Cell death induced by combination of Phthalocyanine photosensitizer and Doxorubicin on MCF-7 breast carcinoma cells.

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Abstract. Cancer is one of the common diseases that affect and threatens our human existence. Breast cancer is an invasive heterogeneous disease and the second most common disease among woman worldwide. For a degenerative disease like cancer to be cured, multiple therapeutic agents that target different pathological processes must be applied. For this reason, combination therapy remains an alternative strategy to combat diseases like cancer. In this study, we evaluated the anticancer effect of sulfonated zinc phthalocyanine (ZnPcS) mediated photodynamic therapy in combination with low dose doxorubicin (0.5 μM) on MCF-7 cancer cells. In addition, we explore the cell death pathway elicited by combination treatment. MCF-7 cells were incubated with low dose doxorubicin for 20 h, afterwards, various concentrations of phthalocyanine were added and further incubated for 4 h. Thereafter, the cells were irradiated with 681.5 nm diode laser at 4.74 wM/cm^2 for 17 min 36 sec (5 J/cm^2), and the cellular responses were measured. Cellular morphology was observed using inverted microscopy while the proliferation of cells was measured with homogenous ATP quantitative assay. The mechanism of cell death was investigated using Annexin V/PI flow cytometric analysis. Findings from this study show that combination of phthalocyanine mediated photodynamic therapy and doxorubicin significantly enhances the anticancer efficacy of phthalocyanine-doxorubicin combination on MCF-7 cells than when used individually. It was observed that this combination treatment led to an apoptotic cell death pathway. Hence, this study suggests a new treatment opportunity for breast cancer to enhance its effectiveness and which warrants further investigation for its potential to reverse multidrug resistance.

1. Introduction

Cancer continues to dominate among the major cause of mortality worldwide despite knowledge of its treatment at the cellular level. Breast cancer is the most frequently diagnosed and the most common cause of cancer death among woman [1]. Global cancer statistics showed that nearly 1.7 million new cases of breast cancer were diagnosed in 2012, which represents about 25% of all cancers in woman [2]. Photodynamic therapy is an emerging attractive treatment regime that has already been used on patients suffering from superficial cancers. It involves the systematic use of a light sensitive chemical called a photosensitizer in the presence of light and oxygen to induce destruction of cancerous cells through the production of superoxide radicals that cause oxidative stress and thus cell death [3]. PDT efficacy greatly depends on the production of reactive oxygen species that attack cellular targets within the cells and leads to cell damage and death [4]. ZnPcS mediated PDT has demonstrated its effectiveness in the treatment of breast cancer cell line [5]. Recently, most therapeutic investigations are now focusing on combining one or two treatment modalities, which can sum the advantages of each individual

treatment, increase treatment efficacy and reduce dose dependent toxicity [6]. Thus, our study aimed to evaluate the anticancer effect of phthalocyanine mediated photodynamic therapy in combination with low dose doxorubicin (0.5 μM) on MCF-7 cancer cells and explore the mode of cell death.

2. Methodology

2.1 Cell culture and Laser Irradiation

MCF-7 breast cancer cell lines (ATCC HTB-22) were used in this study. Appropriately, 5×10^5 cells were seeded in 3.4cm^2 diameter culture dishes and incubated for 4 h to allow the cells to attach and recover homeostatically. Experimental cells were divided into four groups; untreated controls, cells treated with doxorubicin, PDT treated cells and cells treated with a combination of PDT and doxorubicin. Three different concentrations (0.25, 0.5 and 1μM) were used for both doxorubicin and zinc phthalocyanine photosensitizer. For combination treatment, suboptimal concentration of doxorubicin (0.5μM) was used with each of the three concentrations of the zinc phthalocyanine photosensitiser. The choice to use suboptimal concentration was to diminish the possible side effects of chemotherapeutic agent while retaining its efficacy. Laser irradiation was performed using a 681.5 nm diode laser and its parameters are shown in Table 1. Cellular responses were analyzed 24 h after treatment. Cellular morphology was observed using inverted microscopy while the proliferation of cells was measured with homogenous ATP quantitative assay. The mechanism of cell death was investigated using Annexin V/PI flow cytometric analysis. The statistical analysis was performed on four repeats of each sample and significant difference were considered at $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***).

Table 1. Laser parameters.

Parameters	**Laser**
Name and type	Semiconductor (diode)
Wavelength	681.5 nm
Spectrum	Red (visible)
Wave emission	Continuous
Spot size	9.1 cm^2
Power output	43 mW
Power density	4.74 mW/cm^2
Fluence	5 J/cm^2
Irradiation time	17 Minutes 36 Seconds

3. Results

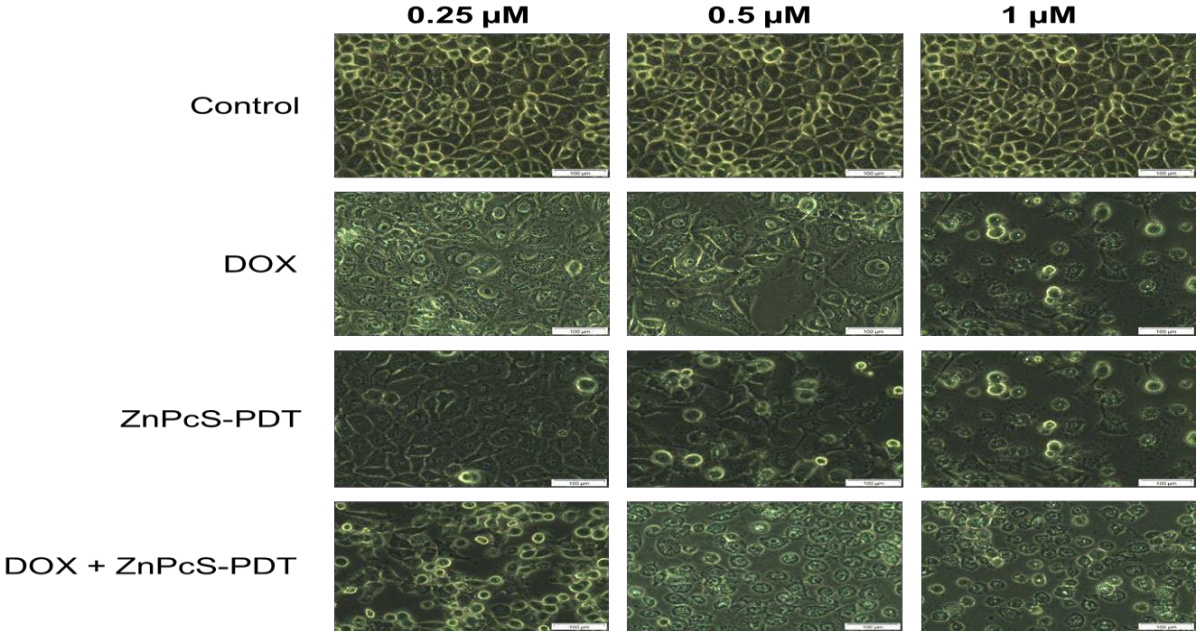

Figure 1. Morphology of MCF-7 cells after treatment investigated using an inverted light microscope with 200X magnification.

The results showed morphological changes like cell membrane damage, rounding up and floating of cells in the culture medium in cell groups treated with either doxorubicin or photodynamic therapy when compared to the untreated control. Combined treated group was observed to have an increased number of rounding and floating cells, indicative of dying cells. Likewise, there was a significant decrease in the cellular viability and proliferation within the combined treated groups. Despite the high antiproliferative efficacy of 1 µM ZnPcS-PDT and 0.5 µM doxorubicin, the percentage of apoptotic cells was approximately 47% which was significant when compared to untreated control. We observed fewer non-apoptotic cells. Altogether, these results showed strong efficacy and enhanced anticancer effects when a low dose doxorubicin chemotherapy is combined with phototherapy mediated by zinc phthalocyanine.

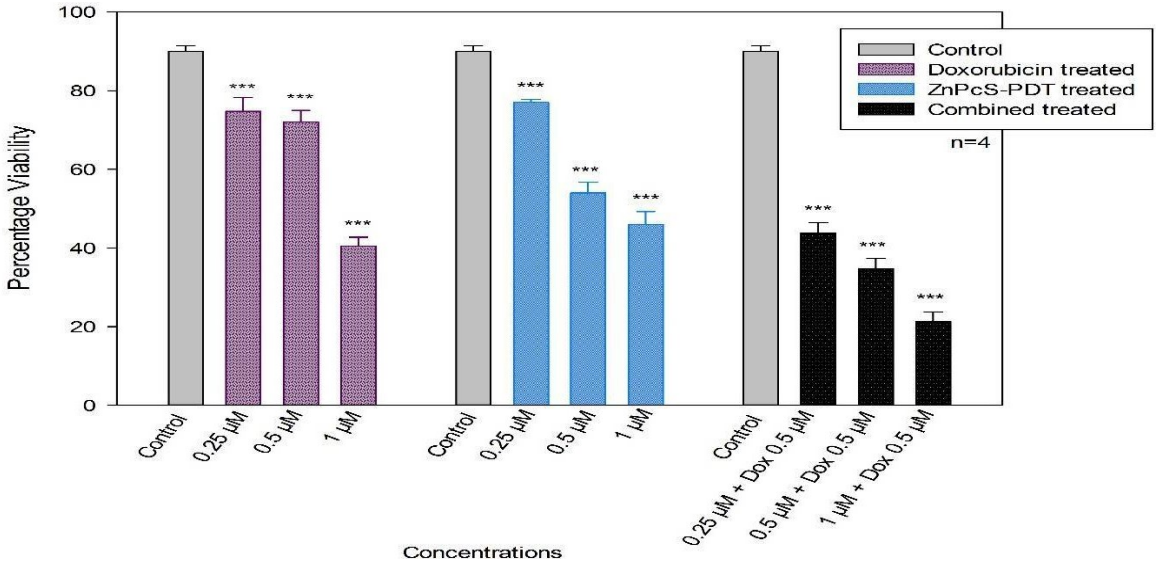

Figure 2 Trypan blue exclusion cell viability assays. There was a significant decrease in the viability of cell across a treated group when compared to the untreated control. Results represents the mean ± standard error of four independent experiments. Significant differences between treated groups and control are shown as ***$P< 0.001$

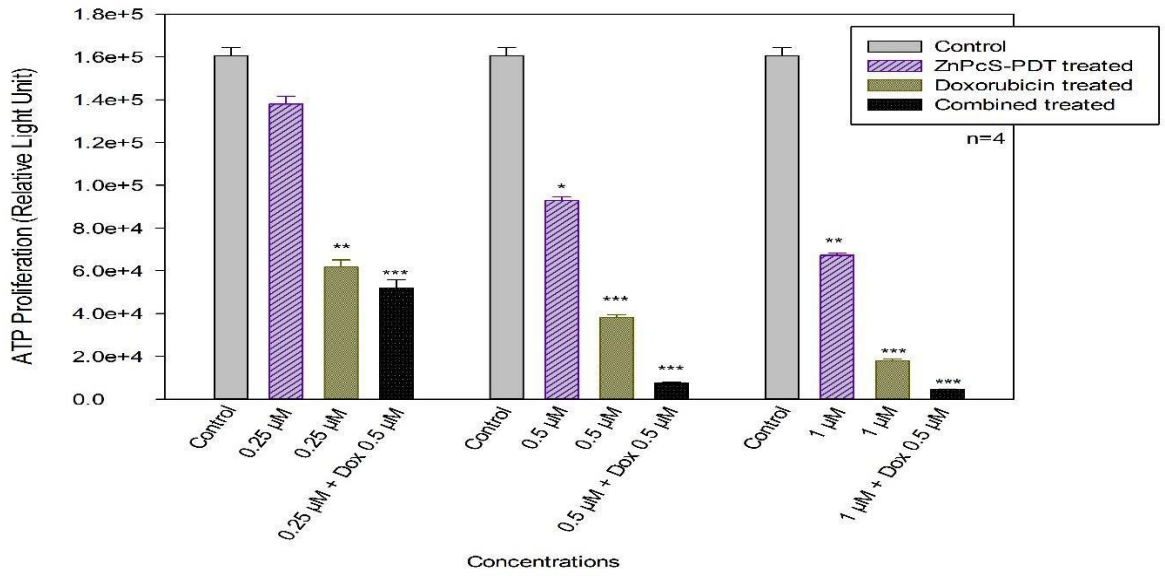

Figure 3 Cell proliferation measured by Adenosine triphosphate luminescent assay. There was a significant reduction in the rate of cellular proliferation within the combination treatement group compared to the untreated control. Results represent the mean ± standard error of four independent experiments. Significant differences between treated groups and control are shown as ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$

Table 2 Flow cytometric assessment of cell death pathway with various concentration of ZnPcS-PDT in combination of 0.5 μM DOX. Cells were stained with Annexin V-FITC/PI and results represent the mean ± SE of three independent duplicate experiment. Significant differences between treated groups and control are shown as ***$P < 0.001$; **$P < 0.01$

Groups	LL-Normal cells (%)	LR- Early apoptotic cells (%)	UR- Late apoptotic dead cells (%)	UL non-apoptotic cells (%)
Control	93.79 ± 0.29	2.11 ± 0.14	3.64 ± 0.20	0.46 ± 0.05
0.25 μM + 0.5μM DOX	70.48 ± 1.70***	17.39 ± 2.33**	12.0 ± 1.17**	0.13 ± 0.03
0.5 μM + 0.5μM DOX	68.61 ± 0.49***	22.48 ± 2.41***	7.89 ± 1.49	1.02 ± 0.54
1 μM + 0.5μM DOX	52.89 ± 2.44***	31.10 ± 2.80***	15.88 ± 1.34**	0.13 ± 0.06

4. Discussion and Conclusion

Degenerative diseases like cancer have shown to involve multiple pathologic processes, which make most mono-therapeutic modalities to be ineffective. Doxorubicin is the most common chemotherapeutic agent used for fighting cancer malignancies but its toxicity and development of drug resistance have been a major problem limiting its use [7]. Photodynamic therapy, directly or indirectly produce an increase reactive oxygen species that kill tumor cells, induces inflammatory and immune response with tumor vasculature shutdown that effectively leads to tumor control [8]. It was hypothesized that the use of another anticancer agent whose mechanism of action also involves the formation of toxic oxygen radicals may also enhance the effectiveness of photodynamic therapy. Hence, we explore the interaction and therapeutic efficacy of combining doxorubicin and photodynamic therapy in cancer treatment. Such combination therapies are currently been investigated as an alternative treatment modality for various cancer types. The sole aim of combination therapy is to reduce the dose of the toxic chemotherapeutic agents and their side effects while still retain its therapeutic value [9]. Recent studies by Ruiz-Gonzalez et al. [10] demonstrated the potentiation in pheiphorbide a mediated photodynamic therapy with the addition of low dose concentration of doxorubicin. Similar studies by De-Freitas et al. [6] showed that photodynamic therapy mediated by either methylene blue or photogem in combination with cisplatin chemotherapy has low mutagenic potential, which elicits the potential of such combined therapy in diminishing the toxicity of antineoplastic drugs. We have demonstrated in MCF-7 cancer cell lines that ZnPcS-PDT tumor killing can be significantly increased with low dose of doxorubicin. Agostinis and colleagues reported that photodynamic therapy could evoke three main cell death pathways with apoptosis being the major mode of cell death [11]. Other evidence suggests that doxorubicin activity can produce reactive oxygen radicals, which trigger the induction of cell death through apoptosis [12,13]. Reactive oxygen species have been known to be a cellular stress factor that can effectively induce an active mode of cell death, apoptosis if produced over a certain level of quantity [14]. Subsequently, we investigated the mode of cell death after the combination treatment and our results showed that majority of cells were undergoing apoptosis. It was observed MCF-7 cancer cells incubated with 1 μM ZnPcS photosensitizer with 0.5 μM doxorubicin have a significant number of cells in the early apoptosis and late apoptotic stage. Morphology alterations induced by this combination therapy were similar to apoptosis as cells gradually loss their morphological characteristic and become rounded. We observed fewer non-apoptotic cells thus suggesting apoptosis as the undergoing mode of cell death. Doxorubicin

and photodynamic therapy have demonstrated the formation of reactive oxygen species hence results obtained in this study are indicative that apoptosis is the mode of cell death which is in consensus with previous studies mentioned particularly the combination of photogem mediated photodynamic therapy with cisplatin [6]. This study has demonstrated that photodynamic therapy may be enhanced by the addition of cytotoxic agents like doxorubicin at lower doses and still provides great efficacy in destroying cancer cell. In an attempt to increase the therapeutic effect of doxorubicin and possibly reduce its side effects and development of resistance, doxorubicin should be combined with photodynamic therapy. Obviously, further studies are needed to compare other cytotoxic agents in combination with photodynamic therapy for possible synergistic, additive or antagonistic effect. Moreover, studies to delineate the mode of action of doxorubicin and photodynamic therapy warrants further studies.

Funding

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