIATIONS

PDT	Photodynamic Therapy
5-ALA	5-Aminolevulinic acid
Caco-2	Human colorectal adenocarcinoma cancer cell line
PC-3	Human prostate adenocarcinoma cell line
ANOVA	Analysis of variance
FDA	U. S. Food an Drug Administration
ROS	Reactive oxygen species
FBS	Fatal Bovine Serum
MTT	3-(4,5-Dimethyiazol-2-yl)-2,5 diphenyltetrazolium bromide)
SPSS	Statistical Package for the Social Sciences
NO	Nitric Oxide
INOS	Inducible Nitric Oxide synthase
DMSO	Dimethyl Sulfoxide
RPMI	Roswell Park Memorial Institute medium
PBS	Phosphate Buffered Saline
CW	Continuous Wave
LASER	Light Amplification by Stimulated Emission of Radiation
EPR	Enhanced permeability and retention
UV	Ultraviolet
VIS	Visible
OXPHOS	Oxidative phosphorylation
siRNA	small interfering RNA
EGFR	epidermal growth factor receptor

1. INTRODUCTION

1.1 Motivation

Cancer is considered one of the leading causes of death worldwide [1]. Moreover, current treatment methods, such as surgical resection, chemo- and radiotherapy, show variable results and have life-threatening side effects. Researchers have always wondered how to improve current treatment methods or how to provide a novel alternative treatment. PDT is considered as a promising treatment method for its auspicious properties. However, due to the resistance of some cancer cell lines to PDT, it has low efficacy and thus limiting its use. Therefore, there is an urgent need for improving PDT results.

Combined treatment is considered one strategy for having higher efficacy and better survival rate. Curcumin, recently, has been extensively studied for its antitumor and antibacterial properties. Moreover, Curcumin suppresses the growth and proliferation of several tumor cell lines and the activation of many transcription factors that are involved in carcinogenesis, such as inducible Nitric Oxide synthase [2]. Furthermore, Nitric Oxide is considered one of the main causes of cancer cells' resistance to PDT [3]. But up to now, there is no study of 5-ALA mediated PDT in combination with Curcumin.

The main goal of this study is to test whether adding Curcumin to 5-ALA mediated PDT overcomes cancer cells' resistance to PDT and therefore, increases PDT's efficacy on PC-3 and Caco-2 cancer cell lines.

1.2 Objectives

In this study our main hypothesis was that adding the natural compound Curcumin to 5-ALA mediated PDT will increase PDT's antitumor efficacy . To test our hypothesis, we:

- First, examined the efficacy of 5-ALA mediated PDT on PC-3 and Caco-2 cells at different energy densities.
- Then, tested the anti-cancer characteristics of different concentrations of Curcumin on PC-3 and Caco-2 cells.
- Finally, investigated the curcumin's potentiation of 5-ALA PDT on PDT non-resistive and PDT resistive cell lines, PC-3 and Caco-2 respectively.

1.3 Outline

Chapter 1: An Introduction including the Motivation and goals of the thesis.

Chapter 2: Background information about PDT, Cancer cell's resistance mechanisms and PDT Potentiators.

Chapter 3: Materials and Methods used during the experimental phase of the thesis.

Chapter 4: The results are presented.

Chapter 5: Discussion on the outcomes of the thesis.

Chapter 6: The conclusion is stated.

1.4 List of publications produced from the thesis

- Şueki F, Ruhi MK, Gülsoy M, The Effect of Curcumin in Antitumor Photodynamic Therapy: *in vitro* experiments with Caco2 and PC-3 Cancer Lines, Photodiagnosis and Photodynamic Therapy (2019), Accepted Manuscript. https://doi.org/10.1016/j.pdpdt.2019.05.012

2. BACKGROUND

2.1 Photodynamic Therapy (PDT)

Photodynamic therapy or PDT refers to the treatment in which administration of photosensitizing agent, called photosensitizer, causes a sequence of photochemical and photobiological processes, following its activation by a light source with a specific wavelength. As a result, these reactions cause irreversible selective damage to the target tissue [4, 5, 6]. For PDT to be effective, an adequate concentration of oxygen molecules must be present.

PDT has many advantages over alternative treatment methods, which make it a very promising method for curing diseases such as cancer. These advantages include selectivity, comparatively non-invasiveness, causes no side-effects and the healing process shows little or no scarring [7].

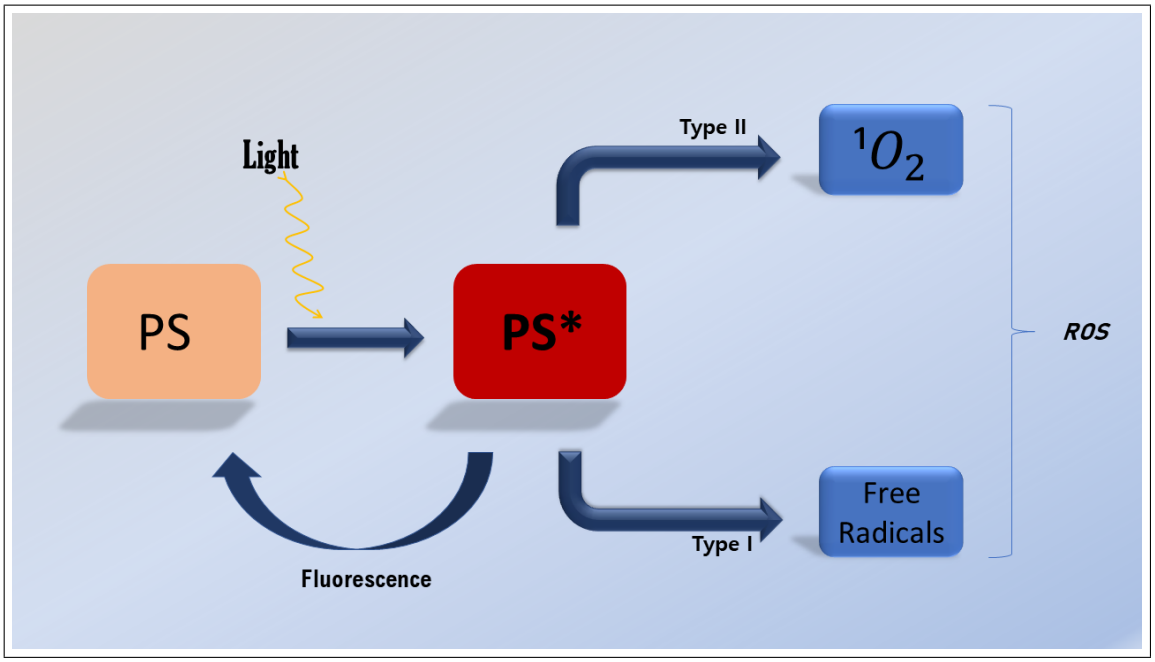

Figure 2.1 Schematic representation of the Mechanism of PDT.

As shown in Figure 2.1, when the excitation light hits the Ground State Singlet Photosensitizer (PS), it passes into an electronic transition to form Excited State Singlet PS, that is short lived (Lasts for nanoseconds). The Excited State Singlet PS can either lose its energy by emitting light (Fluorescence) or undergo a process called intersystem crossing, where it is converted to Triplet PS. The Triplet PS whereby can react with a substrate to form Radicals which in turn react with oxygen to form Reactive Oxygen Species (ROS), which cause irreversable damage to cancer cells. This process is called Type I reaction. Furthermore, the excited Triplet PS can transfer its energy to nearby molecular oxygen to form Excited State Singlet Oxygen, another ROS component. This process is called Type II reaction [8, 9].

Antitumor Photodynamic Therapy starts by injecting or applying the photosensitizer into the patient. After a certain duration the photosensitizer is cleared from the body but is accumulated in the parenchyma of the tumor because of enhanced permeability and retention (EPR) effect. After that the tumor area is irradiated with a light source with a specific wavelength (Suitable with the photosensitizer used). Consequently, the tumor is expected to get suppressed by one of the death pathways.

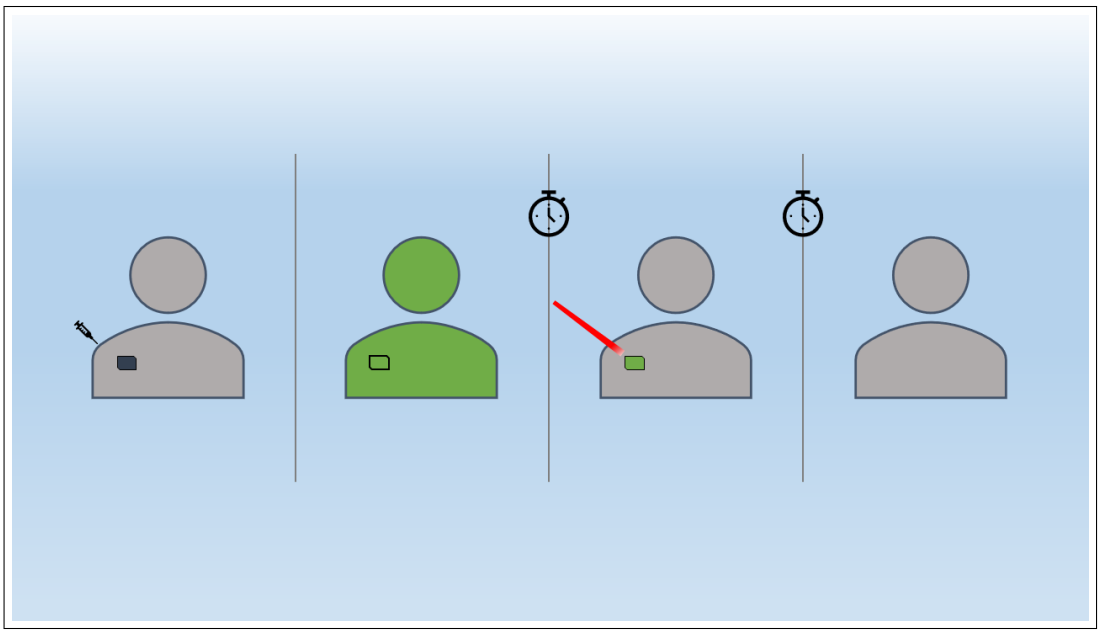

Figure 2.2 Demonstration of Anti-Tumor PDT Administration.

Cancer cells death induced by PDT is a multifactorial mechanism, which include [10] :

1. Cell death by Apoptosis or/and Necrosis caused by direct cancer cell damage.
2. Inhibiton of cancer vasculature causing interrupted nutrition and oxygen sypply to the tumor tissue, rapid vascular shutdown,
3. and Induced anti tumor immunity caused by activating host immune system.

Photosensitizers are one of the influencing elements of PDT's efficacy. Consequently, Photosensitizers must have certain characteristics to work ideally. An ideal PS must accumulate selectively in the treatment area, produce an efficient amount of singlet oxygen, and have low dark toxicity. Moreover, to achieve deeper penetration, it must have a high absorption peak at long wavelength regions. Additionally, an ideal Photosensitizer would be chemically pure and amphiphilic, which means that it must contain a hydrophobic matrix but still be water soluble. Furthermore, it must be stable and easily dissolved in injectable solvents. However, unfortunately, there is still no such photosensitizer with all these ideal characteristics [11].

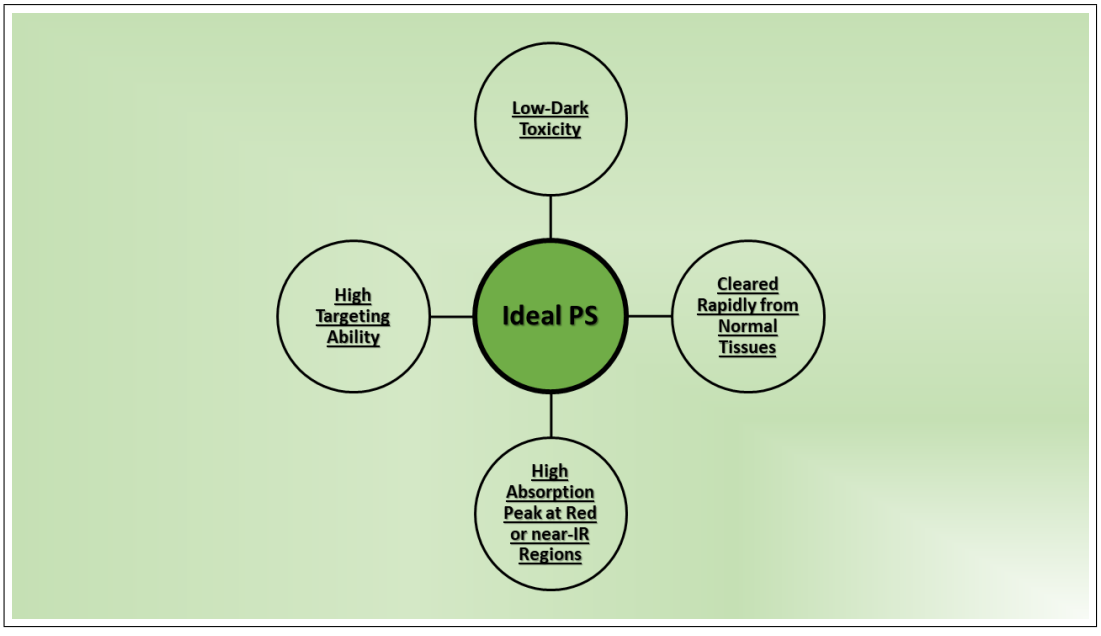

Figure 2.3 The Characteristics of an Ideal Photosensitizer.

5-Aminolevulinic acid (5-ALA) is a naturally occurring compound used as a precursor for protoporphyrin IX (PpIX). 5-ALA is considered as second-generation photosensitizer for its high selectivity and increased ROS generation [12]. 5-ALA is metabolised into PpIX in the Mitochondria via heme biosynthesis pathway [13, 14]. PpIX is photodynamically active and preferencially accumulates within tumor cells [15]. Among all the cancer treatments available today, 5-Aminolevulinic acid mediated PDT can be viewed as the most selective. The reported PpIX Tumor to Normal Tissue (T/N) ratio, that is formed by 5-ALA, varies between 10:1 and 90:1 depending on the dose applied, post-application time and pathologic conditions [16].

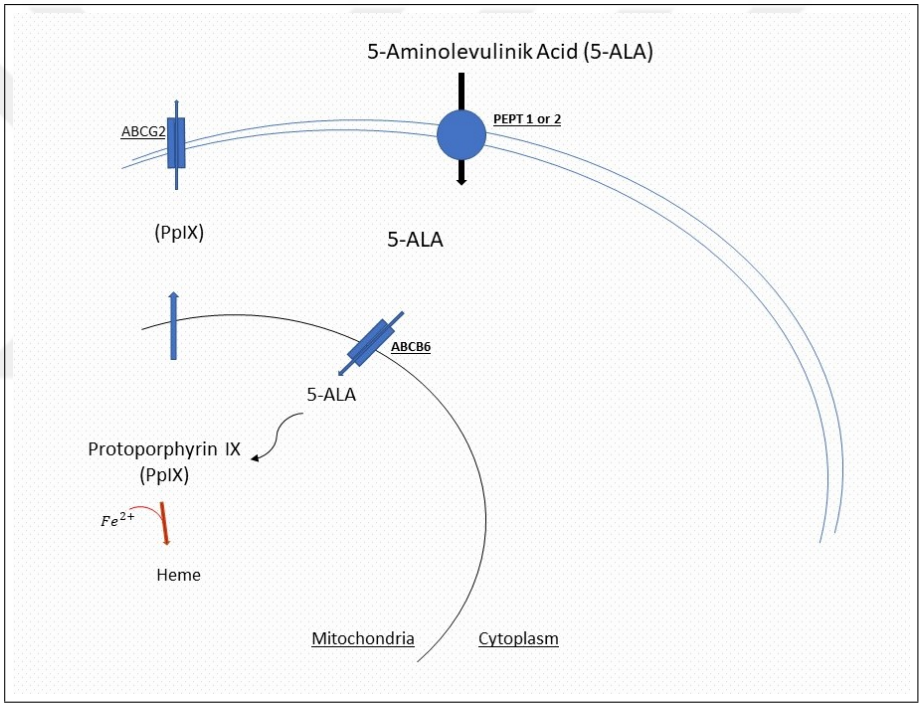

Figure 2.4 Schematic representation of the biosynthesis of Protoporphyrin IX.

2.2 Resistance to PDT

Studies have shown that resistance to PDT is highly dependant on the cell type and the photosensitizer used. However, like chemoresistance, resistance to PDT is a multifactorial phenomenon. When the different cell types are analyzed, identifying cellular characteristics that directly cause variations in resistance to PDT is not possible. On the other hand, different mechanisms associated with the photosensitizer

used, which are common with the drug resistance mechanisms, can be analyzed. These mechanisms may be related to different uptake rate or efflux of the photosensitizer, different intracellular localization of the photosensitizer and reduced activation or increased inactivation of the photosensitizer [17].

Some cell characteristics can be, to a certain degree, related to resistance to PDT. For instance, cells that have an increased cell spreading show higher level of PDT resistance. Moreover, bigger cells with higher protein content are associated with higher resistance to PDT than the smaller counterparts [18]. PDT efficacy is also affected by cell adhesion, invasiveness, and metastasis. For instance, the cell adhesion-mediated drug resistance (CAM-DR) phenomenon is observed when cells adhering to ECM components are protected from the apoptotic effect of chemotherapeutic agents. This phenomenon is also present in PDT, as the cells adhering to the ECM initiate antiapoptotic pathways and thus do not undergo apoptosis when PDT is applied [19]. Cells that are resistant to PDT are associated with signs of disorganized cytoskeleton. A major cytoskeletal protein, called vimentin, is degraded when apoptosis inducers are applied. Vimentin, which is caspase-resistant, partly suppress PDT induced apoptosis [20].

PDT resistive cells show an increased expression of Heat Shock Proteins (HSPs) and glucose-regulated proteins (GRPs), modulating PDT induced cellular damage. HSPs help the cells to recover from PDT damage by stabilizing partially unfolded proteins, preventing unwanted protein aggregation and establishing proper protein conformation [21]. Furthermore, studies have shown that an increase in GRP mRNA levels or GRP protein synthesis is only associated with specific targets of oxidative damage, which are dependent on the photosensitizer used [22].

PDT's efficacy is affected widely by the availability of oxygen in the treated area. Oxygen is needed to form reactive oxygen species (ROS), which can directly harm cancer cells [23]. Moreover, photosensitizer precursors, such as 5-ALA, are metabolized better into active photosensitizer protoporphyrin IX in oxygen-rich environments [24]. PDT and impairment in the vessels of tumor tissues induce hypoxia [25]. Hypoxia is,

therefore, an influencing resistance mechanism to PDT.

Nitric Oxide (NO) molecules are considered as PDT resistance mechanism. Studies have shown that PDT resistance is caused by NO that elicit antioxidant responses or that act as an antioxidant [17]. Moreover, photostress induced Nitric Oxide signals hyper-resistance and a serious increase in growth and migratory aggressiveness [26, 27]. Additionally, opposite to the vasoconstrictive effect of PDT, NO mediates vasodilation [28]. Recent study highlighted the important role played by Nitric Oxide in TIMP-1 down-regulation and MMP-9 activation resulting in an accelerated migration and invasion of cancer cells [29].

2.3 Potentiators

Nowadays, it is known that combination treatments are more effective in treating resistive diseases such as cancer. However, the best combination for each disease is yet to be discovered. Currently, many scientists are trying to find the best coadjuvant added to PDT, making it clinically more applicable and efficient. Potentiating antimicrobial PDT is a widely studied topic with a wide range of potentiators, such as Azide salts, Halogen bearing inorganic salts and natural compounds [30]. On the other hand, potentiating antitumor PDT is achieved by other coadjuvants which make PDT targeted more selectively, induce cell death mechanisms better or overcome cancer cells' resistance.

Table 2.1
Previous *in vitro* Antitumor Potentiated PDT studies.

Potentiator	**PS and λ**	**Cancer cell Line**	**Reference**
Iron Chelating Prodrug (CP94)	5-ALA - 635 nm	Human fetal lung fibroblasts (MRC-5), human skin fibroblasts (84BR) and human epidermal skin carcinoma cells (A431).	Curnow et al. (2019) [31]
Atovaquone (OXPHOS Inhibitor)	ICG - 808 nm	Human cervical carcinoma cells (HeLa)	Xia et al. (2019) [32]
Capecitabine ($Xeloda^{TM}$) (Chemotherapy Medication)	5-ALA - 633 nm	Murine breast carcinoma line (4T1)	Anand et al. (2018) [33]
Dichloroacetic acid (DCA) (Anti-cancer Drug)	5-ALA - 633 nm	Breast cancer cell line (MCF-7)	Alkarakooly et al. (2018) [34]
Epidermal Growth Factor	Curcumin - 460 nm	Human gastric cancer cell line (MKN45) and human gastric epithelial mucosa (non-cancer) cell line (GES).	Tsai et al. (2018) [35]
Biguanide Metformin (Type 2 Diabetes Drug)	5-ALA - 630 nm	Lung cancer cells (KLN205)	OSAKI et al. (2017) [36]

Potentiator	**PS and λ**	**Cancer cell Line**	**Reference**
Hemoglobin	Zinc hexadecafluo-rophthalocyanine (ZnF16Pc) - 671 nm	Human glioblastoma cell line (U87MG)	Tang et al. (2016) [37]
Hypoxia-inducible factor-1 siRNA (To Suppress Hypoxia-related Proteins)	Photosan - 640 nm	Human squamous cell carcinoma cells	Chen et al. (2015) [38, 39]
Perfluorocarbon (Oxygen carrier)	IR780 - 808 nm	MCF-7 & CT26 cells	Cheng et al. (2015) [40]
1,25(OH)2 (calcitriol) (vitamin D3)	5-ALA - 635 nm	Glioma cell lines (U87) and (U98)	Chen et al. (2014) [41]
Curcumin	Photofrin - 630 nm	Human head & neck (AMC-HN3)	Ahn et al. (2012) [42]
Proteasome inhibitors (To induce ER stress)	Photofrin - 632.8 nm	Human cervical cancer (HeLa) and murine breast carcinoma (EMT6) cell lines	Szokalska et al. (2009) [43]
Methotrexate (Chemotherapy agent)	5-ALA - 512nm	Human prostate carcinoma cells (LNCaP)	Sinha et al. (2006) [44]
Cyclooxygenase (COX) 2 inhibitors	Photofrin - 630 nm	Poorly differentiated colon adenocarcinoma cell line (C-26)	Makowski et al. (2003) [45]

Table 2.1 lists some of the previous *in vitro* antitumor potentiated PDT studies. Recently, finding the optimal PDT coadjuvant is a popular topic that is tackled by many scientists to be able to use the promising characteristics of PDT clinically. However, the best PDT coadjuvant is yet to be found.

Curcumin, a natural compound, is extracted from Turmeric (*Curcumin Longa*) plant. Curcumin comes as yellowish orange crystalline powder. It is usually soluble in DMSO and ethanol but not in water nor in ether [2]. For thousands of years, Curcumin has been used in traditional Asian and Indian cuisine and medicine [46]. It also has been described as having antitumor, antimicrobial and anti-inflammatory characteristics [47]. Curcumin also has internal structural resonance stability which provides it with radical chain inducing potential making it an excellent PDT coadjuvant [48].

To conclude, increasing PDT efficacy is important for making the advantageous properties of PDT clinically applicable. Adding Curcumin as a potentiator to 5-ALA mediated PDT seems a potential strategy for better PDT results and applications.

3. MATERIALS AND METHODS

3.1 Cell Culture

Prostate cancer PC-3 cell line (ATCC CRL-1435) and Colon cancer Caco-2 cell line (ATCC HTB-3) were cultured in RPMI-1640 medium (Sigma). PC3 and Caco-2 cells were supplemented with 10% and 20% fetal bovine serum (FBS) (Sigma), respectively. 1% Penicillin-streptomycin solution was added as an antibiotic. The cell cultures were maintained at 37°C in a 5% CO_2 incubator (Nuve EC160). The culture medium was changed every 3-4 days and the cells were passaged when they reached 80% confluence. A 50 mM stock solution of Curcumin (GP8291, Glentham Life Sciences) was prepared by adding 18.42 mg of Curcumin to 1 ml DMSO before each experiment. 5g of 5-Aminolevulinic acid (A7793, Sigma) was dissolved in 500 μl sterile distilled H_2O to prepare 110 mM stock solution, which was stored in the dark at 4°C.

3.2 Curcumin Absorption Spectrum

Using NanoDrop 2000c Spectrophotometer (Thermo Scientific, Barrington, IL, USA), the absorbance spectrum of 10 μM Curcumin was measured. After calibrating the device with a drop of DMSO, a single drop of 10 μM Curcumin was added to the device. The intended wavelength was adjusted to 635 nm, to assure that the photoactivity of Curcumin was not triggered.

Figure 3.1 NanoDrop 2000c Spectrophotometer (Thermo Scientific, Barrington, IL, USA) was used to measure the absorbance spectrum to test the photoactivity of 10 μM Curcumin.

3.3 Power Density Determination

The cells were harvested using 4:1 trypsin-EDTA solution and seeded in a 96-well plate with a density of 15,000 cells/well. After 24 hours incubation, the medium is replaced with a new medium containing 0.5 mM 5-ALA and incubated for 6 h at 37°C in a 5% CO_2 incubator. Subsequently, the medium was replaced with fresh medium and the photosensitized cells were irradiated with 635 nm diode laser system (Thorlabs Inc, NJ, USA) in continuous wave (CW) mode with different laser energy densities (0, 1, 2.5, 5, 10 and 20 J/cm^2). During the experiments, the laser power density was 70-75 mW/cm^2. After irradiation, the cells were incubated in a humidified atmosphere at 37°C and 5% CO_2 for 24 hours, afterwards cell viability was evaluated. All experiments were performed in triplicate.

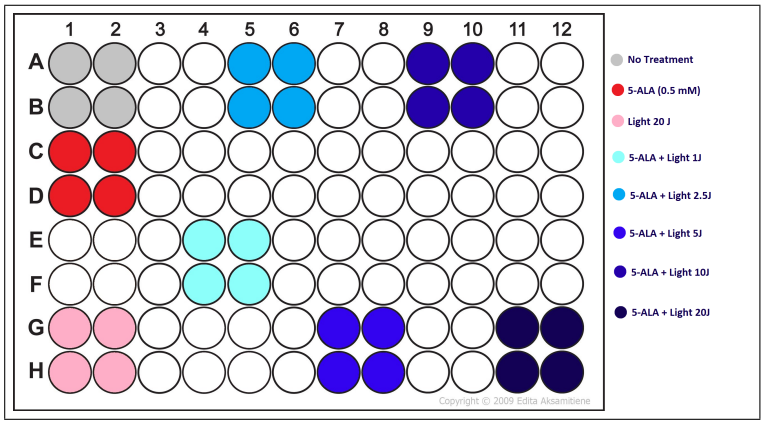

Figure 3.2 Experimental Design for 5-ALA mediated PDT with different fluences.

3.4 Curcumin Concentration Determination

The cells were harvested using 4:1 trypsin-EDTA solution and seeded in a 96-well plate with a density of 15,000 cells/well. After 24 hours incubation, the medium is replaced with a new medium containing different concentrations of Curcumin (0, 10, 25, 50, 100 and 200 μM); an extra concentration of 500 μM Curcumin was added for experiments with Caco-2 cells due to the high resistivity of colon cancer Caco-2 cell line. The cells having the new medium with different concentrations of curcumin were incubated for 6 hours. After that, the medium was replaced with a fresh medium and incubated for 24 hours and then the cell viability was evaluated. All experiments were performed in triplicate.

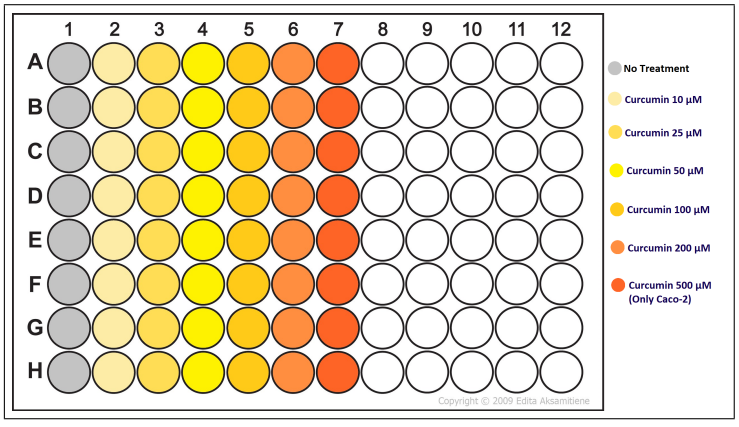

Figure 3.3 Experimental Design for Curcumin's anti-tumor effects at different concentrations.

3.5 The Combination Treatment: Curcumin and PDT

The cells were harvested using 4:1 trypsin-EDTA solution and seeded in a 96-well plate with a density of 15,000 cells/well. After 24 hours incubation, the medium is replaced with a new medium containing 0.5 mM 5-ALA and 5, 10 μM and 25 & 50 μM Curcumin for PC-3 and Caco-2 respectively. After 6-hour incubation, the medium was replaced with fresh medium and illuminated by 635 nm diode laser system (Thorlabs Inc, NJ, USA) in continuous wave (CW) mode with laser energy density of 2.5 and 10 J/cm^2 for PC-3 and Caco-2 respectively. During the experiments the laser power density was 70-75 mW/cm^2. Cell viability was evaluated after 24 hours. All experiments were performed in triplicate.

Figure 3.4 635 nm diode Laser experimental setup.

Experiments were conducted with the following experimental groups:

- No Treatment
- Positive Control (Dark toxicity: Only 5-ALA)

- Positive Control (Dark toxicity: Only Curcumin)
- Positive Control (Dark toxicity: 5-ALA and Curcumin)
- Positive Control (Laser only)
- Positive Control (Laser and Curcumin)
- PDT (Laser and 5-ALA)
- Combined PDT (Laser, 5-ALA and Curcumin)

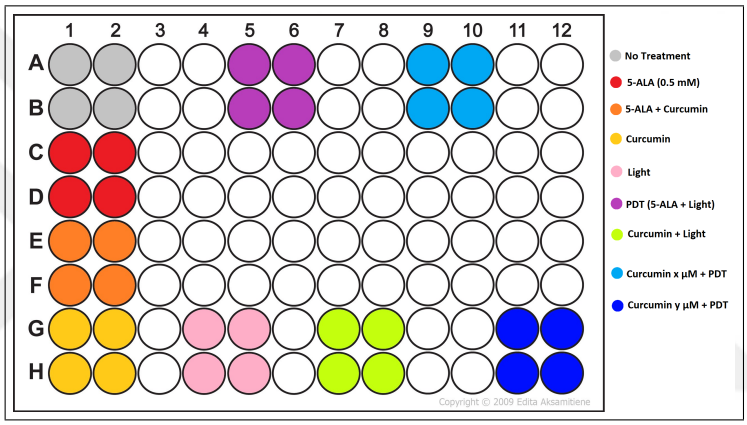

Figure 3.5 Experimental Design for the effect of Curcumin's addition to 5-ALA mediated PDT.

3.6 Cytotoxicity Assay (MTT)

24 hours after treatment or PDT, 10 μl (10%) MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Glentham Life Sciences) solution (5mg/ml PBS) was added to each well and incubated for 3 hours at 37°C in a 5% CO_2 incubator. After that, the media was removed and DMSO 100 μl/well was added and the plate was placed on the shaker for 5 minutes. Optical density at 570 nm was measured using iMark microplate reader (Bio-Rad Labs, Sunnyvale, CA).

Figure 3.6 iMark microplate reader (Bio-Rad Labs, Sunnyvale, CA) was used to measure the optical density.

3.7 Statistical analysis

After normalizing the absorbance values of each group, statistical analysis was performed using IBM SPSS Statistics 25. One-way ANOVA test was performed to check for the presence of significant difference between the groups. For statistically significant difference between groups, Post Hoc Tests, Tukey's-b and Tamhane's T2 tests, were performed. In this study, $p \leq 0.05$ (5%) is considered an acceptable level of significance.

4. RESULTS

4.1 Absorption Spectrum

Because Curcumin is photodynamically active under specific wavelength and can be used as a photosensitizer, the absorbance of Curcumin was measured to make sure the photodynamic activity of curcumin was not triggered. To test the absorbance spectrum of Curcumin, NanoDrop 2000c spectrophotometer was used. At a wavelength of 635nm, the absorbance of 10 μM Curcumin was measured as 0.06, as shown in Fig. 4.1.

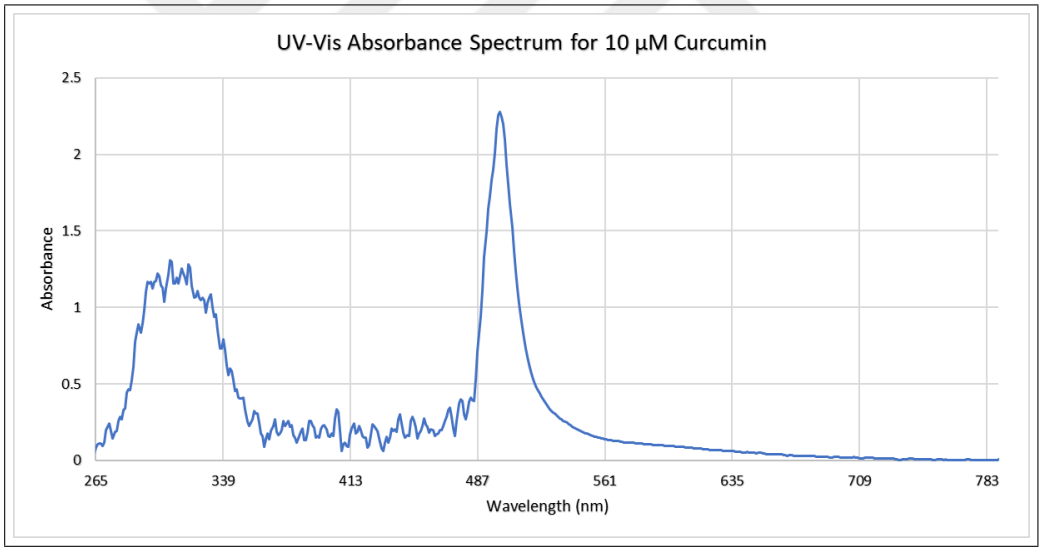

Figure 4.1 The absorbance spectrum of 10 μM Curcumin measured used NanoDrop 2000c. The absorbance at the wavelength of 365 nm is measured to be 0.06.

4.2 5-ALA mediated PDT Results

As a treatment on its own, PDT with different laser energy densities is examined on both PC-3 and Caco-2 using 5-ALA as the photosensitizer. MTT assay was used 24 hours after laser illumination. For PC-3, 5-ALA mediated PDT at 2.5 J/cm^2 inhibited 30% of the cell viability as shown in Fig. 4.2 (a). Alternatively, Fig.4.2 (b) shows the inhibition effect of PDT using 5-ALA as a photosensitizer on Caco-2 cells, 29.3% inhibition of cell viability was achieved at 10 J/cm^2. 2.5 J/cm^2 and 10 J/cm^2 were chosen to be used on PC-3 and Caco-2, respectively.

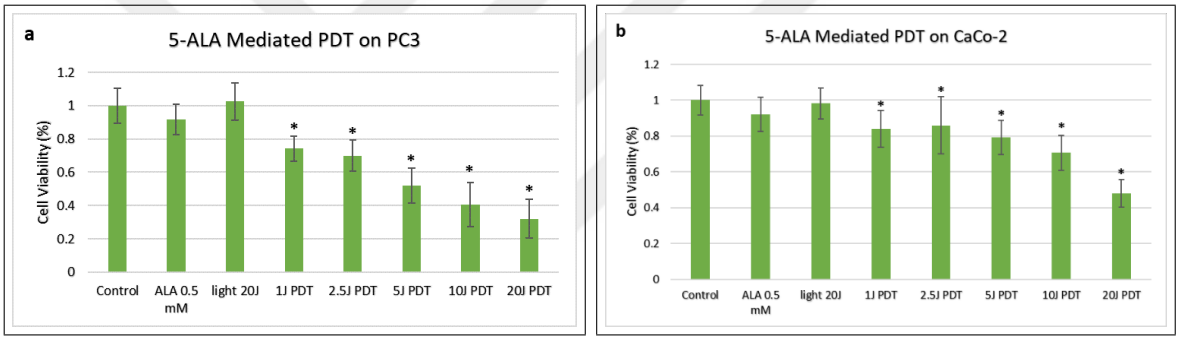

Figure 4.2 The effect of increasing the laser energy density on both cell lines; PC-3 (a) and Caco-2 (b). PDT efficacy increases with increased laser energy densities. The groups that are statistically significantly different from the Control group were labelled (*: $p<0.05$).

4.3 Curcumin Toxicity Results

To assess the toxicity of different concentrations of Curcumin, MTT assay was used 24 hours after changing the medium for both PC-3 and Caco-2 cells. As shown in Fig. 4.3 (b), Caco-2 cells are more resistive to Curcumin, where 16.2% toxicity started at 50 μM. On the other hand, Curcumin showed 11.8% toxicity on PC-3 at a concentration of 10 μM, as shown in Fig. 4.3 (a). As a non-toxic dose, 10 μM and 50 μM of Curcumin were chosen for PC-3 and Caco-2, respectively.

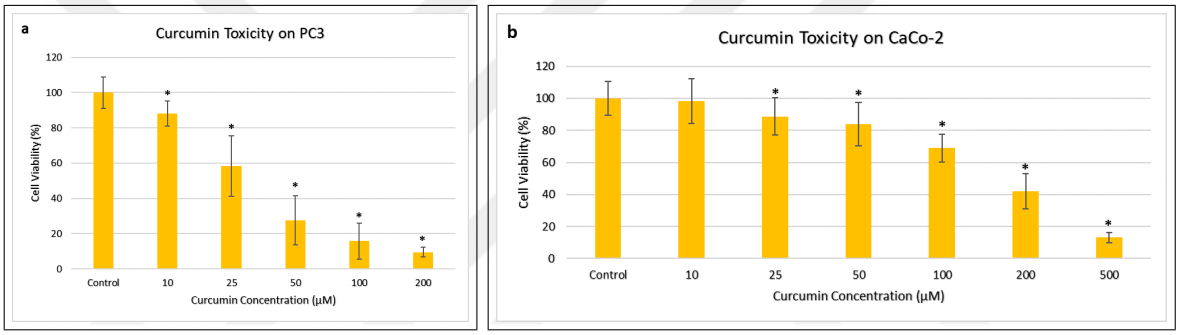

Figure 4.3 Curcumin shows dose-dependent antitumor effect on both PC-3 (a) and Caco-2 cells (b). The groups that are statistically significantly different from the Control group were labelled (*: $p<0.05$).

4.4 Combination Treatment: Curcumin and PDT

To assess the results of the combined PDT, Curcumin and 5-ALA PDT, MTT was used 24 hours after laser illumination on both PC-3 and Caco-2 cells. PC-3 cells didn't show any statistically significant difference between the combined PDT and each treatment alone with 36.2% decrease in cell viability after the combined PDT and 1.5% and 42% decrease after Curcumin and 5-ALA PDT alone respectively, as shown in Fig. 4.4 (a). On the contrary, as in Fig. 4.4 (b), Caco-2 cells showed an inhibited cell viability after the combined PDT with 62.4% decrease which is more than the sum of Curcumin and 5-ALA PDT alone, with 14.6% and 20.6% respectively. This suggests that on Caco-2 the combined PDT had more efficacy than each single treatment alone. A broader display of the data, revealing all positive controls is presented in Supplementary figures S1 and S2.

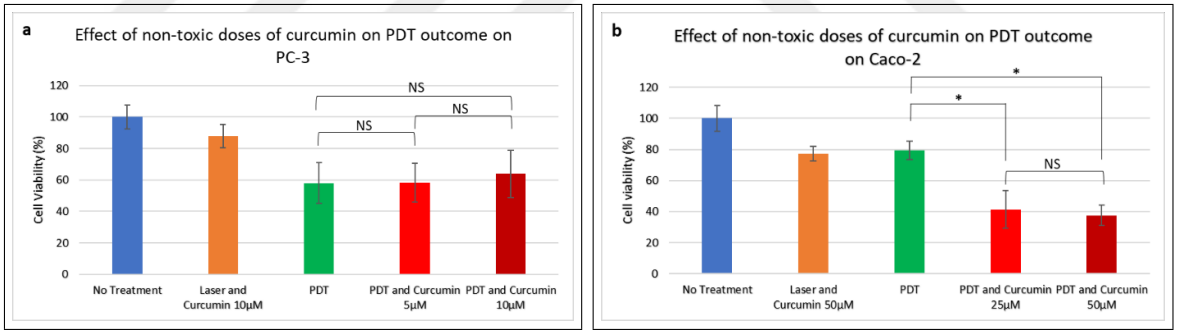

Figure 4.4 (a) Curcumin and 5-ALA mediated PDT combination effect on PC-3 cell line and (b) Curcumin and 5-ALA mediated PDT combination effect on Caco-2 cell line. The combined treatment of Curcumin and 5-ALA-PDT showed better cytotoxicity results than the sum of each treatment alone on Caco-2 cell line (b), but not on PC-3 cell line (a) as there is no statistical significance between PDT group and the Combined Treatment groups. The groups that are statistically significantly different from the No Treatment were labelled (*: $p<0.05$). NS: No Significance.

5. DISCUSSION

Increasing PDT's efficacy is important because it makes PDT more appropriate for clinical use and thus exploiting PDT's advantages, such as selectivity, non-invasiveness and minimal side effects. In our study, curcumin is used as a potentiator to increase the efficacy of 5-ALA mediated PDT by overcoming the resistance of some cancer cell lines to PDT. Aside from being a NO scavenger, curcumin has characteristics making it an excellent coadjuvant to PDT. From these characteristics is its antiproliferative effects. These effects are achieved by suppressing the cell cycle regulatory proteins. Additionally, curcumin demonstrates apoptotic effects by reducing the expression of Bcl-2 members, which are antiapoptotic, and by increasing the expression of procaspase -3, -8 and -9. Curcumin also mediates cytotoxicity by generating ROS [2].

Ahn et al. (2012), investigated the coadjuvant role of curcumin when it is added to Photofrin mediated PDT on human head and neck (AMC-HN3) cell line. They suggested that the enhanced *in vitro* PDT results were achieved due to increased ROS production [42].

Apart from curcumin, many other potentiators where previously used with antitumor PDT. Some has been presented in Table 2.1. These different potentiators vary in the potentiation mechanism. For instance, Hypoxia, which is an influencing PDT resistance mechanism, is mitigated using potentiators. For example, Xia et al. (2019) noticed the contribution of mitochondria-associated oxidative phosphorylation (OXPHOS) to hypoxia and proposed the usage of atovaquone as a potentiator to inhibit OXPHOS and thus achieve better PDT results [32]. Moreover, in a mini review article, Dang et al. (2017) stated multiple methods to manipulate tumor hypoxia to enhance PDT results. From these methods, several potentiators where used; such as, Hemoglobin and Perfluorocarbon (used as oxygen carriers) and Hypoxia-inducible factor-1 siRNA (used to suppress hypoxia-related proteins) [39].

Some potentiators also can be used to enhance the selectivity of PDT. For instance, Tsai et al. (2018) conjugated epidermal growth factor to their fabricated nanoparticles to target the epidermal growth factor receptors (EGFR), which are over-expressed on cancer cells [35]. Moreover, some photosensitizers, such as (PpIX), are metabolized into heme when they react with ferrous ion (Fe^{2+}) and thus become photodynamically inactive. Curnow et al. (2019) suggested the usage of iron chelating prodrug to reduce heme production and thus keep larger amount of PpIX photodynamically active [31].

As shown in Figure 4.4 (b), when added to PDT resistive cell line, Caco-2, Curcumin increased the cell viability's inhibition to 62.4 %, which is more than the sum of both PDT alone and Curcumin alone. We propose that the reason behind this finding is the Curcumin's ability to inhibit nitric oxide, which is one of the main causes of resistance to PDT [49]. Studies have shown that hyper-resistance and a serious increase in growth and migratory aggressiveness are signalled by nitric oxide that is induced by photostress [26, 27]. The exact mechanism making nitric oxide cytoprotective yet to be fully understood, but nitric oxide (NO) is said to cause resistance to PDT by acting as an antioxidant, by causing vasodilation and by activating MMP-9 and down-regulating TIMP-1 causing migration and invasion [50, 51].

On the other hand, PC-3 didn't show any significant increase in inhibition when curcumin is added to PDT, as shown in Figure 4.4 (a). We suggest that because PC-3 is considered as poorly differentiated cell line [52] and it is not as resistive as Caco-2 to PDT, Curcumin didn't significantly enhance PDT's efficacy against it.

6. CONCLUSION

Combination treatment is particularly more efficient in treating malignant diseases. With the antitumor characteristic of Curcumin becoming one of the hot topics to be studied, we investigated an alternative method to increase the efficacy of PDT. The aim of this study was to test whether Curcumin, which is a non-toxic natural compound that has antitumor characteristics, can increase PDT efficacy by overcoming the resistance of cancer cells.

Using non-toxic doses of Curcumin resulted in a significant decrease in PDT resistance in Caco-2 cells and thus increased the efficacy of 5-ALA mediated PDT, but not on PC-3. Adding Curcumin to 5-ALA mediated PDT led to more effective results on Caco-2 with a 62.4 % decrease in cell viability. On the other hand, adding Curcumin to 5-ALA mediated PDT on PC-3 cells didn't produce statistically significant increase in efficacy with a 36 % decrease in cell viability.

In summary, the obtained results give us an insight that Curcumin is promising in increasing PDT's efficacy when it is applied to PDT resistant cell lines. Nevertheless, further analysis must be conducted on other PDT resistant cell lines. We expect the reason behind these results to be the Curcumin's inhibition of nitric oxide, Curcumin's enhancement of cytotoxic and apoptotic effect via mitochondria-dependent pathways or increased generation of reactive oxygen species (ROS).

7. SUPPLEMENTARY FIGURES

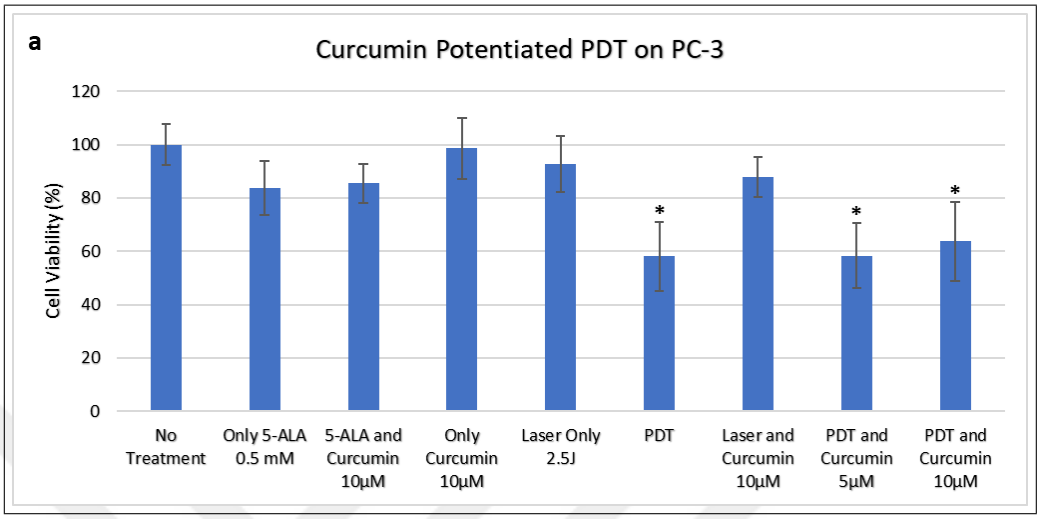

Figure S1 Curcumin Potentiated 5-ALA PDT on PC-3. The groups that are statistically significantly different from the Control group were labelled (*: $p<0.05$).

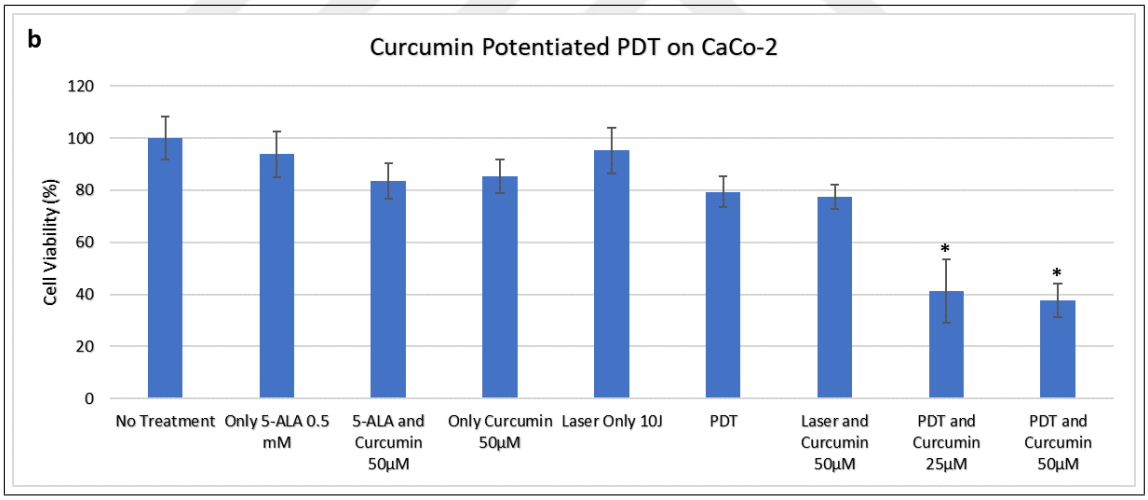

Figure S2 Curcumin Potentiated 5-ALA PDT on Caco-2. The groups that are statistically significantly different from the Control group were labelled (*: $p<0.05$).

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