RESEARCH ARTICLE

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The Effect of NF-κB Deactivation on Cancer Cell Response to ALA Mediated Photodynamic Therapy

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Abstract

Objective: Breast cancer is one of the most widespread tumors among women worldwide, which is difficult to treat due to the presence of chemoresistance and the risk of tumor recurrence and metastasis. There is a pressing necessity to develop efficient treatments to improve response for treatment and increase prolong survival of breast cancer patients. Photodynamic therapy (PDT) has attracted interest for its features as a noninvasive and relatively selective cancer treatment. This method relies on light-activated photosensitizers that, upon absorbing light, generate reactive oxygen species (ROS) with powerful cell-killing outcomes. Nuclear factor kappa B (NF-κB), a transcription factor, plays a key role in cancer development by regulating cell proliferation, differentiation, and survival. Inhibiting NF-κB can sensitize tumor cells to chemotherapeutic agents. Dimethyl fumarate (DMF), an NF-κB inhibitor approved by the FDA for multiple sclerosis treatment, has further shown promise in suppressing breast cancer cell growth in vitro. We hypothesized that combining PDT with Dimethyl fumarate (DMF) could further enhance therapeutic efficacy for both treatment modalities. **Methods:** In the current study, we explored the PDT effect of 1 and 2 mM aminolaevulinic acid (ALA) and low-power He-Ne laser irradiation combined with different concentrations of DMF (2.5, 1.25, or 0.652 µg/ml) against hormone nonresponsive AMJ13 breast cancer cell line that is derived from Iraqi patient. **Results:** Our results demonstrated that co-administration with all tested DMF concentrations significantly enhanced the cytotoxicity of PDT antitumor effect. The combination index analysis showed presence of synergism in combining PDT with DMF. **Conclusion:** This finding suggests that the combination of PDT with DMF could be a promising novel strategy against triple negative breast cancer that could be applied clinically due to the fact that both of these treatments are already clinically approved therapies.

Keywords: Aminolaevulinic acid (ALA)- AMJ13 cell line- dimethyl fumarate (DMF)- photodynamic therapy (PDT)

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Introduction

Breast cancer is a lethal difficult-to-treat type of cancer due to its high recurrent rate and risk of metastasis, as well as developing chemoresistance [1-3]. Therefore, there is pressing need for effective treatment that can overcome the resistance to treatment and attacking cancer cells by multiple therapeutic agents found to be better strategy by utilizing different mechanism for cancer cell death [4, 5]. Photodynamic therapy (PDT) is an emerging non-invasive treatment modality for superficial cancer that consists of two simple steps: the administration of a photosensitive agent (photosensitizer) PS, and the illumination of the tumor to photoactivate the PS drug [6, 7]. PSs are non-toxic inert materials that accumulate in host and tumor cells. The light activation of an applied PS generates a photodynamic reaction in the presence of oxygen. Depending on the formation of reactive oxygen species, this reaction leads to cytotoxic and vasculotoxic shutdown rapidly, which is demonstrated to be highly effective a tumor-ablative therapy [8, 9]. Light dosimetry, oxygen availability and the characteristics of PS are considered as interfering factors that interfere with the effectiveness of PDT [10]. Aminolaevulinic acid (ALA) is a naturally occurring intermediate in the heme-synthesis pathway. It is normally synthesized in mitochondria via the reaction that is catalyzed by ALA synthase [11, 12]. Cells with elevated metabolic effectiveness, such as tumors, preferentially uptake ALA. Rather than the formation of heme, the conversion of ALA in these cells leads to the accumulation of protoporphyrin IX (PPIX) because the absence of ferrochelatase, the enzyme that converts PPIX into heme [13, 14]. Thus, non-photosensitive 5-ALA is metabolized into sensitive protoporphyrin IX which

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accumulates in cancer cells due to their ferrochelatase deficiency. On the other side, a nuclear transcription factor-kappa B (NF-κB) is involved in many cellular processes such as cell proliferation, differentiation, angiogenesis, metastasis and apoptosis [15] [16]. NF-κB is known to regulate the diverse genes of cytokines, growth factors, cell adhesion molecules, and pro and antiapoptotic proteins [17]. NF-κB molecules present in the cytoplasm as homodimer or heterodimer complexed with an inhibitory protein, IκB. After the pathway stimulation, the signaling cascade leads to activation of the IκB kinase (IKK), which phosphorylates IκB. By proteasome degradation, IκB dissociates from the NF-κB dimer and then moves into the nucleus, and stimulates the production of specific proteins [18]. Many reports have highlighted the activation of the NF-κB following PDT treatment and suggested that NF-κB play a crucial role to increase proliferation of cells via the transcription of anti-apoptotic proteins [19, 20]. DMF is a drug that is clinically used to treat relapsing-remitting multiple sclerosis or as a systemic medication for moderate to severe psoriasis [21]. It is believed that the mechanism of action of DMF consists of two pathways, nuclear factor erythroid-derived 2-related factor (Nrf2)-dependent and independent pathways. This leads to an immune response (anti-inflammatory) caused by type II myeloid cell and Th2 cell differentiation that leads to neuroprotection [22, 23]. A number of molecular or immunological factors have been investigated to explore their potency to sensitize breast cancer cells [24-27] alongside other cancers [28, 29] for therapeutic agents. Many researchers indicated the ability of DMF to inhibit NF-κB in many types of cancers and tumors [30, 31]. The current work aims to explore the combination of PDT and DMF for NF-kB inhibition as a novel strategy against triple negative breast cancer, as both treatments are already clinically approved therapies in human patients.

Materials and Methods

Chemicals

The photosensitizing agent, aminolaevulinic acid (ALA), and precursors were purchased from Santa Cruz Biotechnology (Texas, USA). As a solvent, phosphate buffer saline was used to dissolve ALA in a dark room. NaOH (5M) was used to adjust the pH to 7.0. Sixty mM of ALA was prepared as a stock solution and kept at -20 ° C prior to use. DMF Pharmaceuticals (Almirall, S.A.) was purchased from the market pharmacy. This agent was diluted with a phosphate buffer saline just before use for in vitro studies.

Cell culture

The Iraqi breast cancer cell line AMJ13 was obtained from the Iraqi Center for Cancer and Medical Genetics Research (ICCMGR), Mustansiriyah University, Baghdad, Iraq. Cells were maintained in RPMI-1640 medium containing sodium bicarbonate (2.0 g/L) and L-glutamine (0.3 g/L) and supplemented with 10% fetal bovine serum (FBS) and 25 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin. All materials were supplied by Capricorn Scientific, Germany. The cells were grown to reach near 60-70 confluence, they were passaged using Trypsin-EDTA (US Biological, Salem, MA, USA). After that, they were incubated in a 5 % CO_2 incubator at 37˚C [32].

Exposure of the cells to laser

Twenty-four hours after cells seeding in 96 wells microplate, 100 µl of 1 or 2 mM of ALA were added to the wells and incubated for 4 h at 37°C and then, fresh SFM was used instead of the old media after washing the cells with physiologic saline. After that, we employed a low power Helium-Neon (He-Ne) atomic gas laser (Model DL30, LG Lasers, USA) at wavelength of 632.8 nm with output power of 20 mW for the proposed energy doses provided as continuous wave mode application. In terms of control cells, neither irradiation nor ALA was used, instead they were incubated with fresh media. The low-power helium-neon atomic laser lamp was used for irradiation in this study. The laser light was oriented from the top of the plate to ensure uniform delivery to each well. To calibrate the laser density, a power meter was used. We adjusted the exposure time to obtain a laser energy density of 16.2 or 10.8 J/cm^2. Fresh media was added to irradiated wells and cells were incubated for 24 h at 37°C. Subsequently, dimethyl fumarate, at concentrations of 2.5, 1.25, or 0.652 µg/ml was added and then the cells were incubated at 37°C for 72 hours. To detect the inhibition ratio, an MTT assay was used to determine the cell viability. For the MTT assay, the cells in 96 well plates were incubated with 20 µl of 5X MTT and incubated for 3 h. at 37°C. After that, 200 µl of Dimethyl sulfoxide DMSO was added to the cells. The plate was shaken slowly for 15 min and the absorbance was measured with a microplate reader (FLUOstar, Optima, Germany) at 492 nm. The measured values represent the mean of the absorbance values. The cell inhibitory rate was calculated according to the formula:

Cell inhibitive rate (%) = [OD of control cells - OD of treated cells)/OD of control cells] x100% [33].

By plotting x-y and fitting the data with a straight line, IC_{50} was measured. The IC_{50} value was then estimated using the fitted line, that is, Y = a * X + b, IC_{50} = (0.5 - b)/a

Or by Y=Bottom + (Top-Bottom)/(1+10^((X-LogIC_{50}))

Laser irradiation dose measurements

In this study, the He-Ne laser doses (energy density) applied were 16.2 J/ cm^2 or 10.8 J/cm^2 according to the formula (D (Dose in J/cm^2) = P (Power output in watts) × T (irradiation time in sec) /A (area of irradiation spot in cm^2)), where the respective laser exposure times were 68 sec and 102 sec on the irradiation spot of 4mm diameter.

To calculate the photodynamic dose, we used the formula:

Photodynamic dose = $\int t \varepsilon . \varphi (t ') \rho . 0 h \nu$

where:

- 'ρ' is the density of cells (g/cm^3)
- 'φ' is the laser output power (W/cm^2)
- 'hν' is the energy of a photon (J/ph)
- 'c' is the drug concentration in cells (µM)

- 'ε' is the extinction coefficient of the photosensitizer drug (1/cm/μM) [34, 35]

For example, when we used 1mM ALA with 10.8 J/cm^2, the photodynamic dose was 2160 photons/gm.

According to the equations, and as we used two concentrations of ALA, 2 or 1 mM, with two laser doses (16.2 or 10.8 J/cm^2), the photodynamic doses used were 6,172.8, 5,092, 3,164.2 or 2,160 ph/gm respectively.

Combination index and Chou–Talalay Analysis

To analyze the combination of NF-κB inhibitor DMF with PDT (ALA/Laser), this relationship was studied as a non-constant ratio to detect the synergistic effect. Chou–Talalay combination indices (CI) for this combination were determined by CompuSyn software (Combo Syn, Inc., Paramus, NJ, USA). Nonfixed ratios of DMF and phototherapy, as well as mutually exclusive equations, were used to determine the Cis. A CI between 0.9 and 1.1 is considered additive, while CI < 0.9 and CI > 1.1 indicate synergism and antagonism, respectively [36, 37].

Statistical analysis

All results were presented as means ± SEM. One way ANOVA Tukey's multiple comparisons test analysis was performed using GraphPad Prism version 6, version 6.07 (USA). The level of significance was set at $P < 0.05$.

Results

PDT killing effect

In this study, different concentrations of ALA (1 and 2 mM) with different laser doses (16.2 and 10.8 J/cm^2) were used. Cytotoxicity (MTT) was used to evaluate the effect on the AMJ13 cell line. The results showed that increasing the concentrations of ALA with laser doses caused a mild increase in cytotoxicity. Therefore, the cytotoxicity of 6,172 followed by 5,092 ph/gm was 26.9 and 25.6 % with a highly significant difference compared to the control (cell without PDT) as shown in Figure 1.

Chemotherapeutic agent cytotoxicity

Three concentrations of dimethyl fumarate (2.5, 1.25 or 0.652 μg/ml) were used in this experiment. All these three concentrations showed cytotoxicity against AMJ13 (52.4, 46, 43.9 %) respectively. The highest concentration (2.5 μg/ml) reflects the highest cytotoxicity among them,

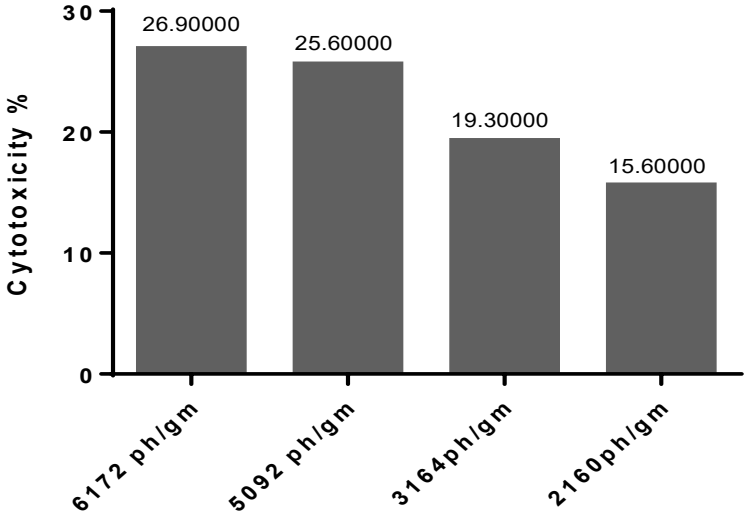


Figure 1. The Percentage of Cytotoxicity of Photodynamic Therapy against the AMJ13 Cancer Cell Line ($p \leq 0.01$).

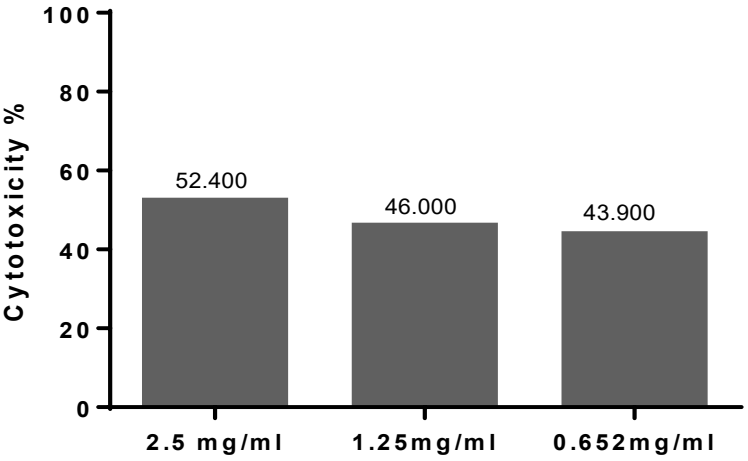
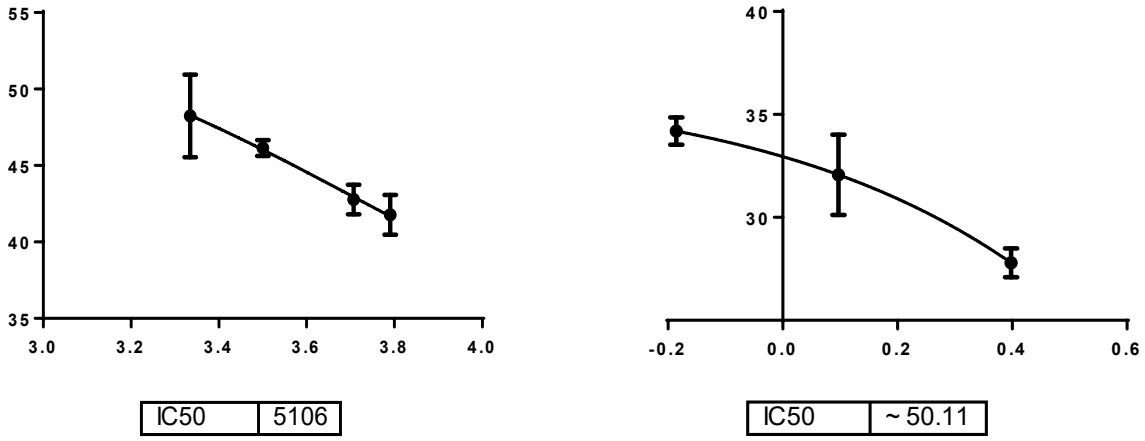


Figure 2. The Cytotoxicity % of Dimethyl Fumarate against the AMJ13 Cell Line ($p \leq 0.01$).



(a) NK- inhibitor (DMF) (b) PDT (ALA/Laser)

Figure 3. a, IC_{50} of NF-κB inhibitor DMF; b, IC_{50} of phototherapy (ALA / Laser) using GraphPad Prism software.

52.4 % (PR=47.6 %) as shown in Figure 2. The difference in optical density is shown in Figure 2.

Determination of IC_{50}

The value of the half-maximum inhibitory concentration (IC_{50}) was estimated to evaluate the effect of each treatment of photodynamic therapy or NF-κB inhibitor on cell proliferation. The results showed that 50 µg/ml was needed to kill the AMJ13 cell line, while the PDT dose needed to kill AMJ13 was 5,106 ph/gm. Therefore, IC_{50}-related doses of PDT and DMF were chosen for the combination study (Figure 3 a and b).

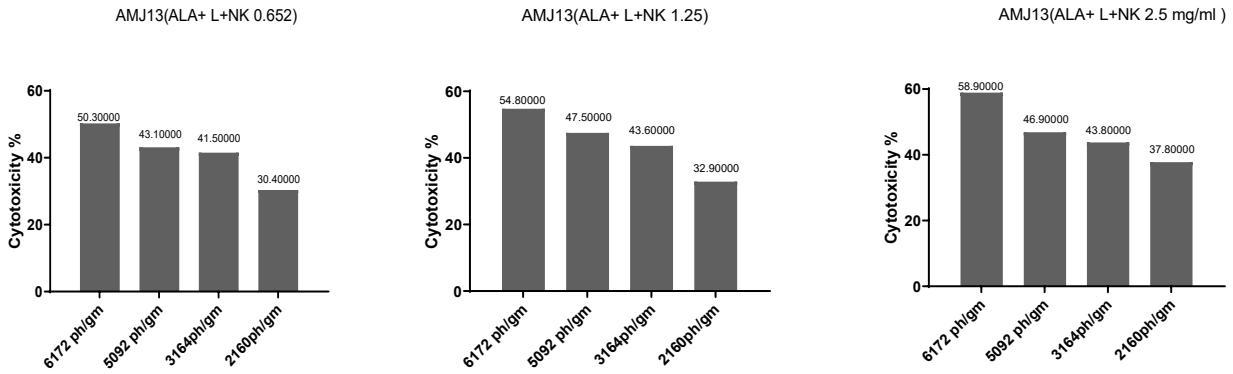
Combination NF-κB inhibitor and phototherapy in vitro

Nearly similar cytotoxicity was achieved at 2.5 and 1.25 µg/ml of DMF when combined with 3,164 ph / g of PDT. Greater cytotoxicity of 58.9 and 46.9 (nearly two folds) were achieved for the synergism of DMF and phototherapy at 2.5 µg/ml followed by 1.25 µg/ml at 6,172 and 5,092 ph/gm respectively. Whereas PDT alone had 26.9 and 25.6 % (PR= 73.1 and 74.4) respectively as shown in Figure 4 a, b, c.

Using the dose-oriented isobologram technique, the AMJ13 cancer cell line had synergism between DMF and phototherapy at 50% growth inhibition doses, as shown in (Figure 5a), which explains the synergism effect at combination points 1(CI: 0.54331), 5 (CI: 0.55110) and 9 (CI: 0.58281) (Figure 5a). The details of the combination point dose are described in Figure 5b.

Discussion

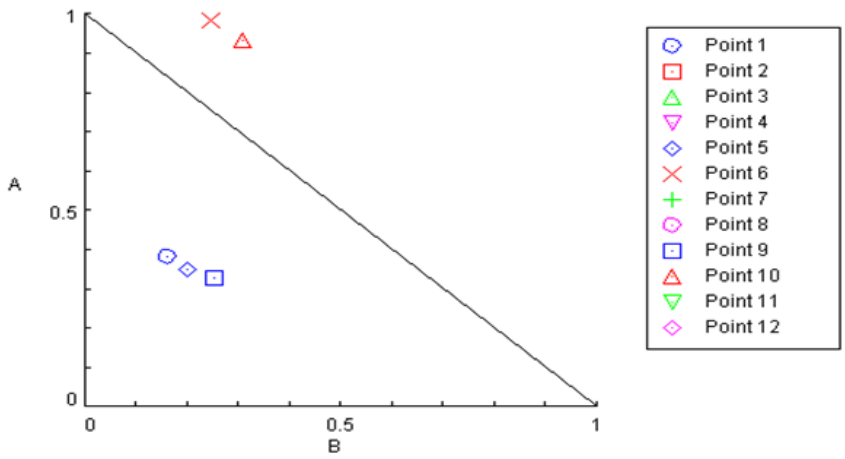
This study focused on developing novel approaches to treat hormone-unresponsive breast cancer derived from an Iraqi patient. Breast cancer is a complex disease that develops chemoresistance to conventional therapies, making it difficult to treat. This complexity manifested by the presence of imbalances in many elements may contribute to its development and progression [38]. There is need for precise diagnostic tools to guide treatment decisions [39]. Novel breast cancer treatment can help overcome resistance to therapy by inducing apoptosis in cancer cells [40, 41]. Developing new treatments, such as biological therapies, is important to overcome the



(a) (b) (c)

Figure 4. Cytotoxicity Percentage (mean ± SD) of Three Concentrations of NF-κB Inhibitor a; 2.5, b; 1.25, c; 0.652 µg/ml combined with 4 doses of phototherapy (6172.8, 5092, 3164.2 or 2160 ph/gm) respectively ($p \leq 0.01$).

(a)



(b)

Points	DMF Dose µm/ml	PDT dose (ph/g)	Effect	CI
1	2.5	6172	0.58	0.54331
2	2.5	5092	0.46	2.5458
3	2.5	3164	0.43	3.77405
4	2.5	2160	0.37	9.23516
5	1.25	6172	0.54	0.5511
6	1.25	5092	0.47	1.23175
7	1.25	3164	0.43	1.9836
8	1.25	2160	0.32	10.5297
9	0.652	6172	0.5	0.58281
10	0.652	5092	0.43	1.24475
11	0.652	3164	0.41	1.4824
12	0.652	2160	0.3	7.86764

Figure 5. Combination Cytotoxicity (AMJ13 Cell Line). According to Isobologram analysis, generally high doses (CI value < 1) represent synergism between DMF and phototherapy, lower doses (CI value > 1) represent antagonism between the agents. The points that are extremely antagonized will not be able to be included as their value is above 2 and will not show in this figure.

complexity of breast cancer [42, 43]. Very recent studies have focused on the gene expression signature to sensitized breast cancer cells to particular chemotherapeutic agents such as Hesperetin [44]. However, more research is needed to fully understand and overcome the challenges in treating this complex disease. Therefore, we choose to combine two FDA-approved treatments against breast cancer, both targeting biological processes inside the cancer cells. Many studies have shown that photodynamic therapy (PDT) may be an excellent alternative in the treatment of different kinds of cancer, including breast cancer. When PS is triggered, acute reactions lead to the activation of cytokines and proteins that react to phosphorylation. Therefore, the ability of these proteins to bind to other proteins will be changed. In addition to many cellular responses in mitochondria. These changes lead to the stimulation of apoptosis and, in certain circumstances, to necrosis [45].

This work was carried out to explore whether PDT can be enhanced by combination with an inhibitor of the NF-κB pathway. However, the mechanism of synergistic activity is not clear, and oxidation of a wide range of biomolecules in cells occurs due to PDT. These molecules include nucleic acids, lipids, and proteins, leading to severe alteration in cell signaling cascades or gene expression regulation [46, 47]. The cycloxygenase-2 (COX-2) enzyme plays a crucial role in the conversion of arachidonic acid to prostaglandin G2 [48]. It was shown that PDT induces proangiogenic factors released in vitro and in vivo and promotes the expression of the inducible cyclooxygenase-2 (COX-2) enzyme which depends on the activation of NF-κB pathway in different mechanisms among different cell types [49, 50]. However, using NF-κB inhibitor might reduce COX-2 enzyme leading to support PDT effectiveness. Matrix Metalloproteinase (MMP) are proteins that digest extracellular matrix which contains collagens, fibronectin, and elastin [51]. They were shown to be involved in the progression of breast cancer [52]. To establish a relationship between PDT and MMP activation, [53] used fibroblasts exposed to conditioned medium from 5-ALA-treated keratinocytes. These results showed an increase in MMP-1 expression in addition to -3 dependent on IL-la release. Furthermore, because IL-la is a well-known inducer of NF-κB, we could conclude that the activation of MMP-1 and -3 is mediated by NF-κB. Hence, using NF-κB inhibitors might prevent the formation of

MMPs and lead to amending the activity of PDT [54, 55]. However, it is believed that the mechanism of action of DMF includes nuclear factor erythroid-derived 2-related factor Nrf2 -dependent pathway which is crucial in an anti-inflammatory immune response [22]. However, increasing Nrf2 can decrease the effect of PDT [56]. Thus, targeting Nrf2 could increase the efficiency of this combination therapy. Moreover, photodynamic therapy (PDT) is shown to activate NF-κB and the activation of NF-κB directly and indirectly enhance several pathways related to cell survival, including proliferation, inflammation, and survival [57]. Another reason in favor of NF-κB suppression is its ability to regulate antiapoptotic genes expression that will help tumor cell survival and prevent the response to proapoptotic therapeutics [58]. Thus, its inhibition is crucial to increase PDT killing activity.

In conclusion, this study suggests a promising combination of He-Ne-PDT with DMF that could be a promising novel treatment against hormone-unresponsive breast cancer cells after photosensitizing by ALA. This novel treatment could be applied clinically as a therapeutic regimen to treat breast cancer patients since all the involved agents are already clinically approved therapies. Further research is needed to optimize the combination to induce higher photosensitization killing rates and to explore the mechanism of action behind this effect.

Author Contribution Statement

A.M. Al-Shammari and A.S.K. Al-Khafaji made substantial contributions to the conception and design of the research project, analysis, and interpretation of data, generated the datasets and drafted the work. M.I. Salman contributed to the acquisition, analysis, and interpretation of literature data, and revised the manuscript. H.A. Hassan contributed to the acquisition of literature data, provided interpretation, and revised the manuscript. A.M. Al-Shammari sufficiently participated in the acquisition, performed data analysis, interpretation of the data and revised the manuscript. All the authors have read and approved the final version of the manuscript for publication.

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Study Approval

The study proposal was reviewed and approved by the scientific committee of the National Cancer Research Centre, University of Baghdad, Iraq.

Ethics Approval

Ethical approval for the present study was obtained from the Research Ethics Committee at the University of Baghdad under the reference no. NCRCEC/01/004.

Availability of data

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest Disclosure

The authors declare that they have no conflict of interests.

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