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Combination therapy application of Abemaciclib with Doxorubicin in triple negative breast cancer cell line MDA-MB-231

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Abstract

Due to lack of clinical biomarkers, Triple Negative Breast Cancer (TNBC) is more likely to have spread to other tissues at time of diagnosis and therapy planning generally involves use of cytotoxic chemotherapy agents, such as Doxorubicin. We aimed to investigate possible advantages of using combination strategy using Doxorubicin alongside Abemaciclib. After determining the IC50 values for Doxorubicin (DOX) and Abemaciclib (ABE); CompuSyn and ComBenefit software were used to reveal the effect resulting from the combination of two drugs. Following the determined effect, cell death was revealed by fluorescence microscopy and a colony forming assay was performed to see the potential of even a single cancer cell with adhesive character to survive over time and form a clone of itself. Detection of changing antioxidant activity following DOX, ABE and DOX+ABE combination therapy in MDAMB231 cells was determined by measuring MDA, SOD and GSH activities. The expression of Cleaved Caspase 3, PARP, Cleaved PARP, Cdk2 and Bax, which changed as a result of DOX, ABE and DOX+ABE application, was shown by Western Blotting.Cyclin-dependent kinase inhibitors appear as promising agents in therapy planning for breast cancer due to their prominent role in cell cycle regulation, where the number of studies interrogating its efficiency in the treatment of cancer such as TNBC is limited. For this reason, in this study, we aimed to determine the impact of the combined use of the CDK4/6 inhibitors ABE and DOX on the cytotoxicity, apoptotic homeostasis, alterations in antioxidative mechanisms, and the molecular pathways that they utilize. Our results showed that when used in combination, Doxorubicin and Abemaciclib showed a synergistic effect on TNBC cell line MDA-MB-231.

Keywords: Triple negative breast cancer, TNBC, Abemaciclib, Doxorubicin, Combination therapy.

1. Introduction

Breast cancer is the most common type of cancer in women and is the second most common cancer in humans, after lung cancer. It is also important to note that it is the leading cause of cancer-related deaths in women, in developing countries [1,2]. One of the most commonly observed mechanisms leading to the swift transformation of the cancer cells to the advanced stages of the disease is the uncontrolled growth and spread of epithelial cells of the mammary gland and milk ducts [3].

Cells of breast cancer may feature all, some or none of the estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptor 2 (HER2) proteins, where clones that display none of these are termed as the triple-negative breast cancer (TNBC). TNBC has been shown to account for 10-15% of all breast cancer cases, and it tends to be more common in younger women or who have a BRCA1 mutation. The cardinal feature of TNBC that differentiates it from other types of breast cancer is that it tends to develop and metastasize faster, stems from a more complex yet intriguing molecular background, hence has fewer therapeutic targets identified and tends to have a worse prognosis.

Despite the presence of numerous chemotherapeutic agents such as doxorubicin (DOX), and cyclophosphamide; hormone treatment using tamoxifen, and anastrozole; radiation therapy and surgery, long term survival rates of these patients still suffer due to possible side effects of these treatments using strong cytotoxic agents as well as molecular resistance developed during the course of treatment. Loss or alteration of Estrogen Receptor (ER) proteins and/or related pathways leading to the resistance to endocrine-based treatments, via insensitivity or development of alternative gateways. This confronts the scientific community with the challenge of developing of novel strategies that aim to minimize the side effects as well as develop resistance to treatment strategies. For example, a strong agent DOX used in the treatment of TNBC leads to cell elimination via inhibition of Topoisomerase II and DNA intercalation. Its action on Topoisomerase II has been studied in detail which also enabled us to understand the possible paths for developments of the resistance to

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it, via export of the agent and alteration of the topoisomerase [4,5]. Cancer cells employed these anti-strategies employed by cancer cells were through the expression of P-glycoprotein (P-gp) and induction of the cellular pumps such as Multiple Drug Resistance Protein 1 (MRP1). These pumps actively export DOX outside the cell reducing the agent's cellular concentration hence hampering its effectiveness. This can be in parallel to other mechanisms lessening the penetration of DOX into the cancer cells. Yet another resistance mechanism may involve the upregulation of DNA repair mechanisms for cells that already have effective DNA repair mechanisms in place, effectively repairing the DNA damage introduced by the action of DOX, which in total lessens the effectiveness of the DOX agent.

Another possible pathway of anticancer agent action may be through the induction of apoptotic death of cancer cells, which is also triggered by the impacts of DOX in the cancer cells. Therefore, cancer cell's ability to alter apoptotic pathways, downregulating or blocking pro-apoptotic pathways and upregulating or activating anti-apoptotic pathways, rendering the cells insensitive to these trigger signals, may also lead to reduced effect of DOX on cancer cells. Last but definitely not least, the heterogeneous nature of the cancer tissue may further lead to the development of resistant cells or sub-clones within the original tumor tissue. When considered, all of these factors stand as essential reasons for developing DOX resistance in the tumor tissue, underlining the importance of developing personalized and novel treatment strategies.

Combination therapy, using a secondary agent alongside DOX, therefore appears as a promising strategy for formulating strategies for reducing the side effects, decreasing the chance of development of resistance, and increasing the impact of the treatment. This becomes further significant when applied to TNBC with its complex and poorly understood molecular biology (6). For instance, Retinoblastoma (Rb) protein appears as a key player altered during the development of the resistance in these cells [7-9]. This was important since the action of Abemaciclib (ABE) involved inhibition of the CDK4/6 (Cyclin Dependent Kinase 4/6) protein and its downstream Rb pathway may also prevent the establishment of the DOX resistance [10].

Recent years witnessed the development of treatment strategies utilizing CDK inhibitors for HR (Hormone Receptor) positive, negative and advanced or metastatic breast cancer cases. Third-generation and novel CDK4/6 inhibitor Abemaciclib received Food and Drug Administration (FDA) approval and commercialization by 2018 (1). Abemaciclib is a cyclin-dependent kinase (CDK) inhibitor that leads to the inhibition of the CDK4 and CDK6 proteins. CDK4/6 mutations or amplifications leading to overexpression of cyclin D protein, in turn, leads to overphosphorylation of Rb protein, and this leads to aberrations in the regulation of the cell cycle and uncontrolled cell division. This is the underlying cause of the highlighted attention on targeting CDK4/6 protein in cancer [11].

Breast cancer cell lines were intensively used in the study of breast carcinogenesis, with interest in the aberrant regulation of the cellular proliferation, advancement of cancer, and alterations of the apoptotic pathways, with the possibility of investigation of regulation and alteration of the genetics, epigenetics and metabolomics. Human breast cancer cell lines extensively studied for investigative purposes are prone to manipulation and provide consistent and measurable responses enabling us to properly interrogate the impacts of agents in carcinogenesis [12]. MDA-MB-231 cell line has been isolated from the pleural effusion of a patient with invasive ductal carcinoma at the MD Anderson Cancer Center. MDA-MB-231 cell line is ER, PR, HER-2 and E-cadherin negative and expresses a mutant p53 protein, giving it a much-earned nickname as the TNBC cell line. Microarray profiling of the MDA-MB-231 cell genome has been associated with the basal subtype of breast cancer. It is also important to note that MDA-MB-231 cells also lack HER2 receptors, which makes them a perfect cell line model to study TNBC [13].

Recent advances in silico have equipped us with advanced tools to model and predict chemical models in cancer studies. One of these instances was the utilization of the computer software CompuSyn and ComBenefit, enabling us to simulate the combined effects of two therapeutic agents, DOX and ABE, simultaneously, providing us with IC50 values while designing our experiments to document alterations in cellular physiology and metabolism.

Therefore, we studied the impact of the ABE and DOX combination therapy in treating TNBC using the MDA-MB-231 cell line.

2. Materials and methods

2.1. Cell line

MDA-MB-231 (ATCC: HTB-26) breast cancer cell line was grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) supplied with 10% fetal bovine serum (FBS) (Gibco, USA) and 10 U/ml penicillin/streptomycin (PanBiotech, Germany), in a 5% CO2 incubator at 37 ^{0}C. Cell growth was checked daily, and passage was performed to a new culture as they reached 85% confluency. Trypsin was added in volumes of 1 ml and 2 ml to T25 (TPP, Sweden) and T75 (TPP, Sweden) flasks, respectively, after washing with PBS. Detached cells were collected in a falcon tube by adding medium at twice the volume of the added trypsin, following a 3-minute incubation. 10 ml of cells suspended in appropriate amount of media were counted on a Thoma lam after centrifuging for 5 minutes at 1,000 rpm, and the remaining cells were transferred to different growth flasks (6 well plates, 96 well plates, T25 flasks and T75 flasks) for different experimental setups (ATCC, USA).

2.2. Cell survival evaluation

MDA-MB-231 cells were grown at 37^{0}C in 5% CO2 until confluency and were used in MTT (3-(4,5-dimetiltiazol–2-yl)-2,5-difeniltetrazolium bromide) analysis to evaluate cytotoxic effects of ABE (Selleckchem, USA) and DOX (Santacruz, USA) application depending on the duration and dose of exposure. The stock concentrations of ABE and DOX were 1 mM each. Cells were spread on 96 well plates (TPP, Sweden) at a concentration of 1 x 104 cells per well, incubated overnight and subjected to 0 – 5 μM DOX and 0 – 5 μM ABE for 24 hours (The Genomics of Drug Sensitivity in Cancer Project) (FDA, USA). Cells were incubated at 37^{0}C for 4 hours in a 5% CO2 incubator upon addition of 10 mL of MTT salt (Sigma, Life Sciences, USA), which was followed by removal of MTT solution at a final concentration of 5 mg/ml and 100 mL of

DMSO (Sigma, USA) was added to each well. Plates were left in the dark for 5 minutes before obtaining the reading of the spectrophotometer (Biotek, USA). Impact of ABE and DOX on relative survival rates of MDA-MB-231 cells was determined by finding the proportion of the Abs 570 nm reading for treated cells in proportion to the absorbance of the control samples. All measurements were done in triplicates and graphical analyses were carried out using Graph Pad V10.

2.3. Formulation of the combination therapy composition

CompuSyn and ComBenefit softwares were used to elaborate on the combinatory effects of ABE and DOX treatment. Impact of ABE and DOX on relative survival rates of MDA-MB-231 cells was determined by finding the proportion of the Abs 570 nm reading for treated cells in proportion to the absorbance of the control samples. All measurements were done in triplicates and graphical analyses were carried out using Graph Pad V10. Dose-response analyses in MTT tests were prepared based on data obtained from both softwares. Drug synergy impact was first evaluated using the Combination Impact Method developed by Chou and Talalay, using the CalcuSyn software [14]. Further analysis was performed using concentrations from the MTT analysis of ABE and DOX, either alone or in combination. CalcuSyn software was used in the calculation of the IC50 values, Combination Indexes (CI) and Dose Reduction Indexes (DRI) (ComboSyn Inc, Paramus, NJ, USA). CI values smaller than 1 indicated a synergistic effect, equal to 1 indicated additional effect, and larger than 1 indicated antagonistic effect. MDA-MB-231 treatment doses were determined using the calculated CI values.

A secondary approach to determine the synergistic, antagonistic and additive effects of ABE and DOX agents was carried out using ComBenefit software (Cambridge, UK) utilizing the cell vitality and proliferation values obtained from MTT analysis using combinatorial information from dose-response squares [15]. The Bliss Model in the ComBenefit software was used to simultaneously evaluate synergistic and antagonistic impact of ABE and DOX treatment. ComBenefit software calculates a Synergy Score for ABE and DOX, for each different combination of doses. A positive score indicates synergy, a score of "0" indicates additive impact and a negative score indicates antagonism. The resulting graphs were evaluated for synergy and antagonism distribution using contour and matrix views. In this view, dose combinations leading to synergy scores higher than 25-50 range are depicted with green or blue areas, and are regarded as significant synergies. Results obtained from ComBenefit software were further analyzed using the Combinatorial Index Method derived from the Median Impact Principle of Chou and Talalay.

In summary, ABE and DOX concentrations that will be used in the further analyses of the MDA-MB-231 cell line, either as a single agent or in combination with the other agent, were determined using the highest synergy scores obtained from ComBenefit and CompuSyn softwares [14, 15].

2.4. Colony Forming Assay

MDA-MB-231 cells grown into colonies during a period of 14 days, were fixed using MetOH (Sigma, USA) at 4^0C, stained using crystal violet (0.5%) and counted using ImageJ software, following procedures of Franken et al. 2006 (16). Statistical analyses of the impact of ABE and DOX application, each and in combination, were carried out using colony numbers and calculated Relative Colony Forming Efficiency (RCFE) on Graphpad Prism V10. Analyses were carried out in triplicates.

2.5. Fluorescent microscope analysis

MDA-MB-231 cells grown in 6 well plates to a concentration of 1x 10^5 cells per well (TPP, Sweden) were grown for 24 hours, which was followed by a 24-hour exposure of the cells to 0.1 µM DOX, 0.5 µM ABE and a combination of 0.025 µM DOX and 0.25 µM ABE. Dioc-6 (3,3′-dihexyloxacarbocyanine iodide) (Invitrogen, USA) and Propidium Iodine (PI, Applichem, USA) were applied to selected cells in dark and incubated at 5% CO^2 at 37^0C for appropriate durations for analyses. For example, PI incubation was 30 minutes and Dioc-6 incubation was 15 minutes per the manufacturer's instructions. Cells were then visualized under fluorescent microscopy (ZEISS AxioVert01) at magnifications of 4x, 10x, 20x and 40x.

2.6. Evaluation of the oxidative stress

The quinone subgroup of the doxorubicin molecule turns into a semi-quinone free radical and this may in turn lead to a cytotoxic effect in cancer cells. Under aerobic conditions, semi-quinone radicals yield to the formation of superoxide radicals and these radicals can turn into hydrogen peroxide and hydroxyl radicals and lead to DNA, RNA, lipid, and protein damage [17]. We, therefore, aimed to investigate the impact of ABE and DOX on MDA-MB-231 cell lines using oxidative stress experiments.

For this purpose, we measured oxidative stress indicators, such as; MDA and SOD, which are final products of peroxidation, and GSH activity, which acts as an antioxidant for the cell. MDA-MB-231 cells grown to confluency were treated with IC50 doses of ABE, DOX and combination of both agents, for 24 hours. Cells were centrifuged at 1,000 rpm for 5 minutes to obtain unattached cells in the upper part of the medium. The supernatant was removed, and attached cells were treated with trypsin-EDTA. Cells were collected by pipetting 5 mL PBS and transferred to 15 mL falcon tubes. Transferred cells were vortexed and centrifuged at $+4^0$C and 1,000 rpm for 5-minutes. The supernatant was removed and the precipitates were collected using 1 mL of PBS, which were stored at -80^0C for further storage.

Lysates of MDA-MB-231 cells that were treated with ABE, DOX, and ABE + DOX combination were analyzed using the Lowry method to show the levels of above-mentioned proteins, and hence the oxidative stress alterations. Spectrophotometric analyses were carried out in the 500 - 700 nm range, using BSA as a standard for measurements, with a BioTek PowerWave XS2 spectrophotometer. Sun's Method was used in calculating the SOD activity [18]. Lysates from untreated control MDA-MB-231 cells, ABE-treated cells, DOX-treated cells and finally cells treated with ABE + DOX combination were mixed with 2,900 µl of reactive mixture and 50 µl of xanthine oxidase. The blank tube contained 1,425 µl reactive mixture, 50 µl distilled water and 25 µl xanthine oxidase. All tubes were incubated for 20 minutes at 25 ^{0}C, which was followed by addition of 0.5 ml CuCl2 and absorbance measurement at

560 nm. Activity determination was carried out by using the protein amount determined by the Lowry Method as unit/mg.

The DTNB method was used to determine the reduced glutathione amount [19]. This method utilizes the spectroscopic measurement of the p-nitrophenol anion which is created in a 1:1 ratio for each thiol molecule created by the reaction of the 5,5 dithiobis-2-nitrobenzoic acid (DTNB, Ellman reactive) with the aliphatic thiol molecules in the samples, under mild-alkaline conditions. 0.2 ml of MDA-MB-231 lysate was mixed with 0.1 ml distilled water, 0.3 ml phosphate buffer and 2.4 ml Ellman reactive buffer, and its absorbance was measured at 410 nm. Corresponding GSH amounts were calculated using a standard GSH concentration 412 nm absorbance curve. In order to prevent any misinterpretation, arithmetic means of triplicate measurements were used to calculate GSH amount in terms of mg GSH / mg total protein.

TBARS method was used to determine Malondialdehyde (MDA) amount (20). Briefly, 1 ml TBA reactive (0.25 M HCl, 15% (w/v) trichloroacetic acid (TCA), 0.375% (w/v) thiobarbituric acid (TBA) was preheated in boiling water bath. The absorbance of the supernatant was measured at 535 nm against a blank solution mixture of 1 ml TBA and 0.5 ml deionized water. TBARS content of the samples was calculated using standard curve measurement of certain concentrations of malondialdehyde (MDA, 1,1,3,3-Tetramethoxypropane) following the manufacturer's instructions, similar to the prior measurement.

Determination of the GSH levels was done by measuring the GSH activity. For this purpose, 300 µl precipitation solution was added to 200 µl cell lysate and incubated at room temperature for 5 minutes. This was followed with a 10-minute centrifugation at 2,000 g, which enabled the collection of 50 µl of supernatant of the sample to each well of the plates. 200 µl phosphate solution and 25 µl of Ellman Reactive were also added before measurement of the absorbance at 412 nm. The glutathione content of the samples was determined using a standard curve prepared using known standards. Analysis of all measurements for antioxidant levels was performed using GraphPad Prism V10.

2.7. Western Blot analysis

MDA-MB-231 cells were treated with 0.1 µM DOX, 0.5 µM ABE and 0.025 µM DOX + 0.25 µM ABE combination therapy. Cells were then washed with cold 1x PBS solution and resuspended in their wells using trypsin. After the removal of supernatant upon centrifugation, the pellet was treated with RIPA (Biosolution, Korea) and protease cocktail (Thermo Fisher, USA). Upon lysis of the cells, the solution was centrifuged at 13,200 rpm for 15 minutes. Total protein concentration was determined using Bradford method for the control group, cells treated with 0.1 µM DOX, 0.5 µM ABE and 0.025 µM DOX + 0.25 µM ABE combination therapy. Protein amounts were determined using the Bovine Serum Albumin (Sigma, USA) standards that were also subject to Bradford Method (BioRad, USA) using absorbance values at 595 nm. A total of 30 µg protein for each sample was loaded onto 12% SDS-PAGE gel that is used in the SureCast™ Gel Handcast Bundle A - Hardware and Reagents System (ThermoFisher, USA), and ran for 90 minutes with 125 V (SureCast Handcast System in the Mini Gel Tank (Thermo Fisher, USA). Transfer to PVDF membrane (Roche, USA) was performed using a Power Blotter system (Thermo Fisher, USA) with a Transfer Semi Dry method at 1.3 A, 25 V for 1 hour.

Transferred PVDF membranes were washed and blocked with 5 % non-fat milk (95 % TBS with 5 % non-fat milk), and incubated for 1 hour at room temperature. This was followed with an immediate wash with TBS-T buffer, and treated with 1:1,000 diluted cleaved Caspase 3 (CST, USA), PARP (CST, USA), cleaved PARP (CST, USA), Bax (CST, USA), Cdk2 (CUSABio, USA) and β-actin (CST, USA) antibodies for 1 hour at room temperature. This treatment was followed by washing with TBS-T solution three times, and another incubation was followed by horseradish peroxidase (HRP) conjugated secondary antibodies (anti-rabbit IgG or anti-mouse IgG) for 1 hour (CST, USA). A repeat triplicate washing with TBS-T was followed by a wash step with TBS and treatment with Lumi-LightPLUS Western Blot Substrate (Roche, USA) and exposed to Hyperfilm-ECL (Hyperfilm ECL, Amersham Pharmacia Biotech, Freiburg, Germany). Expression levels of all proteins were evaluated in relative to β-actin using ImageJ software and obtained values were analyzed using GraphPad Prism V10.

2.8. Statistical analysis

The experimental set-up was prepared so that all biological analyses were performed in triplicates, which enabled us to maximize the accuracy and minimize the variation which was presented as the standard deviation in the results for each measurement. IC50 values for all agents alone and in combination and their corresponding synergistic cytotoxic impacts were tabulated using Biosoft CalcuSyn 2.1 and ComBenefit softwares. Correlation for impact of each agent alone and in combination was performed using ANOVA analysis and was subject to Dunnet's Multiple Comparison Test. For each calculation, P values smaller than 0.05 were regarded as statistically meaningful. GraphPad Prism V10 was used in all statistical analyses.

3. Results

3.1. Abemaciclib and Doxorubicin application impact cell survival in a dose-dependent manner

MTT Survival Test was performed to measure the cell survival rates of MDA-MB-231 cell lines following treatment of these cells with varying concentrations of Abemaciclib (0.01 µM – 5 µM) and Doxorubicin (0.01 µM – 5 µM). Our results showed that, compared to control cells, application of 0.5 µM Abemaciclib and 0.1 µM Doxorubicin showed a decline of 50%, and therefore these concentrations were selected to be used in the following experiments ($P < 0.05$) (Figure 1).

3.2. Combined application of Abemaciclib and Doxorubicin showed synergistic effect on the MDA-MB-231 cell line

After determining the IC50 doses for Abemaciclib and Doxorubicin agents, we utilized the CompuSyn and ComBenefit softwares to tabulate the impacts of these agents when applied as single agents and as a combinatory therapy. For this purpose, Abemaciclib and Doxorubicin was applied in concentrations of 0.1 µM Doxorubicin (DOX) alone, 0.5 µM Abemaciclib (ABE) alone and, 0.1 µM DOX + 1 µM ABE, 0.1 µM DOX + 0.5 µM ABE, 0.1

μM DOX + 0.25 μM ABE, 0.05 μM DOX + 1 μM ABE, 0.05 μM DOX + 0.5 μM ABE, 0.05 μM DOX + 0.25 μM ABE, 0.025 μM DOX + 1 μM ABE, 0.025 μM DOX + 0.5 μM ABE, 0.025 μM DOX + 0.25 μM ABE, 0.025 μM DOX + 0.25 μM ABE in combination (Figure 2).

CompuSyn software (ComboSyn Inc., Paramus, NJ, USA) was used to calculate Combinatorial Index (CI) and Dose Reduction Index (DRI) values, where CI values smaller than 1 show synergistic effect, values larger than show antagonistic effect and value of 1 shows a minor additional effect. Our results showed that 0.025 μM DOX and 0.25 μM ABE application yielded a strong synergistic effect (Figure 3).

Combinatory Index (CI), Isobologram and Nonconstant Combo data also showed similar significant synergistic effects for this combination therapy. It is important to note that the impact of 0.025μM DOX and 0.25 μM ABE combinatory treatment on cell survival was on par with separate applications of 0.5 μM ABE and 0.1 μM DOX, in these high concentrations ($p < 0.05$) (Figure 3).

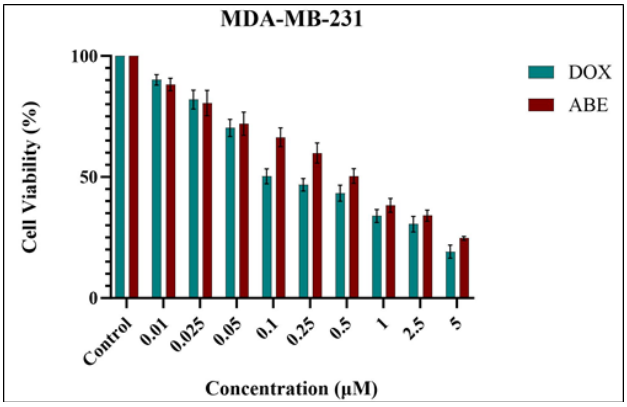

Fig. 1. The cytotoxic effect of DOX and ABE on MDA-MB-231 breast cancer cell line was determined by MTT assay. 24 h incubation of MDA-MB-231 cells with DOX and ABE resulted in a significant dose-dependent decrease in cell viability ($p < 0.05$). Data are shown as mean ± SD of three separate measurements (n = 3).

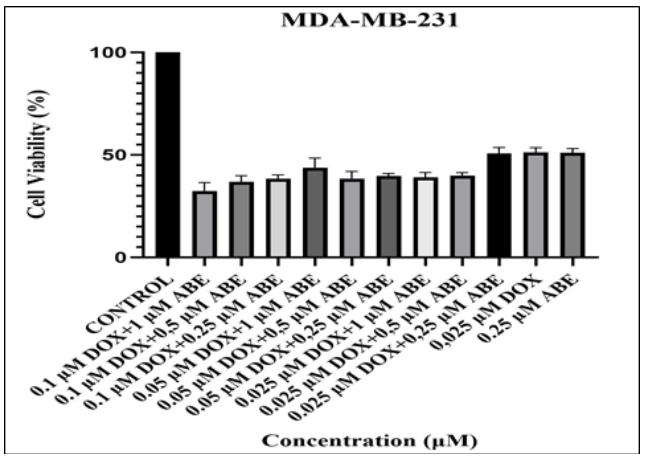

Fig. 2. Cytotoxic effect of DOX and ABE combination therapy on MDA-MB-231 breast cancer cell line determined by MTT assay. 24h incubation of MDA-MB-231 cells with DOX, ABE and DOX+ABE (0.1 μM Doxorubicin (DOX) alone, 0.5 μM Abemaciclib (ABE) alone and, 0.1 μM DOX + 1 μM ABE, 0.1 μM DOX + 0.5 μM ABE, 0.1 μM DOX + 0.25 μM ABE, 0.05 μM DOX + 1 μM ABE, 0.05 μM DOX + 0.5 μM ABE, 0.05 μM DOX + 0.25 μM ABE, 0.025 μM DOX + 1 μM ABE, 0.025 μM DOX + 0.5 μM ABE, 0.025 μM DOX + 0.25 μM ABE, 0.025 μM DOX + 0.25 μM ABE in combination) resulted in significant dose-dependent decrease in cell viability ($p < 0.05$). Data are shown as mean±SD of three separate measurements (n = 3).

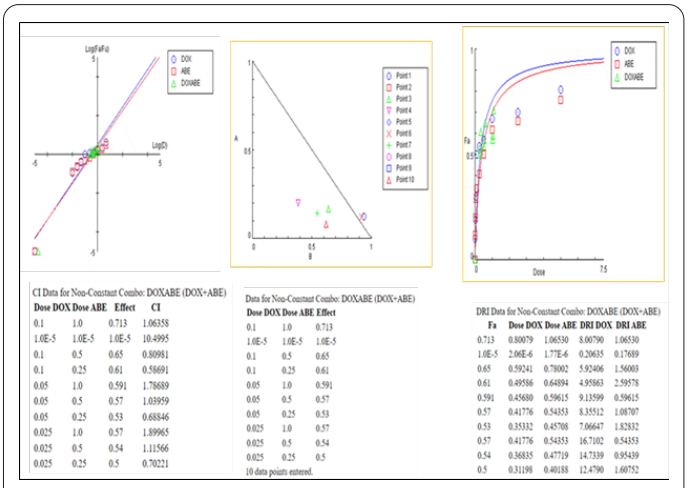

Fig. 3. CompuSyn analysis for DOX+ABE combination treatment. 24 h incubation of MDA-MB-231 breast cancer cells with DOX, ABE and DOX+ABE (0.1 μM Doxorubicin (DOX) alone, 0.5 μM Abemaciclib (ABE) alone and, 0.1 μM DOX + 1 μM ABE, 0.1 μM DOX + 0.5 μM ABE, 0.1 μM DOX + 0.25 μM ABE, 0.05 μM DOX + 1 μM ABE, 0.05 μM DOX + 0.5 μM ABE, 0.05 μM DOX + 0.25 μM ABE, 0.025 μM DOX + 1 μM ABE, 0.025 μM DOX + 0.5 μM ABE, 0.025 μM DOX + 0.25 μM ABE, 0.025 μM DOX + 0.25 μM ABE in combination) resulted in significant dose dependent decrease in cell viability ($p < 0.05$), and furthermore, doses of DOX and ABE in the combination doses change the resulting pattern. Data are shown as mean±SD of three separate measurements (n = 3).

IC50 values for ABE and DOX applications, either separately and in combination, were also calculated using ComBenefit software Bliss Synergy and Antagonism phenomena (Figure 4). Calculated IC50 results were 0.498 μM ABE, 0.101 μM DOX and 0.025 μM DOX + 0.25 μM ABE for Abemaciclib and Doxorubicin application separately and in combination. Based on these results obtained from ComBenefit and CompuSyn calculations, we determined that 0.025 μM DOX + 0.25 μM ABE combinatory doses were to be used for the following experiment to investigate the combinatory effects of these agents.

3.3. Combinatory application of DOX and ABE application decreases colony colony-forming efficiency of MDA-MB-231 cells

Colony forming assay was performed to assess the impact of DOX and ABE application, either alone or in combination on the growth characteristics of the MDA-MB-231 cell line by calculating the reduction in the Relative Colony Forming Efficiency (RCFE). 80% reduction in RCFE was observed when the MDA-MB-231 cells were treated with 0.1 μM DOX, and this reduction increased to 85% when the cells were treated with 0.5 μM ABE alone, and to 88% when the cells were treated with the combinatory treatment of DOX and ABE ($p < 0.05$) (Figure 5).

3.4. Combination Therapy of MDA-MB-231 Cells with DOX and ABE Alters the Antioxidative Properties

We assessed the relative induction of Superoxide Dismutase (SOD) levels in MDA-MB-231 cells upon treatment with DOX and ABE alone and in combination (Figure 6). 0.1 μM DOX treatment of the cells showed an induction of SOD (U/mg) 2 times, 0.25 μM ABE treatment of the cells showed an induction of SOD 1.14 times and combinatory treatment of the cells with DOX and ABE

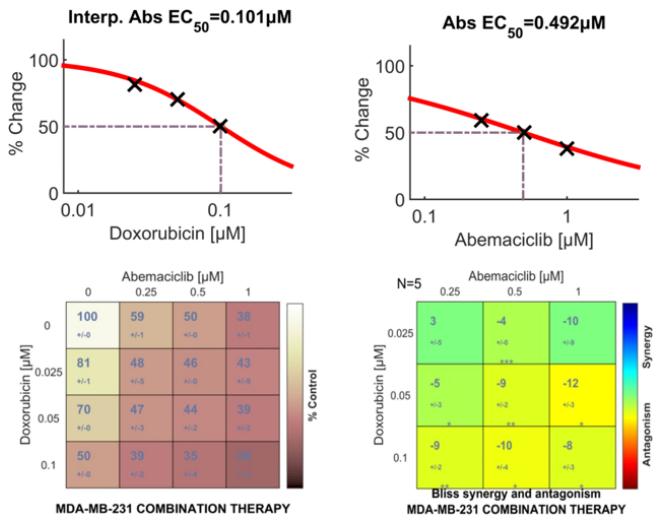

Fig. 4. ComBenefit analysis for DOX and ABE combination treatment on MDA-MB-231 cells compared to control and only DOX and ABE treated cells. Blue and green areas indicate synergy and red to yellow indicates antagonism for the experiment. Data are shown as mean±SD of three separate measurements (n = 3).

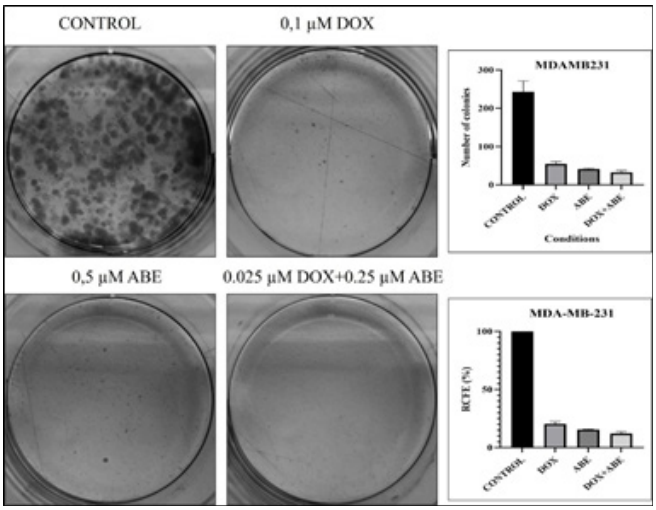

Fig. 5. Effects of combinatory application of DOX and ABE application on colony forming efficiency of MDA-MB-231 cells. Data are shown as mean±SD of three separate measurements (n = 3).

showed a 2.8 induction of SOD (U/mg).

MDA is a characteristic peroxidation product of cellular lipids upon oxidative stress of the cells due to uncontrolled abundance of ROS molecules. Separate application of DOX and ABE showed a reduction in MDA levels, to 25% and 21%, respectively, whereas combinatory application of DOX and ABE showed an induction of the MDA levels to 113%.

GSH homeostasis, owing to its important roles in both prevention of carcinogenesis at stages before and induction of carcinogenesis at stages after its initiation, as well as development of resistance to therapeutic agents. Alterations in total GSH protein (μg/mg) were measured. A 25% increase in total GSH protein levels was observed when MDA-MB-231 cells were treated with 0.1 μM DOX, while GSH levels were lowered to %92 when the ABE was applied compared to the control group. Combination Therapy yielded a 46% increase, relative to the control group.

3.5. Pro-apoptotic Effects of Abemaciclib and Doxorubicin Treatment on MDA-MB-231 Cell Line

We utilized Fluorescence Microscopy to analyze the impact of DOX and ABE treatment, applied alone or in combination, on cellular morphology and survival characteristics of MDA-MB-231 cells by using PI and Dioc6 staining (Figure 7). It is well known that normal cell membranes are not permeable to PI stain, whereas PI stain can easily penetrate pre-apoptotic cells, and for this reason, we assessed the percentage of cells that were positive for PI staining upon treatment with DOX and ABE treatment, at concentrations of 0.1 μM and 0.5 μM, respectively, when applied alone, or 0.025 μM DOX and 0.25 μM ABE when applied as a combination therapy.

Dioc6 staining was performed to assess the mitochondrial integrity of the cells upon treatment, as Dioc6 is not able to bind membranes of the mitochondria if the integrity of the mitochondrial has been compromised. We observed clear disruption of the mitochondrial integrity of the MDA-MB-231 cells upon treatment with DOX and ABE, both in singular and combinational configurations.

3.6. Impact of Abemaciclib and Doxorubicin on Expression of Proteins in the Survival and Apoptotic Pathways

Western Blot analysis was performed to assess the alterations in the expression profiles of Bcl-2 family of proteins as well as intrinsic apoptotic proteins (Figure 8). MDA-MB-231 cells that were subject to 24-hour treatment with 0.5 μM ABE, 0.1 μM DOX and combination of ABE and DOX with concentrations of 0.025 μM and 0.25 μM,

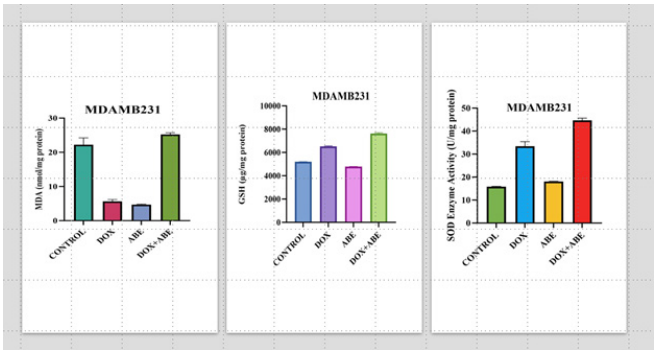

Fig. 6. The effect of DOX, ABE and DOX-ABE treatment on (a) MDA, (b) GSH, (c) SOD activity in the MDA-MB-231 cells relative to the control group. All treatment groups indicate statistically significant differences compared to the control group ($p<0.05$).

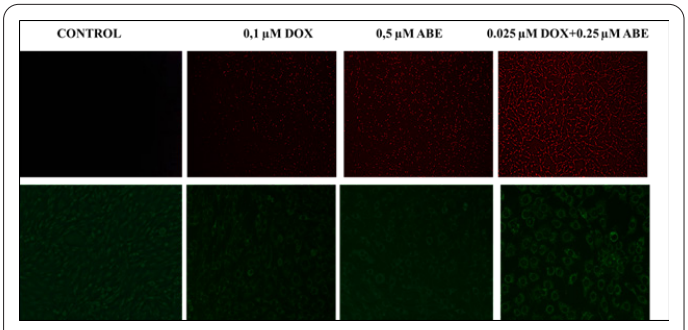

Fig. 7. Determination of Cell Death by PI (red) MOMP (Mitochondrial Outer Membrane Permeability) by Dioc-6 (green) was performed following DOX, ABE and DOX-ABE Combination Treatment on MDA-MB-231 cell line.

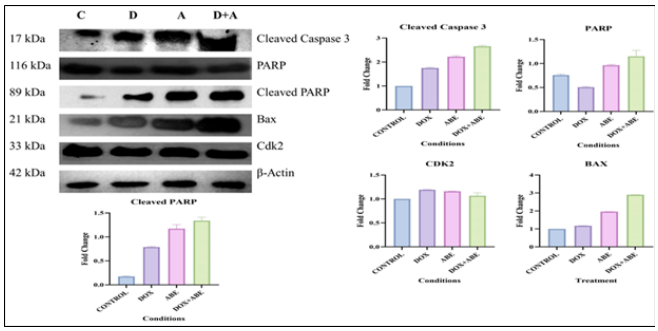

Fig. 8. The effect of DOX, ABE and DOX+ABE treatment on Apoptosis through Modulating Caspase-3, PARP and Bax was analyzed by Immunoblotting. Image J and GraphPad V10 data analysis for immunoblotting assay were provided as column graphs for each protein in MDA-MB-231 cells. Results obtained from all of the groups were statistically different from the control group ($p<0.05$).

respectively, were investigated for changes in the levels of Cleaved Caspase 3, PARP and Cleaved PARP proteins.

Cleaved Caspase 3 expression showed a significant increase for all DOX, ABE and combination therapy, to levels of 1.75 fold, 2.2 fold and 2.6 fold, respectively. Similarly, PARP expression increased 4.5 fold, 7.0 fold and 8.0 fold for DOX, ABE and combination therapy, respectively($P < 0.05$). Bax protein, among proapoptotic members of Bcl-2 family of proteins, has also shown significant increase in their expression upon DOX, ABE and combinational treatment of MDA-MB-231 cells.

Cleaved PARP, which is another important apoptotic biomarker, has also shown a significant increase in its expression. DOX application led to 4.5 fold increase, ABE application led to 7-fold increase, while application of the combination therapy led to 8-fold increase in its expression in the MDA-MB-231 cells.

Bax expression showed 17%, 95% and 190% increase upon DOX, ABE and combinational application on the cells. Bad expression showed 20%, 25% and 40% increase upon DOX, ABE and combinational application on the cells.

Compared to the control group, the change in CDK2 levels was as follows: While only a 19% increase in expression was observed with DOX application and a 15% increase with ABE application; With the DOX+ABE combination, this change was only around 6%.

4. Discussion

MDA-MB-231 cell line, which is among ideal representations for Triple Negative Breast Cancer (TNBC) is Estrogen Receptor (ER), Progesterone Receptor (PR), Human Epithelial Growth Factor Receptor 2 (HER-2) and E-cadherin negative, Rb positive, and expressed a mutant p53 protein [13,21]. It is important to note that 10-20% of the women with Breast Cancer are diagnosed with TNBC subtype and undergo a chemotherapeutic treatment involving an anthracycline, such as Doxorubicin, or cyclophosphamide and taxane group of agents [22]. Doxorubicin is a prominent chemotherapeutic agent for the TNBC and is widely used for a range of cancers, including but not limited to leukemia, and lymphoma. Despite its significant potential in the treatment of several cancers, its effectiveness is hampered by its profound and unwanted cytotoxic side effects, as well as an acquired resistance of the cancer cells to the agent. Even though the underlying mechanisms of this resistance have not been documented in their entirety, a logical strategy to overcome this problem, several groups have postulated to formulate combination therapy strategies mainly aiming to minimize the side effects and target the mechanisms leading to the resistance. For this reason, we elected to use ABE agents as a combination partner for DOX.

Abemaciclib operates through inhibiting one of the major regulatory proteins of the cell cycle, the cyclin-dependent kinase 4/6 (CDK4/6), and in turn inhibiting cell growth. It is also important to note that the primary function of the CDK4 and CDK6 proteins is to phosphorylate the retinoblastoma protein (Rb) which leads to the transformation of the cell from the G1 phase of the cell cycle to the "S" phase, where synthesis of cellular ingredients, such as DNA synthesis, is accelerated and the cellular physiology is prepared for an eminent cell division [21].

Our study showed that increasing DOX and ABE concentrations decrease the cell viability in MDA-MB-231 cells. In our study, we found out that the IC50 doses for DOX and ABE application were 0.1 μM for DOX, and 0.5 μM for ABE. In a similar study, researchers found their IC50 doses as 0.565 μM and 1.61 μM for DOX and ABE, respectively [21]. For the combination therapy doses, we found 0.025 μM for DOX + 0.25 μM for ABE using CompuSyn and ComBenefit softwares. When we used these values for the combination therapy in CompuSyn software, we found out that this combination was the optimal dose for enhancing the Synergistic Effect for DOX. When we further analyzed the combinational effect of these doses in terms of interrelation between DOX and ABE, we calculated Combinatorial Index (CI) lower than 1, which indicated a desired synergistic effect between the two agents. This is in agreement with the calculated values in the 2019 study, where authors performed the same calculation for doses between 1 to 6 μM for ABE, keeping the DOX concentration at 0.565 μM and found CI values all smaller than 1, indicating a desired synergistic relationship [21]. Therefore it is safe to conclude that our three separate analyses, namely MTT, CompuSyn and CompBenefit, yielded the same results within acceptable variation, as well as in accordance with synergistic values reported in the literature. Even though we were not able to find a comparable dose combination in the literature to compare our current results, we calculated the Synergistic impact using the ComBenefit analysis with a safe margin of variation.

It is also important to note that, despite a considerably lower dose for DOX in combination therapy, its impact on the cell viability did not differ for the MDA-MB-231 cells, which can be attributed to the combination therapy. This is especially significant considering the DOX-related cardiotoxicity, which drastically reduces the efficiency and effectiveness of the therapy [23]. Therefore, it is noteworthy that achieving a lower dose of DOX through addition of ABE to the therapy planning can be of significant importance for patients who can not stay on the high-dose treatment plan. On a final note, a lower dose can also delay the development of resistance to the chemotherapy regimen.

Fluorescent microscopic analysis of the experimental groups showed that the combination therapy has a more significant impact on the disruption of the mitochondrial membrane potential, compared to impact of DOX and ABE alone, as well as leads to a higher percentage of cel-

lular demise as observed with the PI staining.

Further analysis of this phenomenon by immunoblotting analyses using the MDA-MB-231 cells of these experimental groups showed that this increase in efficiency of the combination therapy in the elimination of the cancer cells was through alterations in the levels of cleaved Caspase 3, PARP and cleaved PARP.

Our analyses showed significant increase in the expression of Cleaved Caspase 3, Cleaved PARP, and Bax upon treatment of MDA-MB-231 cells with the combination therapy. This may be the underlying reason for the alterations of cellular physiology since Bax protein is among the proapoptotic members of the Bcl-2 family of proteins. Another significant biomarker of apoptosis, cleaved PARP, also showed an important increase upon treatment of the MDA-MB-231 cells with the combination therapy, compared to the application of the agents alone. These increases all point to the importance of the combination therapy in the induction of proapoptotic members, apoptotic mechanisms and cellular death for the cancer cells.

When we analyzed the antioxidant levels in the cells, SOD showed an induction, MDA showed a reduction, and GSH levels increased, which are all showing that the agents are both increasing the treatment efficiency when they are applied alone, but have a further enhanced effect when applied in combination.

In summary, one of the two main points to consider based on the results that we obtained is that we were able to enhance the impact of the DOX agent through the use of the combination therapy, which was also accompanied by the second important point, which was that we were able to achieve these enhanced results with a significantly lower dose of DOX. This is of crucial importance considering the significant unwanted side-effects of the treatment. It is important to note that conventional DOX therapy also targets healthy cells and this monotherapy can be toxic to healthy cells and this causes multiple side effects such as vomiting, nausea, alopecia, fatigue, and cardiotoxicity which may worsen through the course of the therapy, where these effects causes the patients to generally fall off of the treatment plan, limiting its efficiency. Therefore, we believe that it is of crucial importance to achieve the enhanced impacts of DOX with a significantly lower concentration with the inclusion of ABE in the therapy plan.

5. Conclusion

Combination therapy utilizing doxorubicin, which is already widely used in the treatment of Breast Cancer, may enable minimization of its unwanted side effects and stands as a promising potential to increase its efficiency and efficacy. Notwithstanding the molecular potential of the DOX agent, its side effects hamper its use in the clinical setting, and therefore our results shed important light on development of novel strategies utilizing this agent.

For this purpose, we employed molecular biological analyses aiming to decipher the anti-cancer properties of DOX in combination therapy with ABE at the level of protein-protein interactions, cellular metabolism and physiology underlying TNBC, which is among important targets for cancer research. Understanding these metabolomic alterations and interactions will ensure their proper use and even formulation of further improved therapeutic strategies. Furthermore, in turn, this will enable us to determine and evaluate newer target molecules and pathways to enhance cancer prognosis, diagnosis and therapy.

Based on our literature review, our study will be the first to examine the molecular impacts of this combination therapy formulation and its effect on the complex molecular biology of TNBC, enabling us to further our analysis at protein level as well as at clinical level. Our study provides preliminary results for the molecular impacts of DOX and ABE combination therapy on MDA-MB-231 cell lines and documents the cytotoxic and apoptotic pathways of these impacts.

We believe achieving an enhanced effect at a significantly lower dose is a major benefit of the combination therapy. This would significantly improve the patient's quality of life and the possibility of their adhering to the treatment plan. With this regard, we believe that this lower dose would be of great clinical importance. It is plausible to suggest that, based on these results, investigation of the enhanced impact of combination therapy might be a good candidate for clinical application.

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Conflict of interests

The authors declare no competing interests.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

No human or animals were used in the present research.

Informed consent

The authors declare that no patients were used in this study.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request

Authors' contribution

Conceptualization, methodology, validation, investigation was performed by Tugce Nur Eralp; data analysis was performed by Tugce Nur Eralp and Akin Sevinc; writing-original draft preparation, writing-review & editing was performed by Tugce Nur Eralp, Akin Sevinc and Banu Mansuroglu; visualization was performed by Tugce Nur Eralp; supervision was undertaken by Akin Sevinc and Banu Mansuroglu; project administration was carried out by Tugce Nur Eralp and Banu Mansuroglu. All authors have read and agreed to the published version of the manuscript.

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