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The effect of curcumin in antitumor photodynamic therapy: *In vitro* experiments with Caco-2 and PC-3 cancer lines

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ABSTRACT

Background: Photodynamic Therapy (PDT) is a promising antitumor and anti-bacterial treatment method for its high selectivity, non-invasiveness, and minimal side effects. However, cellular mechanisms may lead to PDT resistance and thus effect efficacy. The aim of this study is 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.

Methods: 5-ALA mediated PDT was tested on two cell lines, PC-3 and Caco-2. Curcumin toxicity was evaluated at different concentrations. The determined PDT doses were applied to the cell lines together with two different Curcumin concentrations. Cell viability was evaluated by MTT assay, 24 hs after the treatments. Results were evaluated using One-Way ANOVA followed by post-hoc tests.

Results: 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.

Conclusion: 5-ALA mediated PDT combined with Curcumin synergistically enhanced antitumor PDT efficacy on Caco-2, which is considered a highly resistive cancer cell line.

1. Introduction

Photodynamic therapy or PDT refers to the treatment in which administration of photosensitizing agent 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 [1–3]. For PDT to be effective, an adequate concentration of oxygen molecules must be present. Because of its many advantages over alternative treatment methods, PDT is a very promising method for curing diseases such as cancer. Some of these advantages are comparatively non-invasiveness, targeted accurately, healing process with little or no scarring and no side-effects [4]. However; in some cases, cancer cells' resistance to PDT may decrease the efficacy of the treatment.

Studies have shown that possible resistance mechanisms might be caused by hypoxia, stress responses, antioxidant enzymes or poor cellular uptake, efflux and/or localization of the photosensitizer [5]. Another cause of resistance to PDT is nitric oxide (NO) molecules that are endogenously produced by the cells. Researches have shown that nitric oxide that is present in cancer cells create resistance to PDT by acting as an antioxidant or by eliciting antioxidant responses [6]. Moreover, NO mediates vasodilation which acts in opposition to the PDT's vasoconstrictive effects [7]. A recent study showed that NO plays an important role in MMP-9 activation and TIMP-1 down-regulation resulting in an accelerated migration and invasion of cancer cells [8]. Additionally, Plesková et al., 2006 have shown that the activity of NADPH oxidases, which are important enzymatic sources of ROS [9], can be directly inhibited by nitric oxide [10].

The availability of enough oxygen in the treated area is a very crucial indicator of PDT's efficacy. Hypoxia is, therefore, an influencing resistance mechanism to PDT. Hypoxia is indirectly caused by an impairment in the vessels of the tumor [11]. The formation of reactive oxygen species (ROS), which can directly harm cancer cells, needs molecular oxygen as well as photosensitizer and light; therefore, the amount of oxygen at the targeted area heavily influences PDT efficacy [12]. Additionally, the metabolization of photosensitizer precursors, such as 5-ALA, into active photosensitizer protoporphyrin IX is better in oxygen-rich environments [13].

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One of the strong predictors of PDT efficacy is the photosensitizers' uptake and localization (subcellular distribution), which is determined by the photosensitizer's charge, lipophilicity and 3D structure [14]. Positively charged photosensitizers are more favorable to be uptaken efficiently by cells and to be accumulated intercellularly. The reason behind this favourability is assumed to be the binding of positively charged photosensitizers to the anionic parts of the proteins playing a role in the accumulation of the photosensitizer in the tumor tissue [15]. Additionally, higher lipophilicity is said to be associated with higher uptake, which can be achieved by lengthening the side chains [16]. Furthermore, the molecular structure of the photosensitizer, such as its three-dimensional shape, plays an important role in its uptake by affecting the spatial availability of charges [17].

The aim of this study is to test the effect of adding a non-toxic dose of Curcumin on 5-ALA mediated PDT's efficacy. Curcumin, which is a natural compound extracted from the Turmeric plant, has been described as having antitumor, antimicrobial and anti-inflammatory characteristics [18]. Curcumin also has internal molecular resonance stability which provides it with radical chain inducing potential and consequently better PDT results [19]. Moreover, Curcumin can scavenge nitric oxide directly and inhibit its biosynthesis [20]. For the purpose of our study, two cell lines were used PC-3 and Caco-2, which were previously identified as non-resistant and resistant to PDT, respectively [21].

2. Materials and methods

2.1. 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. The culture medium was changed every 3–4 days and the cells were passaged when they reached confluence.

2.2. Reagents

Before each experiment, 50 mM Curcumin stock solution was prepared by adding 18.42 mg of Curcumin (GP8291, Glentham Life Sciences) to 1 ml DMSO. The stock solution was then diluted with RPMI-1640 to prepare the desired concentrations, making the solutions < 1% DMSO which is less than the threshold value for both Caco-2 and PC-3 cell lines [22,23]. 5 g 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.

2.3. Measurement of Curcumin's absorbance spectrum

10µM Curcumin was prepared in DMSO and the absorbance spectrum of the solution was measured using NanoDrop 2000c Spectrophotometer (Thermo Scientific, Barrington, IL, USA). To assure that the photoactivity of Curcumin was not triggered, the absorbance value at 635 nm was checked.

2.4. Curcumin dose 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 h 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 h. After that, the medium was replaced with a fresh medium and incubated for 24 h and then cell viability was evaluated. All experiments were performed in triplicate.

2.5. Laser dose 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 h 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 h, afterwards cell viability was evaluated. All experiments were performed in triplicate.

2.6. Curcumin and PDT: the combined effect

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 h 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 h 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 h. All experiments were performed in triplicate. In order to have a complete comparative measure of PDT, Curcumin and the combined therapy's efficiencies, 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)

2.7. MTT cytotoxicity assay

24 h after treatment or PDT, 10 µl (10%) MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Glentham Life Sciences) solution (5 mg/ml PBS) was added to each well and incubated for 3 h 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 min. Optical density at 570 nm was measured using *iMark* microplate reader (Bio-Rad Labs, Sunnyvale, CA).

2.8. 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.

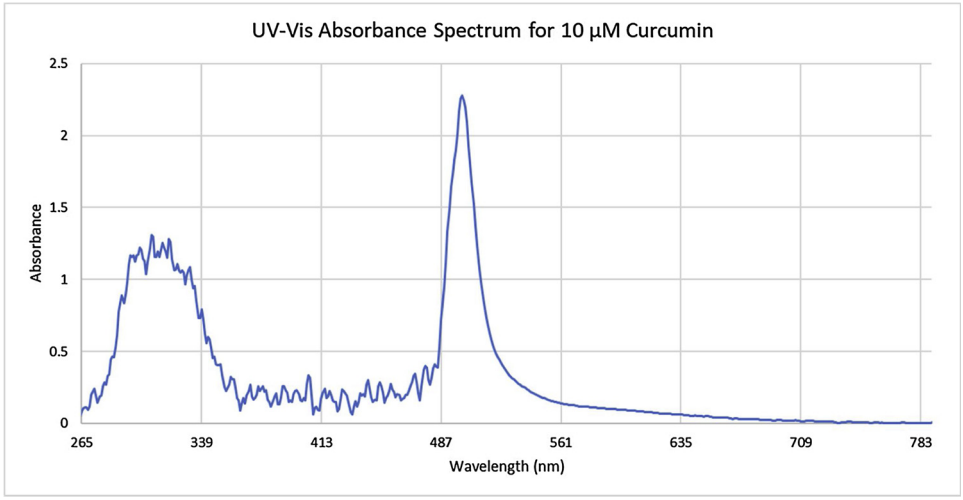

Fig. 1. The absorbance spectrum of 10 µM Curcumin measured used NanoDrop 2000c. The absorbance at the wavelength of 365 nm is measured to be 0.06.

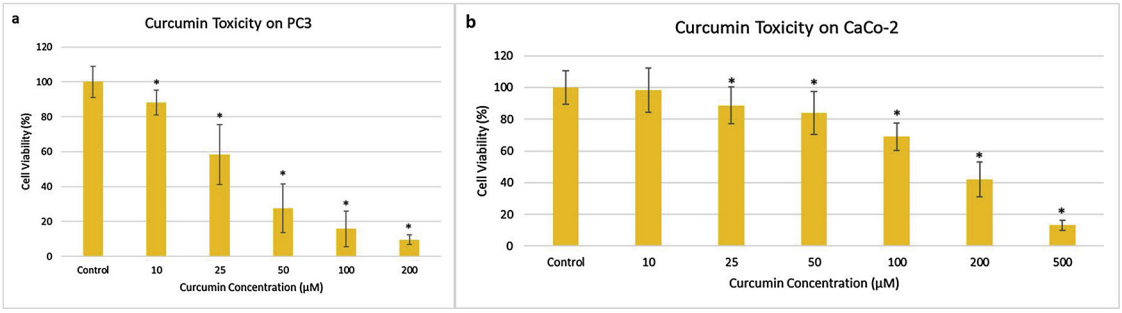

Fig. 2. (a) and (b). Curcumin shows dose-dependent antitumor effect on both PC-3 (a) and Caco-2 cells (b). The groups that are statistically significantly different from the Control group were labelled (*: $p < 0.05$).

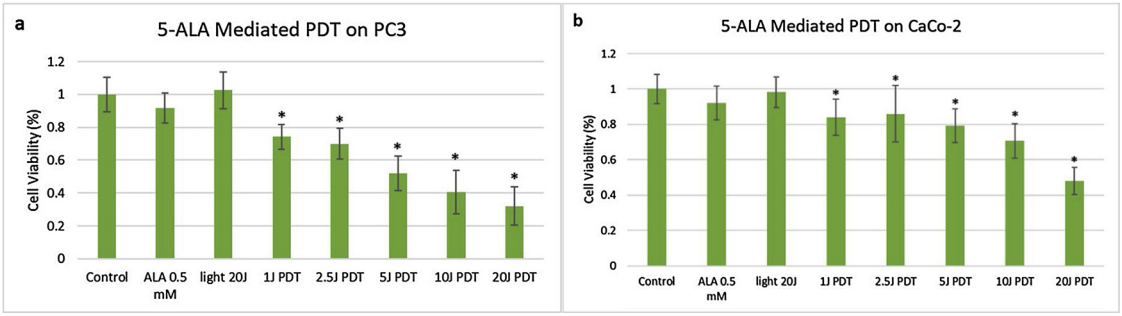

Fig. 3. (a) and (b). The effect of increasing the laser energy density on both cell lines; PC-3 (a) and Caco-2 (b). PDT efficacy increases with increased laser energy densities. The groups that are statistically significantly different from the Control group were labelled (*: $p < 0.05$).

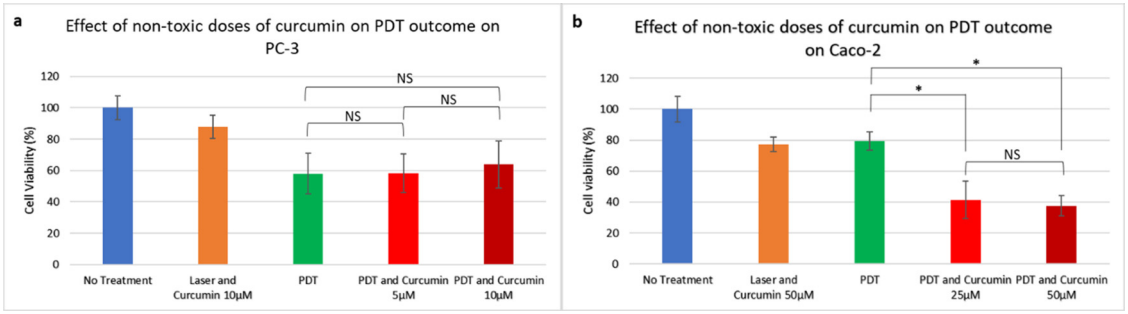

Fig. 4. (a) Curcumin and 5-ALA mediated PDT combination effect on PC-3 cell line and (b) Curcumin and 5-ALA mediated PDT combination effect on Caco-2 cell line. The combined treatment of Curcumin and 5-ALA PDT showed better cytotoxic results than the sum of each treatment alone with on Caco-2 cell line (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.

3. Results

3.1. Absorbance of 10 μM Curcumin solution at 635 nm

Because Curcumin is photodynamically active under specific wavelength range (487 nm–515 nm) 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 635 nm, the absorbance of 10 μM Curcumin was measured as 0.06, as shown in Fig. 1.

3.2. Curcumin toxicity results

To assess the toxicity of different concentrations of Curcumin, MTT assay was used 24 h after changing the medium for both PC-3 and Caco-2 cells. As shown in Fig. 2b, 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. 2a. As non-toxic doses, 10 μM and 50 μM of Curcumin were chosen for PC-3 and Caco-2, respectively.

3.3. 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 h 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. 3a. Alternatively, Fig. 3b 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.

3.4. Combined PDT (Curcumin and PDT) results

To assess the results of the combined PDT, Curcumin and 5-ALA PDT, MTT was used 24 h 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. 4a. On the contrary, as in Fig. 4b, 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 Fig. 1.

4. Discussion

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.

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 lack of 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 [24].

As shown in Fig. 4b, when added to PDT resistive cell line, such as 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 [25]. 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 [28,29].

On the other hand, PC-3 didn't show any increased inhibition when curcumin is added to PDT, as shown in Fig. 4a. We suggest that because PC-3 is considered as poorly differentiated cell line [30] and it is not as resistive as Caco-2 to PDT, Curcumin didn't enhance PDT's efficacy against it.

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 pathway or increased generation of reactive oxygen species (ROS).

As future work, we will be investigating the mechanism in which Curcumin overcame cancer cells' resistance and thus increased PDT's efficacy. Western blot can be done to test the levels of iNOS (inducible Nitric Oxide synthase) levels before and after combined PDT.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.pdpdt.2019.05.012.

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