In vitro antiproliferative effects of berberine in phthalocyanine-mediated photodynamic therapy on MCF-7 breast cancer cells with overexpressed P-Glycoprotein

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Abstract. Multidrug resistance (MDR) is one of the common challenges seen in cancer therapy. This phenomenon has led to the development of novel therapeutic strategies in which chemotherapeutic drugs are administered in combination with photodynamic therapy (PDT). Conventional therapeutic strategies employed in cancer treatment have been reported of yielding good prognosis which is accompanied with undesirable adverse effects. As a result, effective treatments with no potentially catastrophic side effects are required. PDT is a non-invasive phototherapeutic method in which a specific wavelength of light is used to activate photosensitizer (PS), to generate cytotoxic reactive oxygen species (ROS). The combination of PDT with other chemotherapeutic drugs has been studied for many years. The aim of this study was to enhance PDT using a chemotherapeutic drug, berberine (BBR) in combination with zinc phthalocyanine tetrasulfonic acid ($ZnPcS_4$) on MDR MCF-7 breast cancer cells with overexpressed P-glycoprotein (P-gp). MDR MCF-7 breast cancer cells were treated with optimized concentrations of BBR and $ZnPcS_4$ and later irradiated by using a 680 nm diode laser at a fluency of 10 J/cm^2. The established 50 % inhibitory concentration (IC_{50}) was used to evaluate antiproliferative effects induced by individual IC_{50} as well as in combination therapy. Morphological changes, adenosine triphosphate (ATP) proliferation, and live/ dead assay were performed to determine the cell cytotoxicity 24 h post-treatment. The results revealed a dose-dependent cytotoxicity in both monotherapy and combination therapy of BBR and $ZnPcS_4$ mediated PDT on MDR MCF-7 cells with significant morphological changes in combination therapy. In conclusion, the results from the present study suggest the use of BBR as an anticancer agent in $ZnPcS_4$ mediated PDT. Furthermore, the combination of the two IC_{50}'s revealed a dose-dependent cytotoxicity in both monotherapy as well as combination therapy as it led to significant morphological changes accompanied with decreased ATP levels.

1. Introduction

Breast cancer (BC) is the second leading cause of cancer related deaths after lung cancer. This form of cancer primarily originates from the breast tissue. According to the Global Cancer Observatory (GCO) 2020 to 2040 report, the global incidence rate of BC is estimated to increase from 2.26 million to 3.19 million by 2040 while the mortality rate is estimated to increase from 685 thousand to 1.04 million by 2040 [1]. The development of BC can be attributed to several factors e.g., excessive alcohol intake, inflammation, age, genetic mutations, radiation, hormones, body mass index etc. [2]. Surgery, radiation, chemotherapy, hormonal, and immunotherapy are some of the conventional therapeutic options for BC

treatment. However, these modalities have been reported of inducing undesirable side effects [3]. Therefore, novel treatment modalities with improved therapeutic outcomes are worth exploring. It's worth noting that the choice of these therapies is determined by the tumor stages and progression. In order to overcome some of these side effects, many researchers are working on novel therapeutic strategies which include the combination of PDT with chemotherapeutic drugs.

Photodynamic therapy (PDT) is a novel therapy that uses non-ionizing radiation to induce tumor cell death [4]. This form of therapy involves the application, or intravenous injection of a phototoxic drugs known as photosensitizers (PSs) on affected body sites e.g., skin. The principle behind PDT is based on the molecular interactions of preferentially co-localized PS with laser, and molecular oxygen (O_2), thus leading to the generation of cytotoxic reactive O_2 species (ROS) [5]. In addition, there is an increased number of research are focusing on enhancing PDT by use of chemotoxic drugs such as berberine (BBR) [6]. Herein, we evaluated the anticancer effects of BBR in combination with $ZnPcS_4$ mediated-PDT on MDR MCF-7 breast cancer cells using a 680 nm diode laser at 10 J/cm^2 *in vitro*.

2. Methods and materials

2.1. Cell culture, and treatments

MCF-7 human breast cancer cells (ATCC® HTB-22) purchased from American Type Culture Collection (ATCC), were used in the present study. Chekwube et al., developed a doxorubicin (DOX) resistant MCF-7 cell line [7]. MCF-7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 1% Penicillin-streptomycin, 1% Amphotericin B, and 10% Fetal Bovine Serum (FBS). Cells were then incubated for 24 h at 37°C, 85% humidity, and 5% carbon dioxide (CO_2). A seeding density of 3 x 10^5 cells/mL was used to plate cells in a 3.4 cm^2 diameter culture plates for *in vitro* studies. The 50% inhibitory concentration (IC_{50}) for both BBR and $ZnPcS_4$ was calculated 24 h post-treatment using different concentrations of BBR (100, 200, 300, and 400 μM) and $ZnPcS_4$ mediated PDT (10, 20, 40, and 80 μM) respectively.

2.2. Cell proliferation (ATP luminescence assay)

CellTiter-Glo® ATP luminescence assay kit (Promega, G968A) was used in the present study. Briefly, about 50 μL of cell suspension was suspended to an equal of reconstituted ATP reagent, mixed thoroughly and incubated for 10 min at room temperature and pressure (rtp). Post-incubation, the homogenous colorimetric mixture was then measured for ATP luminescence using PerkinElmer, VICTOR Nivo™.

2.3. Morphological analysis and cell viability/ cytotoxicity (LIVE/DEAD™ assay)

Morphological changes were visualized under an inverted light microscopee (Wirsan, Olympus CKX 41) with attached digital camera (Olympus C5060-ADUS). Following the manufacturer's instructions, the LIVE/DEAD™ assay kit (Cat. No. L3224) (Life Technologies Corporation) was used to qualitatively visualize the distribution of viable and non-viable MDR MCF-7 cells 24 post-treatment with IC_{50} concentrations of BBR and $ZnPcS_4$ mediated-PDT. Briefly, the cells (i.e., untreated and treated) were washed thrice by using ice cold 1X PBS (1 mL) and resuspended in 1 mL of 1X PBS. Thereafter, cells were stained with calcein (1 μL), ethidium homodimer-1 (EthD-1) (1 μL) and incubated for 30 min at rtp. Cells were then rinsed thrice with 1 mL 1X PBS after incubation, resuspended in 1 mL 1X PBS, and visualized with Alexa Fluor 488 and EtBr filters using a Carl Zeiss Axio Z1 live imaging microscope.

2.4. Statistical analysis

All experiments were performed four times (n=4). IBM SPSS version 27 software was used to analyse the mean difference and statistical significance between the control and experimental groups. Mean values plotted as mean ± standard error (SE) and statistical significance is represented as ($p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***)).

3. Results and discussion

3.1. Cell proliferation

Energy levels of untreated and treated MDR MCF-7 cells were determined by measuring the levels of ATP luminescence. A dose-dependent decrease in cell proliferation was observed in cells treated with BBR, $ZnPcS_4$ mediated-PDT (Figure 1 A and C) and in combination therapy where established IC_{50}'s was used (Figure 2 B). However, ATP levels were higher in MDR MCF-7 cells that received laser treatment only, and cells that were treated with $ZnPcS_4$ but did not receive laser treatment (Figure 1 C). These findings are in line with those reported by Uprety et al.,[8] and Loonat et al., [9].

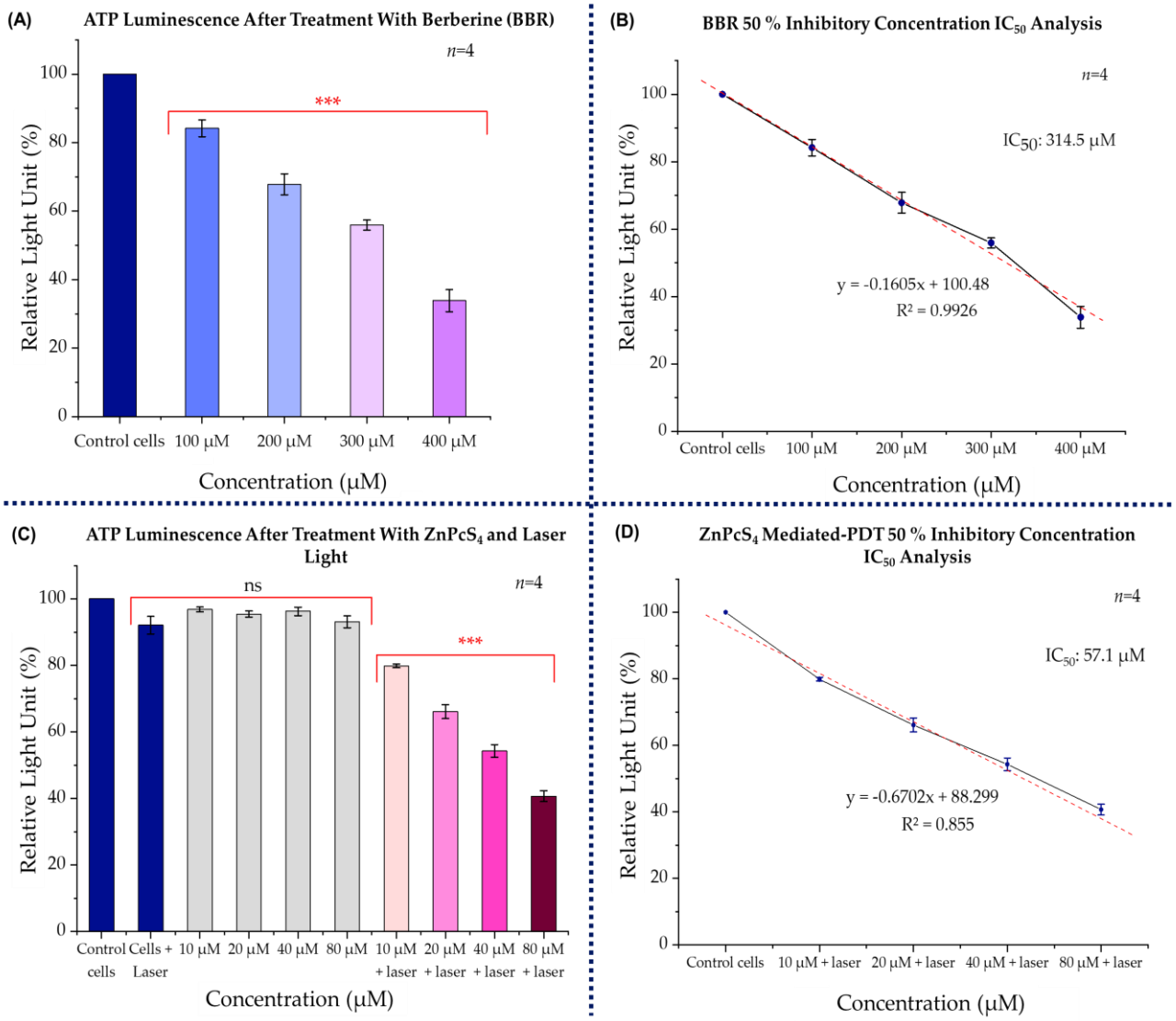

Figure 1. ATP luminescence of the control and experimental groups of BBR and $ZnPcS_4$ 24 h post-treatment **(A** and **C).** Linear regression for IC_{50} calculations; BBR IC_{50} (314.5 µM**) (B)**, and $ZnPcS_4$ mediated PDT IC_{50} (57.1 µM) **(D).** Significance $p<0.001$ (***).

3.2. Morphological analysis and cell viability/ cytotoxicity (LIVE/DEAD™ assay)

Morphology of untreated and treated MDR MCF-7 cells (Figure 2 A). No alterations in morphology were displayed by laser treated cells without a PS (Figure 2, A 2) when compared control cells (Figure 2, A 1). This suggests that 680 nm diode laser alone has no phototoxic effect on cancer cells. However,

changes in morphology were observed in MDR MCF-7 cells treated with BBR IC_{50}, and $ZnPcS_4$ mediated-PDT in monotherapy and combination therapy (Figure 2, A 3-5). The results of the present study are similar to those reported by Tynga et al., [10] and Mkhobongo et al., [11].

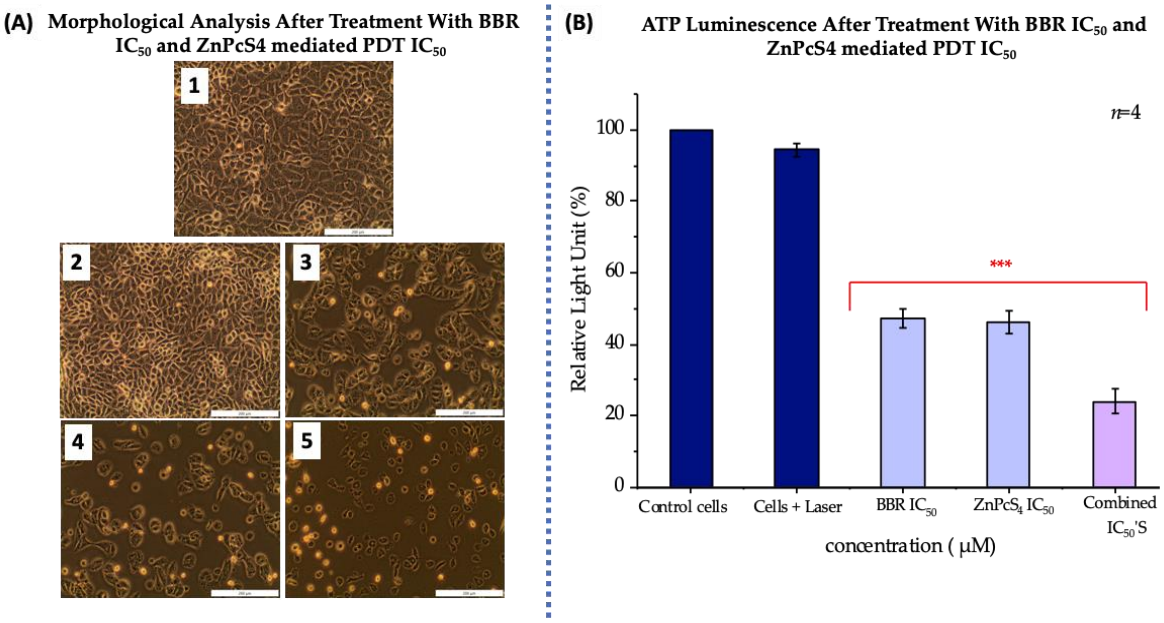

Figure 2. Cellular morphology of untreated cells (**A** 1); cells + laser (**A** 2); cells + BBR IC_{50} (**A** 3); cells + $ZnPcS_4$ IC_{50} + laser (**A** 4); and combination of IC_{50}'s (**A** 5). ATP luminescence of the control, IC_{50}'s of of BBR and $ZnPcS_4$ mediated PDT (**B**). Significance $p<0.001$ (***). (×200 magnification).

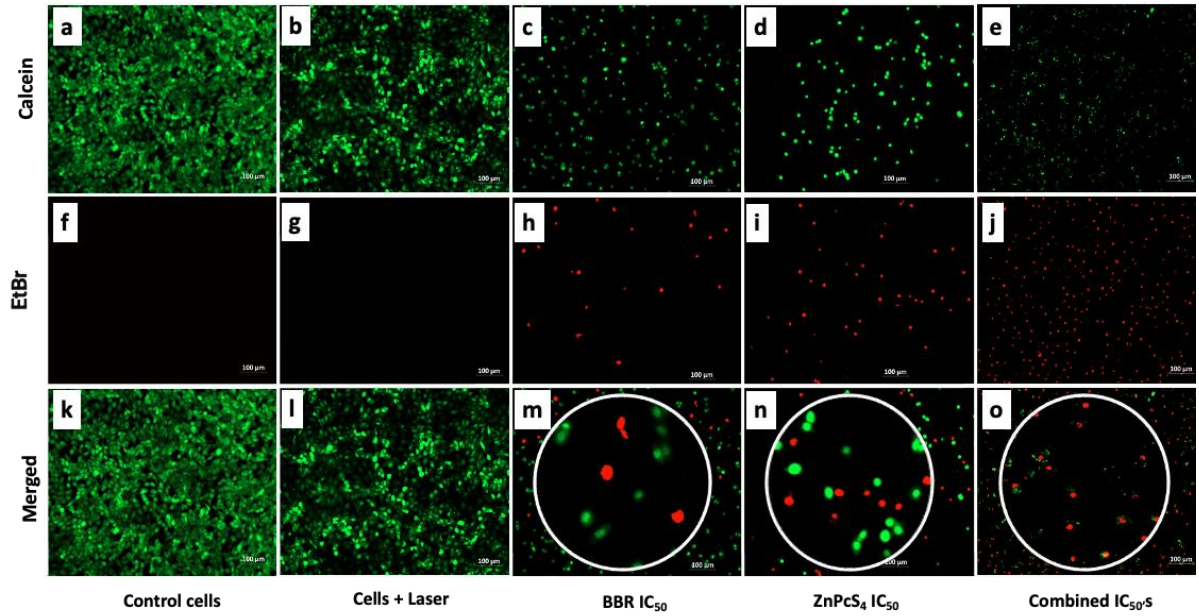

Figure 3. LIVE/DEAD™ assay. Live cells stained with calcein (green fluorescence) (**a-e**) and dead cells stained with EtBr (red fluorescence) (**f-j**). (×200 magnification).

Additionally, the LIVE/DEAD™ assay qualitatively displayed an increase in the number of dead cells in combination of the two IC_{50}'s when compared individual treatments (h-j). Interestingly, MDR MCF-7 cells that were treated with laser light did not stain with EtBr. This suggests that the cells membrane

of the cells was intact and could not allow the penetration of the stain. Overall, the results obtained from this assay are in support with those reported by Hassan et al., [12].

4. Conclusion

In conclusion, BBR has demonstrated antiproliferative effects in individual treatments as well as in combination with $ZnPcS_4$ mediated PDT on MDR MCF-7 breast cancer cells *in vitro*. Although conventional treatment modalities for breast cancer often result in cancer recurrences with undesired side-effects. The present study suggests the use of BBR in combination with $ZnPcS_4$ to overcome the above highlighted limitations seen in breast cancer treatment. In addition, to clearly understand the mechanisms behind BBR and $ZnPcS_4$, a detailed cell death analysis will be warranted to give a comprehensive mode of cell death induced by the two drugs in individual treatments and in combination therapy.

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Conflict of interest

Authors declare no conflict of interest.

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