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Telmisartan Enhances the Accumulation of Doxorubicin as a Combination Therapy for the Management of Triple Negative Breast Cancer

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Abstract

Background: Triple-negative breast cancer (TNBC) is one of the most aggressive tumours with dismal survival and a high death rate. Chemotherapeutic resistance due to P-gp overexpression was shown in MDA-MB-231 human breast cancer cells. The aim of this study was to re-sensitize of MDA-MB-231 human breast cancer cells to Doxorubicin by suggested P-gp inhibitors.

Methods: Screening of around 100 in-house prepared compounds against the crystal structure of the P-glycoprotein was performed using molecular docking tool. The top ranked hits obtained were Verapamil, Benazepril, and Telmisartan.Accordingly, the anticancer activity of Doxorubicin and in combination with suggested P-gp inhibitors were examined on MDA-MB-231 breast cancer cell line, using the [3-(4,5-dimetiltiazol-2-il)-2,5-diphenyl tetrazolium bromide] MTT assay. Accumulation of Doxorubicin was analyzed by flow cytometry.

Results: Telmisartan showed the lowest binding energy (-9.7 Kcal/mol) followed by Benazepril and Verapamil, with -9.2, -7.3 and -6.4 Kcal/mol, respectively. These compounds were chosen for the next phase of studies to evaluate their in vitro biological effects against TNBC cell line (MDA-231). Doxorubicin in combination with Telmisartan significantly inhibited cell proliferation in the MDA-MB-231 breast cancer cell line (IC_{50}=0.08261 μM for MDA-MB-231) more than Doxorubicin alone (IC_{50}=0.2847μM for MDA-MB-231). Flow cytometry examined the accumulation of Doxorubicin inside the MDA-MB-231 breast cancer cell line after 24 hours of treating them with Telmisartan.

Conclusion: current findings suggest that Telmisartan re-sensitize MDA-MB-231 breast cancer cells to Doxorubicin by increasing Doxorubicin accumulation.

Keywords: Triple negative breast cancer, Doxorubicin, Chemotherapy resistance, Telmisartan, P-glycoprotein inhibitors, Molecular docking.

1. Introduction

Breast cancer is the most prevalent type of cancers in women with a high mortality rate. Of all subtypes of breast cancer, triple negative breast cancer (TNBC) is considered the most aggressive one and is associated with very poor prognosis (Yin et al., 2020). Despite the revolution in cancer therapy, the conventional chemotherapy using combination of drugs remains the cornerstone when it comes to TNBC treatment (O'Reilly et al., 2021).

Doxorubicin-based chemotherapy is the most commonly used regimens in the treatment of variety of cancers including TNBC (Denard et al., 2018). Doxorubicin is an anthracycline compound that intercalates with the DNA and inhibits topoisomerases leading to cell cycle arrest and eventually apoptosis (Al-malky et al., 2019). However, regardless its popularity, the emerging resistance to Doxorubicin (also known as chemo-resistance) is a significant issue that has not yet been resolved (Christowitz et al., 2019). When applied at the optimum doses, Doxorubicin can efficiently hamper cancer progression and development. However, following repetitive doses, cancer cells develop chemoresistance towards Doxorubicin leading to diminished antiproliferative effect (Mirzaei et al., 2022). Increasing the concentration of Doxorubicin might not be a wise decision due to the cardiotoxicity of Doxorubicin (Zhang et al., 2020). Therefore, continuous efforts should aim for novel strategies to alleviate Doxorubicin resistance.

Several chemoresistance mechanisms have been proposed in breast cancer with the most extensively being the efflux pumps such as P-glycoprotein (P-gp) (Robey et al., 2018). P-gp (also known as MDR1 or ABCB1) is a

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member of the ATP-binding cassette (ABC) family and a well-known drug transporter presents in the cell membrane that mediate resistance against structurally and functionally different chemotherapeutic drugs (Gottesman et al., 1996). Accordingly, it is not surprising that overexpression of P-gp is associated with poor prognosis and higher incidence of relapse (Waghray & Zhang, 2018). An earlier work conducted by Bao et al. (2011) demonstrated that overexpression on P-gp in breast cancer prevented the internalization of Doxorubicin within the nucleus leading to minimal efficacy and chemoresistance(Bao et al., 2011).

Having its key role in mediating resistance towards chemotherapy, the development of P-gp inhibitors has been a primary focus in the cancer research. The rationale behind combining chemotherapy with P-gp inhibitors is that they can logically enhance the cellular uptake of the chemotherapeutic drugs leading to more prominent effects (Lai et al., 2020). To-date, a large set of small molecules that aim to inhibit the function of P-gp have been developed. The clinical progression of the specific P-gp inhibitors has been rather disappointing due to the failure in clinical trials in addition to the safety issues associated with these small molecules (Chung et al., 2016). Therefore, drug repurposing of the currently FDA approved drugs could be an interesting clinical rationale. This is particularly of huge interest since the FDA approved drugs possess a reasonable safety profile. Moreover, combining these drugs with chemotherapy may result in enhanced anti-cancer effects. For instance, Verapamil, a calcium channel blocker known for its potent P-gp inhibitory effect, has been widely studied in clinical research (Lai et al., 2020). For instance, Bao et al. (2011) reported an increased cellular uptake of Doxorubicin when combined with Verapamil leading to superior anti-cancer effect compared to Doxorubicin alone(Bao et al., 2011). Cyclosporin, an immunosuppressant, is another P-gp inhibitor that was shown to enhance the nuclear contents of Doxorubicin in breast and lung cancer cells (Waghray & Zhang, 2018).

In-silico screening can be used to study and understand the intermolecular interactions between the proposed ligands and the therapeutic target (Al-Najjar 2018, Saqallah et al., 2022). Herein, the current study aims to employ the concept of drug repurposing to investigate the P-gp inhibitory function of a variety of FDA approved drugs and their biological activities. More importantly, the study intends to explore whether combining the potential P-gp candidates with Doxorubicin enhances its cellular uptake and subsequently its anti-cancer effect *in vitro*. This approach has the potential to overcome chemoresistance developed against Doxorubicin leading to better clinical outcomes.

2. Materials and Methods

2.1. Molecular docking

The following software packages were utilized in this project:

a. Avogadro 1.2.0(Hanwell et al., 2012).
b. ACD/ChemSketch, (www.acdlabs.com) (ACD/Labs, 2019).
c. Autodock 4.2 (Morris et al., 2009).

Around 100 ligands were collected according to the availability of the chemicals in the university. These compounds were subjected to molecular docking simulations against the crystal structure of the p-glycoprotein (PDB code: 7A6F) (Nosol et al., 2020). The chemical compounds were created using the ChemSketch software, saved as mol files. A short steepest descent energy minimization procedure was performed to adjust inaccurate bond lengths and bond angles; at this stage, atom types were assigned using the Universal Force Field, pre-packaged with Avogadro' software and converted to pdb format. Atomic charges were added; all hydrogen atoms were combined, and each compound was opened independently. The grid box was cantered at the binding site of the co-crystal inhibitor (Zosuquidar) with the coordinates of 162.15, 160.03, 158.00 as x, y, z, respectively. The box volume was set to 22.5 $Å^3$ with the default grid spacing value of 0.375 Å. Afterward, the default docking parameters were used to conduct the molecular docking of each ligand for 100 Lamarckian genetic algorithm runs using AutoDock 4(Fuhrmann et al., 2010).

2.2. Cell Culture

MDA-231 breast cancer cell line was obtained from the university of Jordan while the origin is "The European Collection of Authenticated Cell Cultures" (ECACC\UK) (ECACC catalogue no. 92020424). MDA-123 cell line is an epithelial, human breast cancer cell line that was established from a pleural effusion of a 51-year-old Caucasian female with a metastatic mammary adenocarcinoma (Cailleau et al., 1978). MDA-231cells were maintained in a high glucose Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% (v/v) fetal bovine serum, 1% penicillin and streptomycin.Unless stated otherwise, all other chemicals, reagents and solvents used in this study were purchased from Invitrogen (USA).

2.3. Colorimetric MTT assay

To measure cell viability, the colorimetric microculture tetrazolium assay (MTT) method was used. The cultured MDA-123 cells were seeded at a density of $1X10^4$ cells per well in 96-well plates(Al-Tawarah et al., 2020). The next day cells were treated at concentrations (10-100 µM) of Verapamil, Benazepril, or Telmisartan as single agent or with Doxorubicin at concentrations (0.001-100 µM), which were purchased from Santa Cruz (USA).

The culture plates were incubated for 72 hours(Lee et al., 2020), then 15 µL of MTT solution was added to each well and for 4 hours. After that 100 µL of DMSO were added to each well. Cell growth was determined by measuring the optical density (OD) at 590/630 nm with Microplate Reader Biotech™ ELx800™.

2.4. Cellular uptakes by Flow Cytometry

Human breast cancer cell line MDA-231 cells were seeded in a 12-well plate at $1x10^4$ cells/well and left over 24 hours to attach. Subsequently, 30µg/mL of Telmisartan were prepared and directly applied to adhered cells and incubated over 1 hour at 37°C. Subsequently, Doxorubicin was added and incubated for 1, 4 and 24 hours. After that, the culture media was removed, and cells were washed two times with PBS. Cells were then detached gently with 200 mL of StemPro™ Accutase™ Cell Dissociation Reagent (Gibco, USA) for 5 min and transferred into 5 mL flow

tubes (BD, USA). 1x10^4 events were counted by FACS Canto II and analyzed using BD FACS Diva™ software version 8.0 (BD, USA).

3. Results

3.1. In-house database virtual screening

The co-crystallised ligandZosuquidar (Structure Code: ZQU1301) was successfully docked against 7A6F crystal structure of the P-glycoprotein with a root-main-square distance (RMSD) of 1.0 Å (**Figure 1**). RMSD is used to quantitatively measure the similarity between two or more compounds. The smaller the RMSD, the more similar compounds (Kufareva & Abagyan, 2012). Generally, molecular docking simulations that produce an RMSD values of less than 2.0Å are considered to have performed successfully (Yusuf et al., 2008).

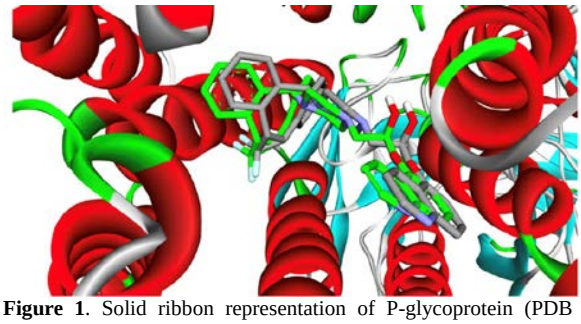

Figure 1. Solid ribbon representation of P-glycoprotein (PDB code: 7A6F). crystal structure bound with the co-crystallized ligand (grey) and the re-docked conformation (green).

Upon screening, around 100 molecules were successfully docked against 7A6F crystal structure of the P-glycoprotein. Eventually, Telmisartan, Verapamil, and Benazepril were amongst top-ranked compounds (**Figure 2**). Investigation of these compounds in the binding site have shown that Telmisartan participates in two hydrogen bond interactions with Gln990 and Phe983 residues, while Leu339, Ile340, Phe343 and Phe983 amino acids perform hydrophobic and aromatic interactions (**Figure 3A**). Additionally, Benazepril, on the other hand, was found to interact with Gln725 and Gln990 by hydrogen bond interaction, while both Ile306, Phe343 interact by hydrophobic and aromatic interactions, respectively (**Figure 3B**). Finally, verapamil mainly performs hydrophobic and aromatic interactions with Leu329, Phe343, Gln725 and Phe728, whereas Trp232 was found to perform hydrogen bond interaction (**Figure 3C**). If we can assume the importance of the residues involved in the interaction with the co-crystallized ligand (Zosuquidar), it will be significant to find a compound that can successfully interact with these key residues. Telmisartan is the only compound among the top-ranked hits that interact with both key residues Leu339, Gln990.**Table 1** shows the top-ranked compounds with their corresponding binding energies, as well as the interacting residues. Telmisartan showed the lowest binding energy (-9.7 Kcal/mol) followed by Benazepril and Verapamil, with -9.2, -7.3 and -6.4 Kcal/mol, respectively. These compounds were chosen for the next phase of studies to evaluate their *in vitro* biological effects against TNBC cell line (MDA-231).

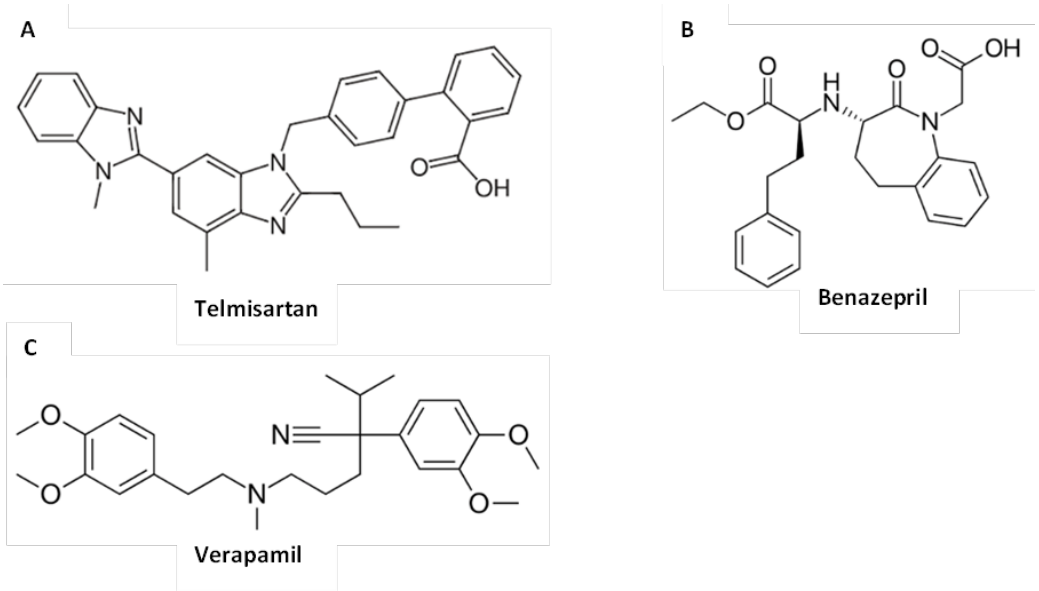

Figure 2: The chemical structure of Telmisartan (A), Benazepril (B), and Verapamil (C).

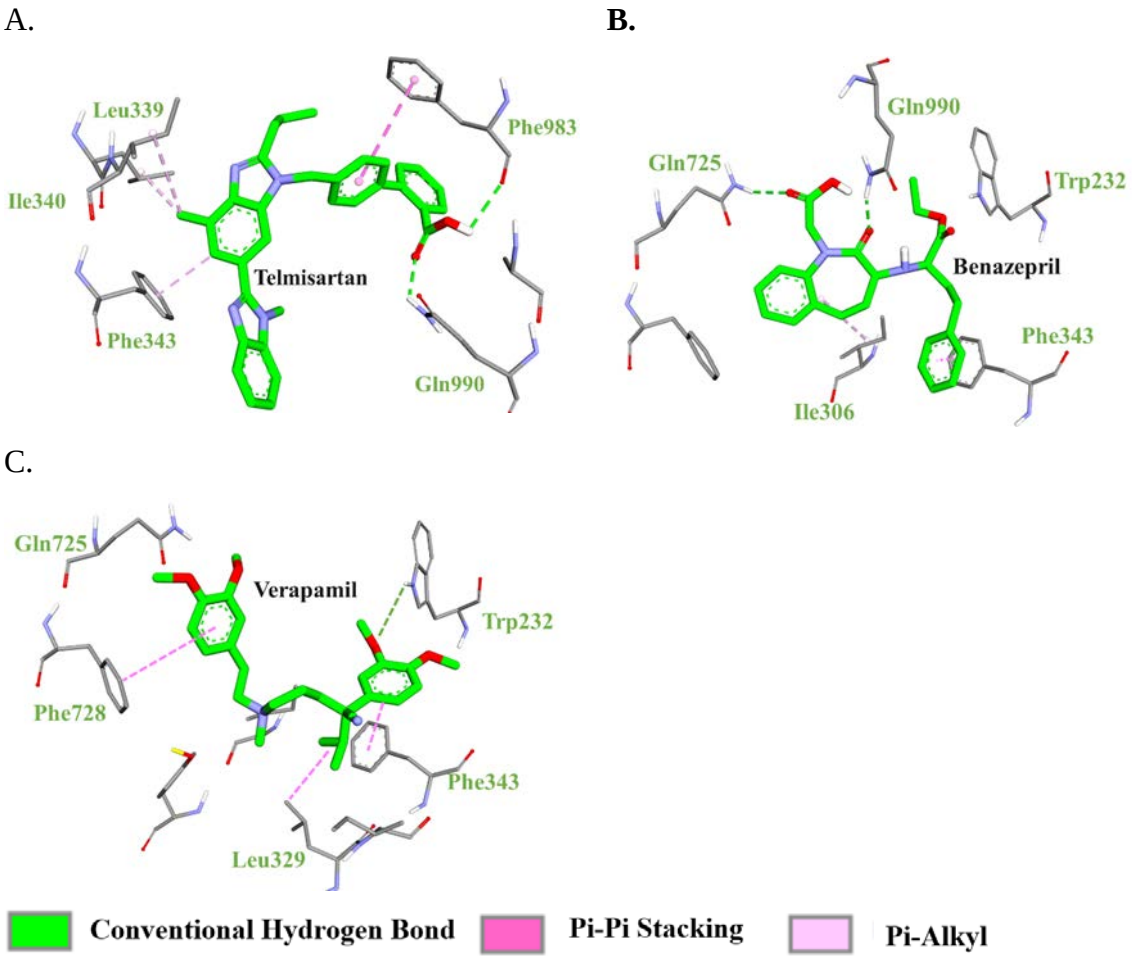

Figure 3. Stick representation of A. Telmisartan, B. Benazepril, and C. Verapamil in the P-gp binding site.

Table 1. The lowest binding energies obtained from AutoDock 4.2 in-house database 7A6F crystal structure and interacting amino acids.

Compound	Lowest Binding Energy (Kcal/mol)	Interacting amino acids
Telmisartan	-9.7	Leu339, Ile340, Phe343, Phe983, Gln990
Benazepril	-7.3	Ile306, Phe343, Gln725, Gln990
Verapamil	-6.4	Trp232, Leu329, Phe343, Phe728
Zosuquidar	-6.5	Phe303, Leu339, Gln990

3.2. Telmisartan enhances the antiproliferative effect of Doxorubicin against MDA-231 TNBC cellline

Following successful docking, it was postulated that the inhibition of P-gp by these compounds may improve the cellular response towards Doxorubicin-induced cell death. Accordingly, the potential synergy between the tested compounds and Doxorubicin against MDA-231 cell line was evaluated using MTT assay. For this purpose, MDA-231 cells were treated with increasing concentrations of Doxorubicin, Benazepril, Verapamil,as monotherapy or in combination with Doxorubicin for 72 hours prior to viability testing using MTT assay. The results revealed that Doxorubicin treatment resulted in a dose-dependent reduction in cell viability over the period of 72 hours with an IC_{50} of 0.2847 µM (Figure 4A). On the contrary, monotherapy treatment with Benazepril, Verapamil, and Telmisartan resulted in modest reduction of cell viability (Figure 4B-D).

The combination treatment of Doxorubicin/benazepril had no superior efficacy compared to monotherapy of Doxorubicin illustrated by no significant reduction in the IC_{50} value (Figure 5A). In contrast, Verapamil appeared to enhance the antiproliferative effect of Doxorubicin. However, this synergistic effect was only observed at certain concentrations but not the full panel (Figure 5B). Interestingly, the combination therapy of Doxorubicin/Telmisartan resulted in the strongest synergy demonstrated by significant reduction in the IC_{50} compared to cells treated with either agent alone (Figure 5C) (Table 2).

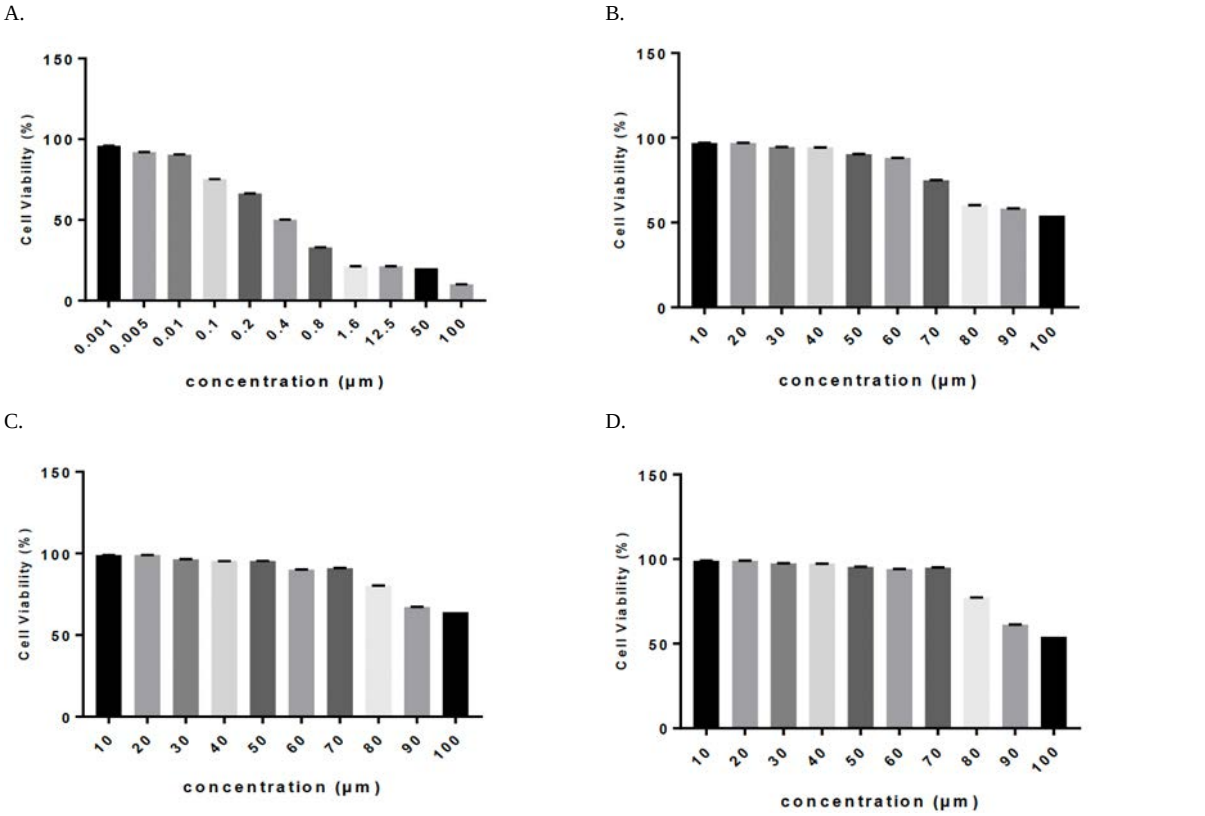

Figure 4. The anti-proliferative effect of Doxorubicin, Benezepril, Verapamil, and Telmisartan on breast cancer. MDA-231 breast canncer cells were treated with increasing concentrations of Doxorubicin (A), Benezepril (B), Verapamil (C), or Telmisartan (D) as monotherapy for 72 hours prior to MTT proliferation assay analysis. Data represent mean ± SD (n=3).

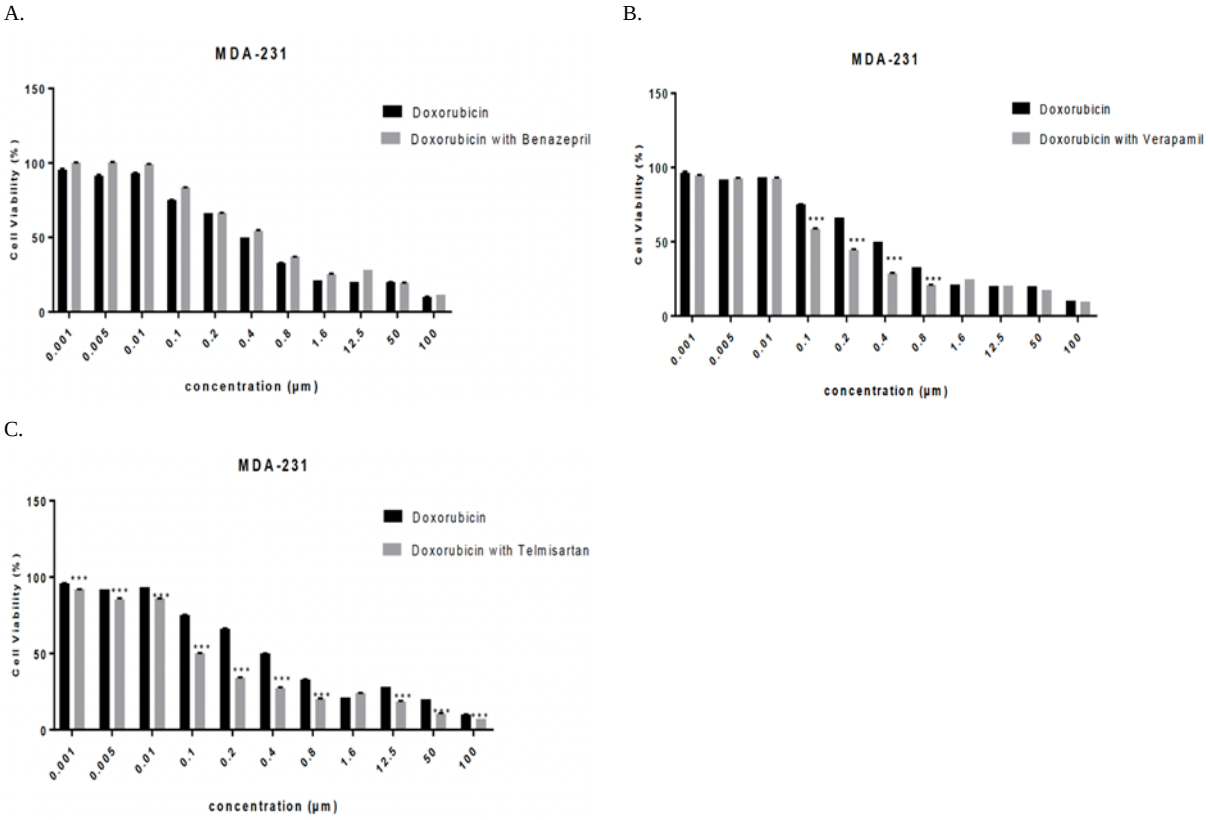

Figure 5. Assessment of the anti-proliferative effect of combination therapy. MDA-231 cells were treated with different concentrations of Doxorubicin (0.001-100 µM) alone or in combination with 70µM Benazepril (**A**), 70µM Verapamil (**B**), or 60µM Telmisartan (**C**) for 72 hours prior to cell viability testing using MTT assay. Data represent mean ± SD (n=3). P values *: p<0.05, ** p<0.01, ***, p<0.001 calculated using *t-test*.

Table 2. IC_{50} values (µM) of the *in vitro* antiproliferative activity of Doxorubicin with Verapamil, Doxorubicin with Benazepril, and Doxorubicin with Telmisartan on MDA-231 cell line.

Treatment	IC_{50} (uM)	p-value
Doxorubicin	0.2847	
Doxorubicin/Verapamil	0.1175	0.1528
Doxorubicin/Benazepril	0.262	0.8226
Doxorubicin/Telmisartan	0.08261	0.0089***

The results were expressed as mean ± SD (n = 3) and analysed using t-test, *: p<0.05, ** p<0.01, ***, p<0.001 compared to their respective Doxorubicin (control).

3.3. Telmisartan enhances the cellular uptake of Doxorubicin

The significant reduction in IC_{50} upon dual drug therapy with Doxorubicin and Telmisartan but not with other combinations raised a question whether Telmisartan was more potent P-gp inhibitor than the other compounds which may led to enhanced Doxorubicin accumulation and therefore improve cytotoxic effect. For this purpose, cellular uptake of Doxorubicin was performed using flow cytometry analysis, thanks to the fluorescent properties of Doxorubicin. MDA-231 cells were exposed to a concentration of 30 µg/mL of Telmisartan for 1 hour prior to the treatment with the IC_{50} concentration of Doxorubicin for 1, 4, and 24 hours. The analysis revealed no significant uptake of Doxorubicin either alone or in combination with the Telmisartan at 1 and 4 hours' time-point (Figure 6A and B). However, at 24 hours' time-point, while there was a notable cellular uptake of Doxorubicin in cells treated with Doxorubicin alone, the Doxorubicin/Telmisartan co-therapy resulted in superior accumulation indicated by more prominent increase in the fluorescence signal (Figure 6C).

The synergistic effect observed between Telmisartan and Doxorubicin along with the superior cellular uptake of Doxorubicin might be attributed to the potent P-gp inhibitory effect of Telmisartan compared to any other compounds tested.

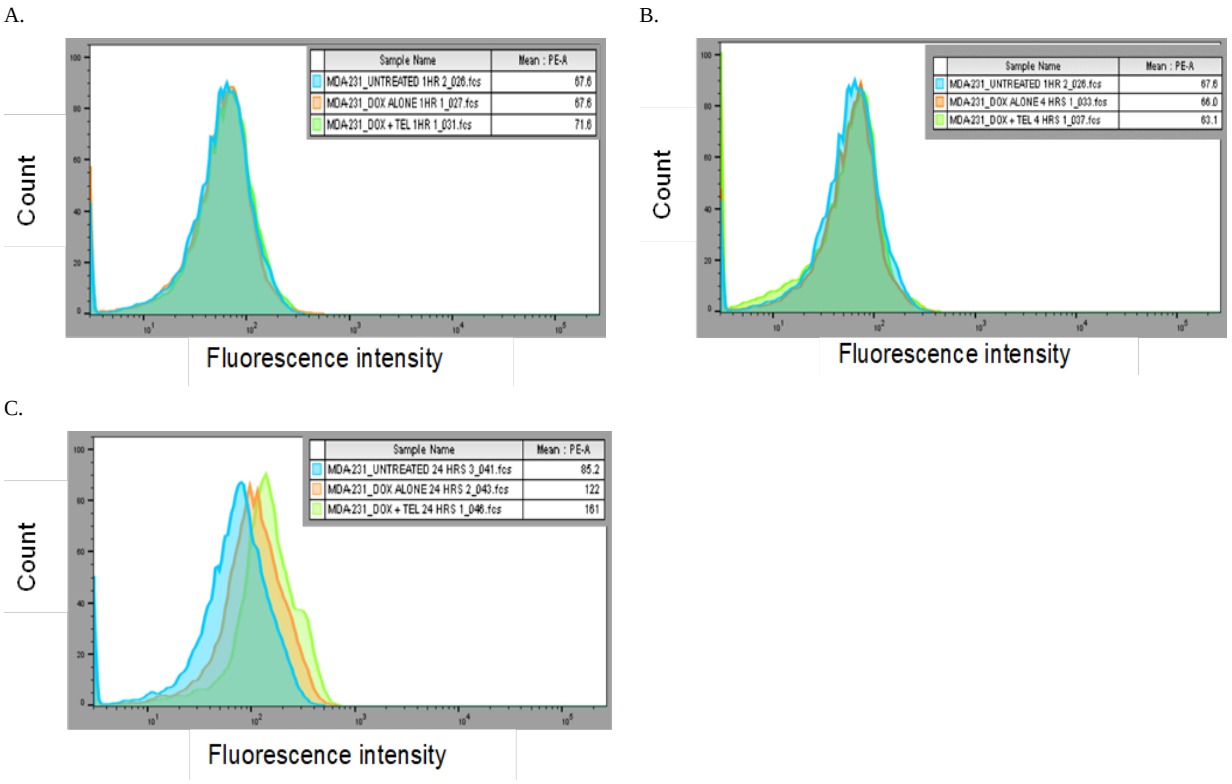

Figure 6. Assessment of cellular uptake of Doxorubicin in combination with Telmisartan. MDA-231 breast cancer cells were treated with 30µg/mL of Telmisartan for 1 hour prior to the treatment with 0.285 µM of Doxorubicin for 1, 4, and 24 hours (A, B, and C respectively) prior to flow cytometry analysis. The results are summarized by measuring the fluorescence intensity relative to that of untreated (control) cells.

4. Discussion

Regardless the huge ongoing research and efforts, breast cancer continues to be a major health problem. The incidence and mortality rates have dramatically increased during the past few years and are expected to worsen in the future. Recently, breast cancer has been recognized as the most common cancer and the leading cause of cancer-related death especially in young women under 45 years-old of age (Anastasiadi et al., 2017; Karzai et al., 2022).

TNBC counts for almost quarter of newly diagnosed breast cancer cases and relatively to other BC subsets TNBC has higher incidence of metastasis to visceral organs such as brain, lungs, and bones, which usually occurs within 5 years. Also, TNBC has higher incidence of recurrence and relapse that is characterized by chemoresistance and aggressive course (Yin et al., 2020). Accordingly, it is unsurprising that TNBC is the most aggressive type of breast cancer.

Due to the aggressive etiology of TNBC, treatment progression is still a challenge. Systemic chemotherapeutic

agent is the cornerstone treatment for TNBC (Omarini et al., 2018).

Doxorubicin is an anthracycline related chemotherapeutic agent, known to be the most effective cytotoxic agent while using it with adjuvant setting to decrease relapse and mortality in TNBC (Shah & Gradishar, 2018). However, chemoresistance is a major concern. Among variety of proposed mechanisms, the overexpression of a multi-drug resistant (MDR) protein called P-gp represents a major cause of chemoresistance (Trédan et al., 2007). P-gp acts as an efflux pump to efflux the chemotherapeutic agents such as Doxorubicin outside the cancerous cells leading to the development of resistance towards chemotherapeutic drugs (Ughachukwu & Unekwe, 2012). In this context, Bao et al. (2011) have shown that the overexpression of P-gp in breast cancer cells prevented the accumulation of Doxorubicin within the nucleus, and thus the intercalation of Doxorubicin with the DNA was hindered. Therefore, molecules aim to inhibition of P-gp efflux mechanisms represents an attractive clinical concept(Bao et al., 2011).

A plethora of studies were conducted to evaluate the efficacy of P-gp inhibitors in combination with chemotherapeutic agents to re-sensitize the cells to many chemotherapeutic agents. For instance, Kopecka et al.(2020) have shown that Tariquidar (a P-gp inhibitor) re-sensitized TNBC cells towards Doxorubicin-induced apoptosis by inhibiting the efflux pump and increase the accumulation then the cytotoxic effect of Doxorubicin(Kopecka et al., 2020). However, most of the P-gp inhibitors candidates have failed in clinical trials where the combination of chemotherapeutic agents with P-gp inhibitors resulted in no improvements but increased toxicity (Carlson et al., 2006).

Herein, we aimed to explore and investigate potential candidates as P-gp inhibitors that may alleviate the chemoresistance against Doxorubicin in TNBC.

Computational docking studies were done to discover a P-gp inhibitors on cultures of MDR cancerous cells and showed that the inhibitors were not just substrates for P-gp and enhanced the accumulation of chemotherapeutic agents but also some of them activate the immune response, eventually, resulting in enhanced cell death (Nanayakkara et al., 2018).

In this study, we found that many compounds have a high fitting value by the docking study, then Telmisartan, Benazepril, and NCS were selected to bioavailability study on TNBC cells. Amongst all compounds tested, Telmisartan was the only compound found to interact with the key amino acid residues within the P-gp binding site which are Leu339, Gln990. Our findings are supported by data from *in silico* studies where two pharmacophore modelling indicated the exceptional P-gp inhibitory effect of Telmisartan with IC_{50} values of 3.9 and 1.2 μM (Chang et al., 2006; Weiss et al., 2010). Also, and in agreement with our findings, Telmisartan was shown to exhibit superior potency compared to Verapamil as a P-gp inhibitor rendering it more clinically relevant P-gp inhibitor (Chang et al., 2006).

In cell culture model, the co-treatment of MDA-231 TNBC cells with Doxorubicin and Telmisartan resulted in a significant reduction in the IC_{50} of Doxorubicin suggesting that Telmisartan successfully sensitized the TNBC cells to Doxorubicin potentially through P-gp inhibition(**Figure 7**). Further studies using flow cytometry analyses demonstrated that the efficacy of Telmisartanis is attributed to enhanced accumulation of Doxorubicin in the Doxorubicin/Telmisartan treated cells compared to untreated cells. While the exact mechanism of synergy was not evaluated during the course of this study, Chang et al. (2006) indicated that Telmisartan did not result in reduction in mRNA expression of P-gp. Rather, the inhibition is thought to be a direct interaction between P-gp and Telmisartan(Chang et al., 2006).

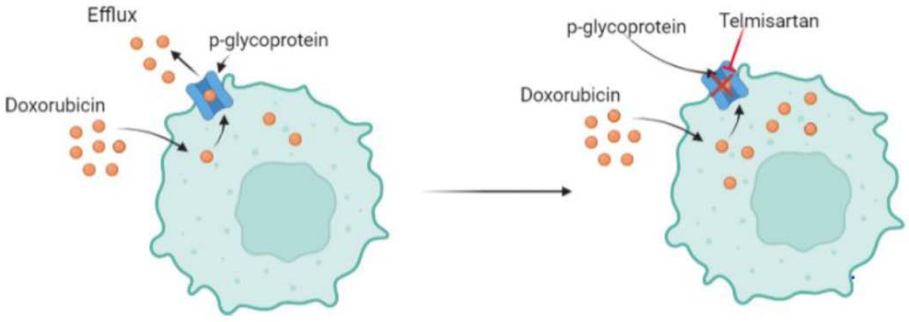

Figure 7. The proposed mechanism of synergy of Doxorubicin and Telmisartan combination therapy.

5. Conclusion

In conclusion, this study shows a potential synergistic effect between Doxorubicin and Telmisartan indicated by a significant reduction in IC_{50} values of combination therapy compared to either agent alone. The mechanism of synergy is proposed to be mediated at least in part by p-glycoprotein inhibition by Telmisartan . This potential mechanism was based on the observation that pre-treatment with Telmisartan resulted in enhanced Doxorubicin accumulation within breast cancer cells. However, more investigation is still needed to elucidate the exact mechanism of synergy.

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