Empagliflozin and Doxorubicin Synergistically Inhibit the Survival of Triple-Negative Breast Cancer Cells via Interfering with the mTOR Pathway and Inhibition of Calmodulin: *In Vitro* and Molecular Docking Studies

Shenouda G. Eliaa, Ahmed A. Al-Karmalawy,* Rasha M. Saleh, and Mohamed F. Elshal

Cite This: *ACS Pharmacol. Transl. Sci.* 2020, 3, 1330–1338

Read Online

ACCESS |

Metrics & More

Article Recommendations

Supporting Information

ABSTRACT: Triple-negative breast cancers (TNBCs) comprise 10–15% of all breast cancers but with more resistance affinity against chemotherapeutics. Although doxorubicin (DOX) is the recommended first choice, it has observed cardiotoxicity together with apparent drug resistance. The anti-hyperglycemic drug, empagliflozin (EMP), was recently indicated to have *in vitro* anticancer potential together with its previously reported cardioprotective properties related to calmodulin inhibition. In this study, we carried out molecular docking studies which revealed the potential blocking of the calmodulin receptor by EMP through its binding with similar crucial amino acids compared to its cocrystallized inhibitor (AAA) as a proposed mechanism of action. Moreover, combination of DOX with EMP showed a slightly lower cytotoxic activity against the MDA-MB-231 cell line ($IC_{50} = 1.700 \pm 0.121$) compared to DOX alone ($IC_{50} = 1.230 \pm 0.131$), but it achieved a more characteristic arrest in the growth of cells by 4.67-fold more than DOX alone (with only 3.27-fold) in comparison to the control as determined by cell cycle analysis, and at the same time reached an increase in the total apoptosis percentage from 27.05- to 29.22-fold, compared to DOX alone as indicated by Annexin V-FITC apoptosis assay. Briefly, the aforementioned *in vitro* studies in addition to PCR of pro- and antiapoptotic genes (mTOR, p21, JNK, Bcl2, and MDR1) suggest the chemosensitization effect of EMP combination with DOX which can reduce the required therapeutic dose of DOX in TNBC and eventually will decrease its toxic side effects (especially cardiotoxicity), along with decreasing the chemoresistance of TNBC cells to DOX treatment.

KEYWORDS: TNBC, EMP, calmodulin antagonist, docking, apoptosis

1. INTRODUCTION

Cancer is a diverse category of diseases characterized by uncontrolled cell proliferation that represents the greatest cause of mortality and morbidity worldwide.¹ It is a long-term process that starts with a single mutation and builds up through the years to make the first out-of-control cell and thereafter the tumor.² Cancer has continued through the past centuries to become the second leading cause of death worldwide.³

Triple-negative breast cancers (TNBCs) are a subtype of breast cancers with an aggressive phenotype (high metastatic capability and poor prognosis) which comprise 10–15% of all breast cancers.⁴ TNBCs do not express estrogen or progesterone receptors, nor do they contain an amplified HER2/Neu gene. The downregulation in the previously mentioned genes slows down the treating process due to the shortage of curing targets which leaves TNBC as the poorest breast cancer type in prognosis.⁵

Doxorubicin (DOX) is an anthracycline antibiotic that has a vital role in chemotherapy since was approved by the FDA in

1974 for cancer treatment.⁶ It has been used in the treatment of different types of cancer including breast cancers.⁷ However, DOX has poor selectivity against normal and cancerous cells. This problem causes damage and toxicity to many healthy cells, especially cardiac cells that become gravely affected leading to cardiotoxicity (Figure 1).^{8,9} The second evident problem of using DOX as a chemotherapeutic agent is the acquired tumor resistance against it.¹⁰ Multidrug resistance (MDR) is considered a major obstacle that hinders the use of several potent chemotherapeutics including DOX.¹¹ DOX drug-resistance is developed as a result of increased expression of the ATP-dependent efflux pump *ABCB1* (MDR1),¹² which encodes the membrane drug transporter P-glycoprotein and

Received: September 18, 2020

Published: November 11, 2020

Figure 1. Graphical representation showing that adding EMP enhances the anticancer effects of DOX at a reduced concentration, suggesting that EMP could minimize adverse effects of DOX while preserving its therapeutic value.

often contributes to poor prognosis and development of metastatic tumors resistant to chemotherapy such as TNBCs.¹³ One of the first approaches to overcome drug resistance is by using chemosensitizers or efflux pump modulators that could enhance the therapeutic effect of the chemotherapy drugs at a lower concentration to lower their adverse effects;^{14,15} therefore, it is important to select the chemosensitizer agents which are less toxic and more beneficial to the cancer patients.

Drug repurposing (or drug repositioning) is an accelerated tool for drug development that involves seeking new indications for drugs that are already FDA-approved rather than discovering new compounds and currently constitutes 30% of the newly marketed drugs in the United States.^{16,17} Many successfully repurposed drugs have been introduced in the market as in the case of aspirin for the treatment of stroke and/or myocardial infarction and topiramate for the treatment of obesity.¹⁸

Empagliflozin (EMP) is an anti-hyperglycemic drug that belongs to the class of sodium-glucose cotransporter 2 (SGLT2) inhibitors which are a recently approved class of drugs with an insulin-independent mode of action.¹⁹ The European Medicines Agency Committee for Medicinal Products for Human Use recommended the granting of a marketing authorization for EMP.²⁰ Recently, it was indicated that EMP has an *in vitro* anticancer potentials against both breast cancer cell lines MCF-7 and lung cancer cell lines A549.²¹ Moreover, EMP has shown cardioprotective properties due to its role as an inhibitor of calmodulin.²² Mustroph et al. proposed that EMP reduces Ca^{2+} /calmodulin-dependent kinase (CaMKII) activity in isolated murine ventricular myocytes.²³ Also, the diastolic function of heart failure was improved in a nondiabetic rodent model by using EMP.²⁴ Calmodulin is a calcium-binding protein which is regulating many of the intracellular actions of calcium. It is proposed that calmodulin is responsible for the regulation of cellular proliferation and that its function may be altered in malignancy. Besides, calmodulin antagonists are cytotoxic and can restore the sensitivity of resistant cells to drugs such as DOX and vincristine.²⁵ Consequently, calmodulin has been suggested as an emerging target for anticancer therapeutic intervention.²⁶

Therefore, we sought to explore the potential of EMP as an inhibitor of calmodulin which could be a probable mechanism to sensitize TNBCs to DOX and to investigate the underlying mechanism using *in silico* and *in vitro* techniques.

2. METHODOLOGY

2.1. Molecular Docking Studies. A molecular docking study of EMP at the calmodulin receptor using MOE 2019.0102 drug design software²⁷ was performed to evaluate the activity of our tested compound compared to the cocrystallized inhibitor (AAA) as a potent calmodulin antagonist (Protein Data Bank PDB, <http://www.rcsb.org/>, code 1QIW).²⁸

2.1.1. EMP and AAA Preparation. The chemical structure of EMP was downloaded from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). It was converted to its 3D form and checked for both its chemical structure and formal charges on atoms. Then, energy minimization and automatic calculation of the partial charges were also done. Finally, EMP was imported with the isolated calmodulin antagonist (AAA) in the same database and saved as an MDB file before docking calculations with the target calmodulin receptor.

2.1.2. Target Calmodulin Receptor Optimization. The X-ray structure of the target calmodulin receptor complex was downloaded from the Protein Data Bank (<http://www.rcsb.org/>, PDB code 1QIW, resolution of 2.64 Å).²⁸ It was prepared for docking studies as follows: adding hydrogen atoms with their standard 3D geometry, checking for any errors in the atom's connection and the type through automatic correction, and fixing the potential of the receptor and its atoms. The applied force field was CHARMM, and site spheres were used for the selection of the same active sites of the cocrystallized ligand to show the different interactions with it in the complex structure. The target calmodulin receptor was found to be composed of two subunits (namely, A and B), and two molecules of the inhibitor (AAA) bound to the A subunit, while only one inhibitor molecule bound to the B subunit. Therefore, we performed two different docking processes, one for each of subunits A and B, respectively.

2.1.3. Docking of the Tested Molecule to the Target Calmodulin Receptor Active Sites. The prepared database containing both EMP and AAA was docked using the MOE 2019 suite. The applied methodology was as follows: Docking was initiated as a general process for each subunit after loading the file for each prepared subunit (A and B, respectively) of the target protein active site. The docking site was specified using dummy atoms, and triangle matcher was chosen as the placement methodology. London dG was selected as the scoring methodology. The refinement methodology selected as a rigid receptor, and the scoring methodology was GBVI/WSA dG for the best poses selection. The two-ligand MDB file was generally docked automatically. After the end of the two docking processes, the obtained poses were carefully studied, and the best ones for each having the best interactions and scores with the protein pockets were selected.

2.2. In Vitro Studies. EMP was obtained from Cayman Chemical (Ann Arbor, MI). DOX (MW = 543.5, purity > 98.0%, HPLC) was purchased from Sigma (cat. no. D1515, St. Louis, MO). RPMI 1640 medium and fetal bovine serum (FBS) were purchased from GIBCO (Invitrogen, CA). MDA-MB-231 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 50 U/ml penicillin/

streptomycin, and 2 mM L-glutamine in a humidified incubator at 37 °C and 5% carbon dioxide.

2.2.1. MTT Cytotoxicity Assay. The cytotoxic activity of EMP was measured *in vitro* against TNBC cell lines compared to DOX as a reference drug. MDA-MB-231 cells were treated with DOX, EMP, or DOX combined with EMP, respectively, for 24 h at different concentrations. Multiwell plates were used in the MTT method, and the final number of cells should not exceed 10^6 cells/cm² in the log phase of growth for the best results. Also, untreated cells were included for each experiment as control cells. Cells were treated with EMP or DOX at different concentrations (from 100 to 0.39 μ M) for 24 h, and the killing effect of different concentrations was recorded. The half-maximal inhibitory concentration (IC₅₀) values were analyzed and used to determine the concentrations to be used in EMP/DOX combinations, comprising the ratio of IC₅₀ DOX/IC₅₀ EMP. The effects of the combination of EMP on the antitumor activity of DOX on MDA-MB-231 cells were also recorded.

2.2.2. Combination Index Analysis. Drug synergism studies were carried out using CompuSyn software version 1.0 (Ting Chao Chou and Nick Martin, Paramus, NJ). The combination index (CI) was measured based on the mass action law of degree of drug interaction according to Chou and Talalay. CI calculation is based on the formula $CI = (D_1)/(D_x)_1 + (D_2)/(D_x)_2$, where (D_x)₁ and (D_x)₂ represent the doses of EMP and DOX in a combination which was required to achieve the same efficacy as that of EMP (D₁) and DOX (D₂) when used alone.²⁹ CI < 1 indicates synergism, where CI = 1 indicates an additive effect and CI > 1 indicates antagonism. Also, the drug reduction index (DRI) values above 1 imply a favorable dose reduction in the drug combination compared to the monotherapy.

2.2.3. Cell Cycle Analysis. Cell cycle phases and apoptosis detection in samples of untreated or treated MDA-MB-231 cell cultures were analyzed using flow cytometry as previously described.³⁰ Briefly, MDA-MB-231 breast cancer cells were seeded at 8×10^4 cells/well and incubated overnight at 37 °C and supplied with 5% CO₂. MDA-MB-231 cells were treated by the IC₅₀ of the three treatments (DOX, EMP, and EMP/DOX combination), and their impact on the cell population was recorded and compared to the control (media). After 48 h of treatment, cell pellets were collected and centrifuged at 300g for 5 min. For cell cycle analysis, cell pellets were fixed in 70% ethanol on ice for 15 min. The collected pellets were incubated with propidium iodide (PI) staining solution (50 μ g/mL PI, 0.1 mg/mL RNase A, and 0.05% Triton X-100) at room temperature for 1 h. Stained cells were kept in the dark at 4 °C until analysis using flow cytometry.

2.2.4. Annexin V-FITC (Anx V) Apoptosis Assay. Apoptosis detection was performed by FITC Annexin-V/PI kit (Becton Dickinson, Franklin Lakes, NJ) following the manufacture's protocol. The samples were analyzed by fluorescence-activated cell sorting (FACS) as we previously described.³¹

2.2.5. Reverse Transcription and Quantitative Real-Time PCR. Quantitative PCR to investigate the effects of combining EMP on DOX mRNA expression of five target genes (mTOR, p21, JNK, Bcl2, or MDR1) and the housekeeping gene (GAPDH) in TNBC cells was performed. The primer sequences for the genes analyzed in the current study is tabulated in (Table 1). Briefly, RNA from MDA-MB-231 breast cancer cells was extracted after different treatments for 48 h. The extraction of the total mRNA from MDA-MB-231

Table 1. Primers Sequences of the Target Genes (mTOR, p21, JNK, Bcl2, and MDR1) and the Housekeeping Gene (GAPDH)

gene	primer sequence
mTOR	F 5'-GCTTGATTGTTGCCAGGACAGT-3'
	R 5'-GTGCTGAGTTTGCTGTACCCATGT-3'
p21	F 5'-TGGAGACTCTCAGGGTCGAAA-3'
	R 5'-GGCGTTTGGAGTGGTAGAAATC-3'
JNK	F 5'-ATGAGCAGAAGCAAGCGTGAC-3'
	R 5'-AAGAAGTAGCTCTCTGTAGGC-3'
Bcl2	F 5'-GACTTCGCCGAGATGTCCAG-3'
	R 5'-CAGGTGCGGTTCCAGTACT-3'
MDR1	F 5'-CCCATCATTGCAATAGCAGGx-3'
	R 5'-TGTTCAAACCTCTGCTCCTGA-3'
GAPDH	F 5'-GGCAAATTCACGGCACAGT-3'
	R 5'-AGATGGTGATGGGCTTCCC-3'

cells was done using Trizol, and purification was performed using the Qiagen RNA kit. About 1 μ g of cDNA was synthesized, and real-time PCR was performed using the Rotor-Gene Q-Pure Detection system. Fold change was calculated using the Rotor-Gene Q software package following the manufacturer's instructions (Qiagen). The method used was as follows: 95 °C for the 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Data analysis was carried out using the 2^{−ΔΔC_t} method³² which was expressed as the ratio between the expression of each gene. Triplicate measurements were done, and the average of all was analyzed in our results.

2.3. Statistical Analysis. The experimental results are expressed as mean $\pm$ standard error of the mean (SEM). Data analysis was performed using the one-way ANOVA, and a *p* value < 0.05 was considered significantly different.

3. RESULTS AND DISCUSSION

3.1. Docking Results. First, a validation process was performed for the target receptor by running a redocking process for only the cocrystallized inhibitor, and a low RMSD value indicated the valid performance (RMSD = 1.46, Figure 2).^{33,34}

The calmodulin receptor pocket was found to be composed of two subunits (namely, A and B) occupied by the cocrystallized inhibitor (AAA). Two molecules of AAA antagonists were required for the interaction with A subunit and the key amino acids were found to be Glu84, Glu11, Glu127, and Met144. However, only one molecule was required to be bound with Glu11 in B subunit.²⁸

Regarding A subunit, the docked AAA binding mode showed a score value of −8.18 kcal/mol and 1.74 RMSD. It formed five H-bonds with Glu11, Glu127, and Met144 amino acids, two H-bonds with Glu11 at 2.92 and 3.06 Å, and one H-bond with Glu127 at 3.23 Å, and the remaining two H-bonds were with Met144 at 3.71 and 3.85 Å, respectively. Concerning the B subunit, the calmodulin antagonist (AAA) formed three H-bonds with a binding score of −8.52 kcal/mol and 2.05 RMSD: one H-bond with Glu11 at 3.37 Å, a second H-bond with Met144 at 3.50 Å, and a third one with Met145 at 3.80 Å. The 3D positioning for the cocrystallized calmodulin antagonist (AAA) was represented for different selected poses in subunits A and B, respectively, showing the best and proper fitting inside the previously described protein pockets (Figure 3).

Figure 2. 2D and 3D representations for the redocked cocrystallized inhibitor (AAA) inside the calmodulin receptor pocket. The cocrystallized inhibitor is represented in green and the redocked one is represented in red.

Figure 3. 3D representations and positionings for the docked cocrystallized calmodulin antagonist (AAA) inside A and B subunits of the calmodulin receptor.

Figure 4. 3D representations and positionings for empagliflozin inside A and B subunits of the calmodulin receptor.

EMP was found to form four H-bonds through its terminal hydroxylated pyran moiety inside the A subunit, three with Glu11 at 3.07, 3.13, and 3.14 Å and a fourth one with Glu114 at 2.71 Å, respectively, with a binding score of -6.61 kcal/mol and RMSD value of 1.50, in addition to an H- π interaction through its terminal tetrahydrofuran ring with Phe92 at 4.20 Å. Furthermore, EMP was stabilized in subunit B with a binding score of -6.89 kcal/mol and an RMSD value of 1.72. It formed three H-bonds through its terminal hydroxylated pyran ring as well: two with Glu11 and a third one with Glu14 at 2.70, 2.73, and 3.08 Å, respectively (Figure 4).

3.2. In Vitro Results. **3.2.1. Evaluation of Drugs Cytotoxicity and Drugs Synergism.** By analyzing the MTT cytotoxicity assay records, the cytotoxic order of our tested compounds on the MDA-MB-231 cell line was as follows: DOX > DOX combined with EMP > EMP (Figure 5A). The

IC₅₀ values of DOX combined with EMP and of DOX alone on the MDA-MB-231 cell line were very close (1.70 and 1.23, respectively; Figure 5B), so EMP is not greatly affecting the cytotoxic potency of DOX. Furthermore, it will decrease its dose in the combination and eventually will decrease its side effects (especially cardiotoxicity). Combined treatment of EMP and DOX yielded significantly greater growth inhibition in a dose-dependent manner. The combination index (CI) was computed for the combination of EMP/DOX according to the method developed by Chou³⁵ to confirm and quantify the synergism observed with DOX and EMP. The two drugs were combined in a constant ratio (1:1) to calculate CI and DRI (dose-reduction index) values using CompuSyn software. Figure 5C shows a CI range of 0.26–0.15 with corresponding to fraction affected (Fa) values from 0.5 to 0.95 which indicate a synergism between the two drugs in inhibiting the proliferation of MDA-MB-231 cells. We also calculated the

Figure 5. Graphical representations showing (A) the cytotoxic effects of DOX, EMP, and DOX/EMP combination, (B) IC₅₀ of each treatment, (C) combination index (CI) of EMP and DOX, and (D) dose-reduction index (DRI) of DOX as analyzed with CompuSyn software. Data are average of three independent experiments $\pm$ SD.

DRI which represents the actual fold-change of dose attenuation in a synergistic combination at a given effect level compared with the drug alone. The Fa-DRI plot and Fa-log (DRI) plot demonstrates whether the influence of synergistic treatments may ameliorate side effects caused by cytotoxicity to normal cells. Figure 5D demonstrates that the DRI of DOX values were higher than 1, which indicates favorable dose reduction when combined with EMP.

3.2.2. Cell Cycle Analysis. Most anticancer agents act by arresting the cell cycle at definite stages of growth to exert their antiproliferative effect. Flow cytometry is used in cell cycle analysis to distinguish cells at different phases of the cell cycle. In this study, we tested the effect of the IC₅₀ of DOX, EMP, and DOX/EMP combination to determine the definite phase at which cell cycle arrest takes place in the MDA-MB-231 breast cancer cell line, and their impact on the cell population was recorded and compared to the control treated with solvent dimethyl sulfoxide (5% DMSO) as a vehicle. Treatment of MDA-MB-231 cells with DOX, EMP, and DOX/EMP combination resulted in a significant decline in the cell population at the G2/M phase with 2.41 and 1.69% for DOX and DOX/EMP combination respectively, compared to that of the control (DMSO), which was 7.89% (Figure 6). EMP showed a clear decline in the cell population at the S phase with 35.18% compared to 42.76% of the control. This indicates that DOX/EMP combination acts on the same phase of the cell cycle as DOX, and at the same time, it achieved a more characteristic arrest in the growth of cells by 4.67-fold in comparison to the control. However, DOX alone showed only

Figure 6. Impact of conjugates DOX, EMP, and DOX/EMP combination on the cell cycle phases of MDA-MB-231 cells. Data are average of three independent experiments $\pm$ SD. *, §, and # represent significance ($p < 0.5$) compared with control, EMP, or DOX alone, respectively.

3.27-fold arrest compared to the control. This greatly clarifies that the DOX/EMP combination halted the cell cycle proliferation of MDA-MB-231 cells in the G2/M phase with a 1.42-fold increase in activity compared to DOX alone.

3.2.3. Annexin V-FITC (Anx V) Apoptosis Assay. Annexin-V/PI based flow cytometry apoptosis assay is a helpful tool that can clarify whether cell death is due to programmed apoptosis or uncontrolled necrosis (Figure 7). It enables

Figure 7. Combined effect of DOX and EMP on the apoptosis of MDA-MB-231 cells. Representative dot plot presenting the flow cytometry analysis of cells stained with Annexin V and PI following treatment with vehicle alone (A), 1.3 μ M DOX alone (B), 50 μ M EMP alone (C), or a combination of 1.3 μ M DOX + 50 μ M EMP (D) for 24 h. Data are representative of three individual experiments.

researchers to identify the early apoptotic cells within a cell population. EMP is also tested for its apoptotic effect on the MDA-MB-231 cell line, compared to Dox and Dox/EMP combination. Since the combination of EMP with DOX achieved the highest antitumor activity against the MDA-MB-231 cell line, it was further evaluated for its effect on the cell cycle of the aforementioned cell line compared to both DOX and EMP alone.

Treatment of MDA-MB-231 cells with IC_{50} concentration of DOX, EMP, and DOX/EMP combination showed a marked increase in the AnxV-FITC apoptotic cells percentage in both early (from 0.33 to 2.51, 8.26, and 4.26%, respectively) and late apoptosis (from 0.12 to 19.65, 6.60, and 25.12%, respectively) phases (Figure 8). This indicates an increase in

Figure 8. Distribution of apoptotic cells in the AnnexinV-FITC/PI apoptosis assay in MDA-MB-231 cells after treatment with DOX, EMP, and DOX/EMP combination. Data are average of three independent experiments $\pm$ SD. *, §, and # represent significance ($p < 0.5$) compared with control, EMP, or DOX alone, respectively.

the total apoptosis percentage by 27.05-, 14.90-, and 29.22-fold, respectively, compared to the control. This confirmed that the cytotoxic activity of either DOX, EMP, or DOX/EMP combination is due to physiological apoptosis, not nonspecific necrosis. Furthermore, as reported in all parts of our research, DOX/EMP combination achieved the best increase in the percent of apoptotic cells confirming the concept of chemosensitization of the combination compared to DOX alone.

3.2.4. Reverse Transcription and Quantitative Real-Time PCR. To unravel the underlying molecular mechanisms of the found synergistic antitumor effects of EMP and DOX on TNBCs, we studied the expression of apoptosis-related genes. mTOR is a well-known regulator of cell proliferation either directly or indirectly. It can stimulate cell cycle regulators or produce nutrient transporters that promote angiogenesis. Besides, its activation may activate the antiapoptotic proteins which contribute also to tumor progression.³⁶ In preclinical studies, inhibiting mTOR resulted in antiproliferative effects in several cancer models.³⁶ In line with these data, we found that the combined treatment of TNBC cells with EMP/DOX produced a synergistic negative effect on mTOR mRNA expression (Figure 9A). Bcl2, an antiapoptotic gene that is reportedly regulated by mTOR,³⁷ is classified as a poor prognostic marker in patients with TNBCs.³⁸ In our results, Bcl2 expression was significantly decreased in EMP/DOX combination therapy compared with either treatment alone (Figure 9B). Moreover, the JNK gene is responsible for many roles in cellular death, proliferation, and inflammation which cause many diseases, like cancer.³⁹ It was considered an important signal transduction gene in Ras-mediated oncogenesis.⁴⁰ We found EMP synergistically inhibits JNK mRNA with DOX (Figure 9C). However, p21 is a gene that is responsible for cell cycle inhibition and antiproliferation in normal healthy cells and is classified as a tumor-suppressor

Figure 9. Analysis of gene expression profiles of mTOR (A), Bcl2 (B), JNK (C), and p21 (D), and of MDA-MB-231 cells treated with DOX, EMP, and DOX/EMP combination compared to blank by using real-time PCR. Data are average of three independent experiments $\pm$ SD. *, §, and # represent significance ($p < 0.5$) compared with control, EMP, or DOX alone, respectively.

gene.⁴¹ Our data (Figure 9D) revealed a significant increase in p21 gene expression in cells treated with combined EMP and DOX.

Finally, the multi-drug-resistance gene MDR1 is associated with the expected previously mentioned drug resistance, and its overexpression is observed in responses to some anticancer agents like DOX.¹¹ Again, our results (Figure 10) showed that EMP could not affect greatly the cytotoxic effect of DOX on MDA-MB-231, and cotreatment of EMP and DOX decreased

the expression of MDR1 and consequently increased sensitivity of MDA-MB-231 TBNCs to DOX, suggesting that adding EMP to DOX treatment protocols would decrease its therapeutic dose required for the treatment of TNBC and its toxic side effects as well.

4. CONCLUSION

In summary, EMP is a promising drug to be combined with DOX and repurposed against the drug-resistant TNBC subtype due to its chemosensitization effect on DOX as proved by the reduction in the MDR1 gene and reflected by the enhanced cytotoxicity and apoptotic properties of DOX as indicated by the MTT, DNA cell cycle, and Annexin V/PI apoptosis assays. Moreover, the molecular mechanisms of these effects were revealed by the downregulation of proliferation genes (mTOR, Bcl-2, and JNK) and the upregulation of a pro-apoptotic gene (p21). Collectively, these findings together with the recommended mechanism of action for EMP as a calmodulin receptor antagonist proposed its chemosensitization effect on DOX. The chemosensitizing effect of the DOX/EMP combination will achieve two important outcomes. The first one will decrease the required therapeutic dose of DOX and consequently decrease its toxic side effects, especially cardiotoxicity, which hinder DOX use. Second, it will participate in the repression of DOX drug resistance and resensitize tumor cells to DOX. Finally, we recommend further *in vivo* studies for EMP in combination with DOX as a new potential chemotherapeutic combination for treating TNBC.

Figure 10. Analysis of gene expression profiles of the multidrug resistance gene MDR1 in MDA-MB-231 after treatment with DOX, EMP, and DOX/EMP combination compared to blank by using real-time PCR. Data are average of three independent experiments $\pm$ SD. * and § represent significance ($p < 0.5$) compared with control, EMP, or DOX alone, respectively.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acspstsci.0c00144>.

2D and surface and maps of both docked calmodulin antagonist (AAA) and docked EMP inside subunits A and B (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Ahmed A. Al-Karmalawy – Department of Pharmaceutical Medicinal Chemistry, Faculty of Pharmacy, Horus University-Egypt, New Damietta 34518, Egypt; orcid.org/0000-0002-8173-6073; Email: akarmalawy@horus.edu.eg

Authors

Shenouda G. Eliaa – Department of Molecular Biology, Genetic Engineering and Biotechnology Research Institute, University of Sadat City, Sadat City 32897, Egypt

Rasha M. Saleh – Department of Physiology, Faculty of Veterinary Medicine, Mansoura University, Mansoura 35516, Egypt

Mohamed F. Elshal – Department of Molecular Biology, Genetic Engineering and Biotechnology Research Institute, University of Sadat City, Sadat City 32897, Egypt

Complete contact information is available at:

<https://pubs.acs.org/doi/10.1021/acspstsci.0c00144>

Notes

The authors declare no competing financial interest.

■ REFERENCES

- (1) Watson, I. R., Takahashi, K., Futreal, P. A., and Chin, L. (2013) Emerging patterns of somatic mutations in cancer. *Nat. Rev. Genet.* 14 (10), 703–718.
- (2) Tate, J. G., Bamford, S., Jubb, H. C., et al. (2019) COSMIC: the catalogue of somatic mutations in cancer. *Nucleic Acids Res.* 47 (D1), D941–D947.
- (3) Heron, M., and Anderson, R. N. (2017) Changes in the leading cause of death: Recent patterns in heart disease and cancer mortality. *NCHS Data Brief* 254, 1–8.
- (4) Chavez, K. J., Garimella, S. V., and Lipkowitz, S. (2011) Triple negative breast cancer cell lines: one tool in the search for better treatment of triple negative breast cancer. *Breast Dis.* 32 (1–2), 35.
- (5) Ismail-Khan, R., and Bui, M. M. (2010) A Review of Triple-Negative Breast Cancer. *Cancer Control* 17, 173–176.
- (6) Yu, A. F., Chan, A., and Steingart, R. M. (2019) Cardiac Magnetic Resonance and Cardio-oncology—Does T2 Signal the End of Anthracycline Cardiotoxicity? *J. Am. Coll. Cardiol.* 73 (7), 792.
- (7) Arcamone, F. (2012) *Doxorubicin: Anticancer Antibiotics*, Elsevier.
- (8) Tacar, O., Sriamornsak, P., and Dass, C. R. (2013) Doxorubicin: An update on anticancer molecular action, toxicity and novel drug delivery systems. *J. Pharm. Pharmacol.* 65 (2), 157–170.
- (9) Ghanem, A., Emara, H. A., Muawia, S., Abd El Maksoud, A. I., Al-Karmalawy, A. A., and Elshal, M. F. (2020) Tanshinone IIA synergistically enhances the antitumor activity of doxorubicin by interfering with the PI3K/AKT/mTOR pathway and inhibition of topoisomerase II: in vitro and molecular docking studies. *New J. Chem.* 44, 17374.
- (10) Thorn, C. F., Oshiro, C., Marsh, S., et al. (2011) Doxorubicin pathways: pharmacodynamics and adverse effects. *Pharmacogenet. Genomics* 21 (7), 440.
- (11) Tada, Y., Wada, M., Migita, T., Nagayama, J., Hinoshita, E., Mochida, Y., Maehara, Y., Tsuneyoshi, M., Kuwano, M., and Naito, S. (2002) Increased Expression of Multidrug Resistance-Associated Proteins in Bladder Cancer during Clinical Course and Drug Resistance to Doxorubicin. *Int. J. Cancer* 98, 630–635.
- (12) Abolhoda, A., Wilson, A. E., Ross, H., Danenberg, P. V., Burt, M., and Scotto, K. W. (1999) Rapid activation of MDR1 gene expression in human metastatic sarcoma after in vivo exposure to doxorubicin. *Clin. Cancer Res.* 5 (11), 3352–3356.
- (13) Marquette, C., and Nabell, L. (2012) Chemotherapy-resistant metastatic breast cancer. *Curr. Treat Options Oncol.* 13 (2), 263–275.
- (14) Osman, A.-M. M., Al-Harthy, S. E., AlArabi, O. M., Elshal, M. F., Ramadan, W. S., Alaama, M. N., Al-Kreathy, H. M., Damanhour, Z. A., and Osman, O. H. (2013) Chemosensitizing and cardioprotective effects of resveratrol in doxorubicin-treated animals. *Cancer Cell Int.* 13 (1), 52.
- (15) Osman, A.-M. M., Telity, S. A., Damanhour, Z. A., Al-Harthy, S. E., Al-Kreathy, H. M., Ramadan, W. S., Elshal, M. F., Khan, L. M., and Kamel, F. (2015) Chemosensitizing and nephroprotective effect of resveratrol in cisplatin-treated animals. *Cancer Cell Int.* 15 (1), 6.
- (16) Pushpakom, S., Iorio, F., Eyers, P. A., et al. (2019) Drug repurposing: Progress, challenges and recommendations. *Nat. Rev. Drug Discovery* 18 (1), 41–58.
- (17) Park, K. (2019) A review of computational drug repurposing. *Transl Clin Pharmacol.* 27 (2), 59–63.
- (18) Polamreddy, P., and Gattu, N. (2019) The drug repurposing landscape from 2012 to 2017: evolution, challenges, and possible solutions. *Drug Discovery Today* 24 (3), 789–795.
- (19) Inzucchi, S. E., Bergenstal, R. M., Buse, J. B., et al. (2012) Management of hyperglycaemia in type 2 diabetes: a patient-centered approach. Position statement of the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD). *Diabetologia* 55 (6), 1577–1596.
- (20) Committee for Medicinal Products for Human Use (CHMP). (2017) *Guideline on the Evaluation of Anticancer Medicinal Products in Man*, European Medicines Agency, London, U.K..
- (21) Uzma, F., Al-Mutairi, F., Humaira, P., and Sahar, K. (2020) An in-vitro and in Silico Anticancer Study of FDA Approved Antidiabetic Drugs Glimepiride and Empagliflozin. *Int. J. Pharma Bio Sci.* 10 (2), 52–57.
- (22) Mustroph, J., Wagemann, O., Lucht, C. M., et al. (2018) Empagliflozin reduces Ca/calmodulin-dependent kinase II activity in isolated ventricular cardiomyocytes. *ESC Heart Fail.* 5 (4), 642–648.
- (23) Anker, S. D., and Butler, J. (2018) Empagliflozin, calcium, and SGLT1/2 receptor affinity: another piece of the puzzle. *ESC Heart Fail.* 5, 549–551.
- (24) Connolly, K. A., Zhang, Y., Visram, A., Advani, A., Batchu, S. N., Desjardins, J.-F., Thai, K., and Gilbert, R. E. (2019) Empagliflozin Improves Diastolic Function in a Nondiabetic Rodent Model of Heart Failure With Preserved Ejection Fraction. *JACC Basic Transl Sci.* 4 (1), 27–37.
- (25) Hait, W. N., and Lazo, J. S. (1986) Calmodulin: A potential target for cancer chemotherapeutic agents. *J. Clin. Oncol.* 4 (6), 994–1012.
- (26) Brzozowski, J. S., and Skelding, K. A. (2019) The multi-functional calcium/calmodulin stimulated protein kinase (CaMK) family: Emerging targets for anti-cancer therapeutic intervention. *Pharmaceuticals* 12 (1), 8.
- (27) Chemical Computing Group, Inc. (2016) *Molecular operating environment (MOE)*, Montreal.
- (28) Harmat, V., Böcskei, Z., Náray-Szabó, G., et al. (2000) A new potent calmodulin antagonist with arylalkylamine structure: Crystallographic, spectroscopic and functional studies. *J. Mol. Biol.* 297 (3), 747–755.
- (29) Chou, T.-C. (2006) Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol. Rev.* 58 (3), 621–681.
- (30) Osman, A.-M. M., Bayoumi, H. M., Al-Harthy, S. E., Damanhour, Z. A., and Elshal, M. F. (2012) Modulation of doxorubicin cytotoxicity by resveratrol in a human breast cancer cell line. *Cancer Cell Int.* 12, 47.

- (31) Mohamed, S., Elshal, M., Kumosani, T., Aldahlawi, A., Basbrain, T., Alshehri, F., and Choudhry, H. (2016) L-asparaginase isolated from *Phaseolus vulgaris* seeds exhibited potent anti-acute lymphoblastic leukemia effects in-vitro and low immunogenic properties in-vivo. *Int. J. Environ. Res. Public Health* 13 (10), 1008.
- (32) Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta$ CT method. *Methods* 25 (4), 402–408.
- (33) Davis, I. W., and Baker, D. (2009) RosettaLigand Docking with Full Ligand and Receptor Flexibility. *J. Mol. Biol.* 385 (2), 381–392.
- (34) McConkey, B. J., Sobolev, V., and Edelman, M. (2002) The performance of current methods in ligand-protein docking. *Curr. Sci.* 83 (7), 845–856.
- (35) Chou, T.-C. (2010) Drug combination studies and their synergy quantification using the Chou-Talalay method. *Cancer Res.* 70 (2), 440–446.
- (36) Advani, S. H. (2010) Targeting mTOR pathway: A new concept in cancer therapy. *Indian J. Med. Paediatr. Oncol.* 31 (4), 132.
- (37) Asnaghi, L., Calastretti, A., Bevilacqua, A., D'Agnano, I., Gatti, G., Canti, G., Delia, D., Capaccioli, S., and Nicolin, A. (2004) Bcl-2 phosphorylation and apoptosis activated by damaged microtubules require mTOR and are regulated by Akt. *Oncogene* 23, 5781–5791.
- (38) Eom, Y. H., Kim, H. S., Lee, A., Song, B. J., and Chae, B. J. (2016) Breast Cancer BCL2 as a Subtype-Specific Prognostic Marker for Breast Cancer 19 (3), 252–260.
- (39) Wood, R. A., Barbour, M. J., Gould, G. W., Cunningham, M. R., and Plevin, R. J. (2018) Conflicting evidence for the role of JNK as a target in breast cancer cell proliferation: Comparisons between pharmacological inhibition and selective shRNA knockdown approaches. *Pharmacol. Res. Perspect.* 6, e00376.
- (40) Tournier, C. (2013) The 2 Faces of JNK Signaling in Cancer. *Genes Cancer* 4, 397–400.
- (41) Shamloo, B., and Usluer, S. (2019) p21 in Cancer Research. *Cancers* 11, 1178.