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Adhesion G-protein-coupled receptor (GPCR) and Triple-negative breast cancer (TNBC): A new perspective for the therapeutic effect of GPR116 in TNBC

Indiraleka Muthiah[1]*, Karthikeyan Rajendran[1] & Premnath Dhanaraj[2]

[1]Department of Biotechnology, Mepco Schlenk Engineering College, Sivakasi-626 005, Tamil Nadu, India

[2]Department of Biotechnology, School of Agriculture and Biosciences, Karunya Institute of Technology and Sciences (Deemed to be University), Coimbatore-641 114, Tamil Nadu, India

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Research on finding a novel molecular target for the treatment of triple-negative breast cancer (TNBC) is getting more attention nowadays because of the complex nature of the cancer and issues with the existing therapeutic strategies. G-protein-coupled receptor116 (GPCR116) is an orphan adhesion receptor known for its role in cell invasion and promotion of metastasis. In this study, GPR116 was chosen as the target and the effect of quercetin and doxorubicin on the expression of GPR116 was examined. MDA-MB 231 cells were used to evaluate the effect of quercetin and doxorubicin on cell proliferation, invasion, and apoptosis assays. Expression of GPR116 in mRNA and protein levels were evaluated by qRT-PCR and Western blotting, respectively. Quercetin inhibited MDA-MB 231 cell proliferation similar to the standard drug doxorubicin and a cell cycle analysis study disclosed that quercetin leads to cell death *via* apoptosis and doxorubicin induces more necrotic effect. These ligand candidates inhibit the expression of GPR116 in mRNA and protein levels. qRT-PCR results showed that quercetin efficiently inhibits the expression of GPR116 than doxorubicin and 2-fold variations were observed between quercetin and doxorubicin. Western blotting results also found the selected ligands effectively inhibit the GPRR116 protein. Tran swell migration study showed quercetin inhibits migration effectively compared with untreated cells. Inhibition of cell migration may be due to the downregulation of GPR116 expression in treated cells. Thus, the findings of this experimental study revealed that quercetin and doxorubicin could target the GPR116 in the treatment of TNBC.

Keywords: Doxorubicin, GPR116, G-protein-coupled receptor, MDA-MB 231 cells, Quercetin, Triple-negative breast cancer (TNBC)

Triple-negative breast cancer (TNBC) is described by the absence or lack of expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2)[1]. TNBC accounts for 20% of breast cancer in women[2-4]. TNBC pursues different patterns of metastatic spread compared with other breast cancer subtypes. The lung and brain were the most important sites for metastasis in TNBC-positive patients[5]. Because of hormone receptor-negative, the existing treatment strategy against hormonal and other molecular receptors is ineffective in TNBC[6,7]. For this reason, the treatment of TNBC mostly relies on neoadjuvant therapy like radiotherapy and systemic chemotherapy. Though these methods produce promising results in a few types of cancers; one of the biggest limitations is relapse[8,9]. Relapse usually occurs in TNBC patients after 3~5 years of clinical intervention and then develop resistance to chemotherapy. The number of neoadjuvant chemotherapeutic drugs including anthracyclines and taxanes have shown comparatively better response rates (RR) to the neoadjuvant chemotherapy for TNBC than other breast cancer (BC) subtypes. However, the overall survival (OS) rate is relatively poor[10,11]. Further, radiotherapy is harmful since it can induce carcinogenesis. Thus, finding common druggable targets is a critical demand in TNBC treatment[12].

Plant phytoconstituents generally possess the properties of anti-microbial, antioxidant, anti-inflammatory, and anti-cancer activity. Quercetin is a dietary phytochemical widely present in apples, berries, green tea and onions[13,14]. The anti-cancer research on phytochemicals is constantly increasing because these natural products show advantages including no or less effect on normal cells and delayed cancer relapse [15,16]. However, the anti-cancer mechanisms of quercetin on TNBC are poorly understood[17].

*Correspondence:
Phone: +91-7402389414 (Mob)
E-mail: indirajith1812000@gmail.com

G-protein-coupled receptors (GPCRs) are transmembrane proteins known for their role in the regulation of numerous physiological processes by transducing signals from a diverse range of ligands. Abnormal activation of GPCRs interferes with different phases from stages I to IV of cancer development, as well as several cancer-associated signaling pathways. However, the clinical utility of GPCRs as molecular targets in cancer therapy is still poorly explored[18-20]. The genomic profile of TNBC showed that orphan adhesion receptor GPR116 is over-expressed specifically in TNBC. GPR116 promotes BC cell migration and invasion *via* $G_{\alpha q}$ signaling and p63RhoGEF ($aG_{\alpha q}$ effector) mediated activation of the RhoA and Rac1. Knockdown of GPR116 in human metastatic TNBC cell lines showed reduced cell proliferation and cell migration[21].

GPR116 adhesion sub-type differs from other GPCRs by their long N-terminal region with several structural domains and this facilitates cell and extracellular matrix (ECM) interactions. GPR116 is an orphan receptor, no known ligands are available. Since the adhesion with the ligands and migration of cells are the inherent properties of the adhesion molecules such as integrins, cadherins, selectins, *etc*.[22,23], it is reasonable to consider adhesion GPCRs as a molecular target for treatment. GPR116 activates Rho family GTPases, including RhoA, Rac1, and Cdc42 and plays an essential role(s) in cancer cell migration[24,25]. Activated Rho GTPases provoke multiple downstream signaling pathways during cancer cell migration, such as ROCK1/2 signaling[26]. Therefore, this research intends to evaluate the anti-cancer effect of quercetin and doxorubicin on TNBC and the impact of this drug on GPR116 expression. Our results suggest that quercetin effectively inhibits the expression of GPR116, thus it reduces the cell migration in the metastatic TNBC cell line. Hence, GPR116 could be used as a potential target for cancer metastasis.

Materials and Methods

Cell culture

MDA-MB-231 (human TNBC cells) cell line was purchased from American Type Culture Collection (ATCC) and it was cultured in liquid medium [Dulbecco's Modified Eagle Medium (DMEM)) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and incubated under an atmosphere of 5% CO_2 at 37°C. Cells were sub-cultured periodically and monitored frequently to identify phenotypic change and contamination[27,28].

Cell viability assay and IC_{50} determination

Quercetin and doxorubicin were tested for *in vitro* cytotoxicity, using MDA-MB-231 cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay[29-31]. The cells were harvested at 90% confluent stage using the trypsinization method and then the cells were transferred to a 15 mL centrifuge tube and centrifuged at 3,000 rpm for 10 min. Then, the supernatant was discarded, and the cell pellet was resuspended in a DMEM medium containing 10% FBS and 1% antibiotic solution. The cells were seeded in a 96-well tissue culture plate at a density of 1×10^5 cells/mL (200 µL/well), then the plate was incubated for 24-48 h at 37°C. Then the medium was removed, and the cells were washed with sterile phosphate-buffered saline (PBS) and varying concentrations of quercetin and doxorubicin were added to a serum-free DMEM medium and incubated at 37°C in a humidified 5% CO_2 incubator for 24 h. Then, MTT (20 µL of 5 mg/mL) was added to each well and the plate was incubated for a further 2-4 h until purple precipitates formed. Then MTT was aspirated off along with the medium without disturbing the formazan crystals. Then, the formazan crystals were dissolved with 100 µL of dimethyl sulfoxide (DMSO) and absorbance was measured at 570 nm using a microplate reader (Thermo Fisher Scientific, USA), and the percentage cell viability and IC_{50} values were determined (as below).

% Cytotoxicity = (1–absorbance of an experimental well) / absorbance of negative control well × 100

Annexin V / propidium iodide (PI) staining for apoptosis assay

Quercetin and doxorubicin were tested for apoptosis analysis, using MDA-MB-231 cells by Annexin V and propidium iodide (PI) staining methods[32,33]. Briefly, the cells were harvested and seeded in DMEM medium containing 10% FBS and 1% antibiotic solution at a density of 1×10^6 cells/mL in a 6-well tissue culture plate and incubated for 24-48 h at 37°C. After incubation, the wells were washed with sterile PBS and treated with 18.28 µM of doxorubicin and 20.76 µM of quercetin in a serum-free DMEM medium and incubated at 37°C in 5% CO_2 incubator for 72 h. Then, the cells were trypsinized and centrifuged at 1500 rpm for 5 min. The cell pellet was washed with PBS and resuspended in 1X annexin-binding buffer. 5 µL Alexa FluorR 488 annexin V and

1 µL 100 µM PI solution were added to each 100 µL of cell suspension. The cells were incubated for 15 min at room temperature. Then, 400 µL 1X annexin-binding buffer was added and mixed gently, and the samples were kept on ice. Finally, flow cytometry was performed, and the stained cells were analyzed by measuring the fluorescence emission at 530 nm and 575 nm (or equivalent) using 488 nm excitation[34,35].

Transwell migration and cell invasion assay

The effect of quercetin and doxorubicin on MDA-MB-231 cell invasion was assessed *in vitro* using Transwell chambers (8 µM pore size, Corning, USA)[36,37]. Briefly, cells were resuspended in migration buffer containing serum-free DMEM medium, treated with 18.28 µM of doxorubicin and 20.76 µM of quercetin, and plated at a density of 1×10^6 cells/mL on the upper chamber of the transwell plate. In the cell invasion assay liquefied Matrigel was added to the upper chamber and then cell suspension was added to this Matrigel ECM. DMEM containing 10% FBS in the lower chamber served as the chemoattractant. Tans well chamber is incubated at 37°C and the atmosphere contains 5% CO_2 for 24 h. After the incubation period, non-invasive cells present in the upper chamber were removed using a cotton swab and the invasive cells present in the lower chamber were fixed with 75% ethanol and then stained with 0.5% crystal violet and images were captured.

Analysis of gene expression using quantitative real-time PCR (qRT-PCR)

Quercetin and doxorubicin were tested for GPR116 gene expression, using MDA-MB-231 cells. The cells were cultured in DMEM medium containing 10% FBS and 1% antibiotic solution and incubated at 37°C for 24 h. After the incubation period, the cells were washed with sterile PBS and treated with 18.28 µM of doxorubicin and 20.76 µM of quercetin in a serum-free DMEM medium and incubated at 37°C in a humidified 5% CO_2 incubator for 24 h. After incubation, the cells were harvested, and total RNA was isolated using the TRIZOL method[38]. The MDA-MB-231 cells were centrifuged at 5000 rpm for 10 min using diethylpyrocarbonate (DEPC)-treated centrifuge tubes and finally, the cell pellet was isolated. TRIZOL (700 µL) was added to the cell pellet (1X 10^7 cells) for cell lysis. The lysate was collected and 300 µL of chloroform was added and mixed well for 5 min at room temperature. Then the mixture was centrifuged at 12,000 rpm for 20 min at 4°C and the aqueous layer was collected in a separate fresh 1.5 mL tube. 700 µL of isopropanol was added to the aqueous layer to precipitate the RNA and it was pelleted by centrifugation at 12000 rpm for 20 min at 4°C. The pellet was washed with 70% ethanol and allowed to air dry. The dried RNA pellet was dissolved into 30 µL double distilled autoclaved water and stored at -80°C till its next use. The quantity and quality of the isolated RNA were evaluated by Labman UV- Vis Spectrometer and resolved in 1.5% agarose gel, respectively. DNA contamination, if any seen with RNA preparation, was separated by the DNase treatment and the reaction volume was set up to 20 µL containing 1U of DNase. Then, the sample was incubated for 30-45 min at 37°C then 2 µL of 20 µM ethylene glycol tetra-acetic acid (EGTA) was added and further incubated at 66°C for 10 min. Sodium acetate (1/10 V) and absolute ethanol (2 V) were added and incubated at -20°C for 60 min. Then, it was centrifuged at 12000 rpm for 20 min at 4°C, and the pellet was isolated and washed with 500 µL of 75% ethanol. Then, it was kept air dry and dissolved in 20 µL of Double autoclaved milli-Q grade water and stored till further process.

1.5 µg of total RNA was converted to cDNA by adding M-MLV Reverse transcriptase. The cDNA synthesis was performed at 25°C for 10 min followed by 37°C for 120 min. cDNA & RNA hybrid denatured at 85°C for 2 min. The prepared cDNA was used as a template to detect metastasis. Quantitative Real-Time-PCR (qRT-PCR) by the Power Syber Green kit (Applied Biosystems, USA) in ABI StepOne Plus (Applied Biosystems, USA)[39]. The expression levels of the selected genes were assessed by qRT-PCR using the relative quantification ($2^{-\Delta\Delta CT}$) method. Expression was normalized using the endogenous control (β-actin) and clinically control cells were used as the calibrator[40].

The PCR condition was:

The initial melting temperature was 94°C for 5 min, followed by 40 cycles of 94°C for 30 sec Annealing at 52°C or 20 sec, and the extension temperature was 72°C for the 30 sec. The real-time data was captured at the end of each extension stage, followed by melting curve analysis that was performed as per the default temperature profile of the thermal cycler.

The Primers used were:

Human GPR116: Forward- 5′-CCGCTCGAGT GGACAGGCCAACCAACTC-3′, Reverse-5′-ATA AGAATGCGGCCCGGAGGTCACGTAGGTTGG -3′

Human β-actin: Forward- 5′ G ATG GAT GAT GAT ATC GCC GCG 3′, Reverse- 5′ G CTA AGG TGT GCA CTT TTA TTC AAC 3′

GPR116 expression analysis by Western blotting

MDA-MB 231 cells were treated with doxorubicin and quercetin and incubated for 48 h. Then, the cells were harvested using the trypsinization method and washed with sterile PBS. Then ice-cold lysis buffer consisting of 150 mM sodium chloride, 1% Triton X-100, 50 mM Tris pH 8.0 was added for cell lysis. Then the cells were homogenized by brief sonification at 4°C and centrifuged at 10,000 g for 2 min at 4°C. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to resolve the protein and then blotted on the nitrocellulose membrane using the electro-transfer method. Then, the membranes were blocked with TNT buffer [*i.e.*, 10 mM Tris (pH 8.0), 150 mM NaCl, 0.05% Tween 20] containing 3% skimmed milk powder and incubated for 30 min at room temperature. The membrane was washed thrice each 7 min with TNT buffer. Then, the membrane was incubated with the primary antibodies at 4°C overnight. After that, the membranes were washed with TNT buffer and incubated with alkaline phosphatase (AP)-labeled goat anti-rabbit secondary antibody (1:5,000) for 1 h. Then, the membrane was washed and incubated with 5-bromo-4-chloro-indolyl phosphate (BCIP) substrate for 15-20 min and finally, blue colored band was observed on the membrane[41,42].

Results

Evaluation of cytotoxic effect using MTT test

The MTT assay is an *in vitro* assay for the evaluation of the cytotoxic effect of drugs. Herein, the anti-proliferative effect of quercetin was evaluated on the MDA-MB-231 cell line. The results were compared with the standard drug doxorubicin and untreated cells. MDA-MB-231 cells were treated with varying concentrations of quercetin and doxorubicin (0.5, 1, 5, 10, 15, 20, 10, 60, 80, and 100 µM). No difference was found between untreated cells and those treated with 0.5 µM quercetin and doxorubicin, but a significant difference in cell proliferation was observed from the concentration of 1 µM and greater (Fig. 1). Quercetin showed a good cytotoxic effect similar to the standard drug doxorubicin. The IC_{50} value was found to be 18.28 µM and 20.76 µM for doxorubicin and quercetin, respectively. Though quercetin showed a high IC_{50} value, the dose-dependent anti-proliferative effect was better than doxorubicin beyond 20.76 µM.

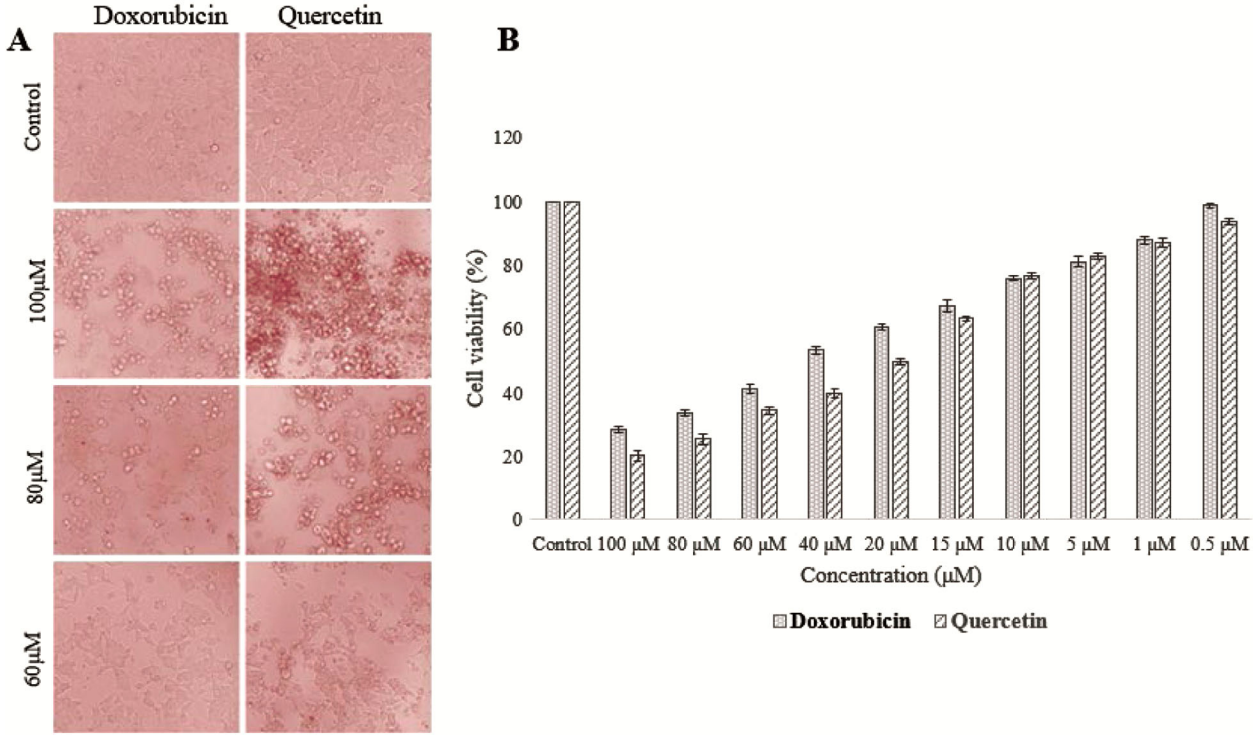
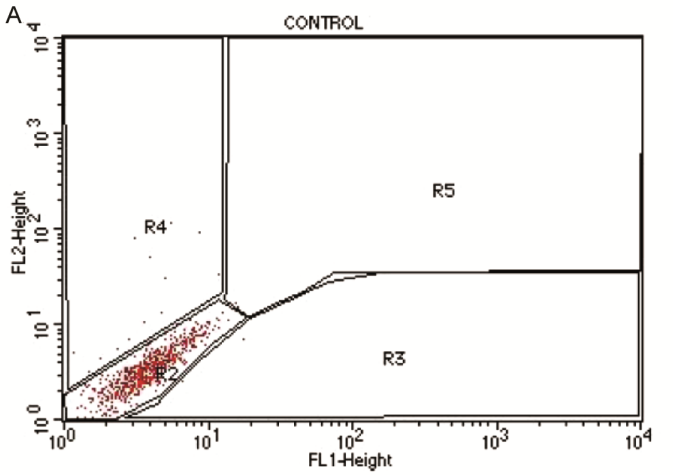


Fig. 1 — Cytotoxic effect of doxorubicin and quercetin. (A) Effect of doxorubicin and quercetin on the proliferation of MDA-MB-231 cells; and (B) Dose-response curve exhibiting linear behavior in the concentration range from 0.5 µM to 100 µM

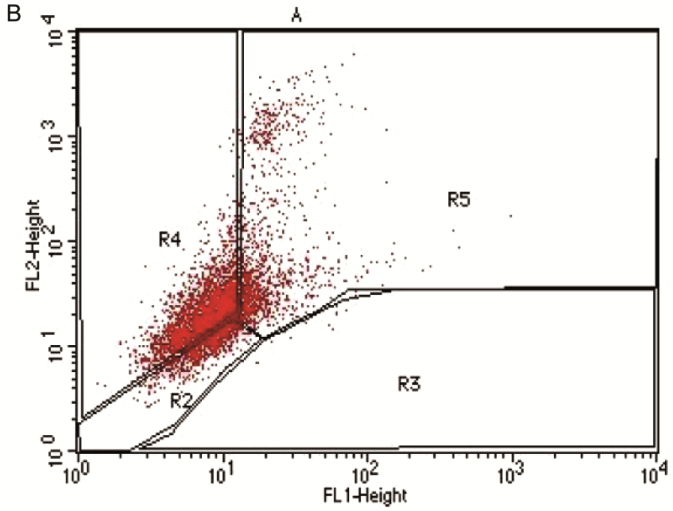
Quercetin induces apoptosis of MDA-MB-231 cells

In this study, the apoptotic effect of quercetin and doxorubicin on MDA-MB-231 cells was evaluated and the results were compared with untreated cells. Results showed that the number of apoptotic cells was significantly higher in the quercetin group (58.96%) than in doxorubicin treated (21.82%) and control group (0.6%) (Figs 2 and 3). The percentage of the necrotic cell population (50.78%) was found to be significantly higher in doxorubicin-treated cells than



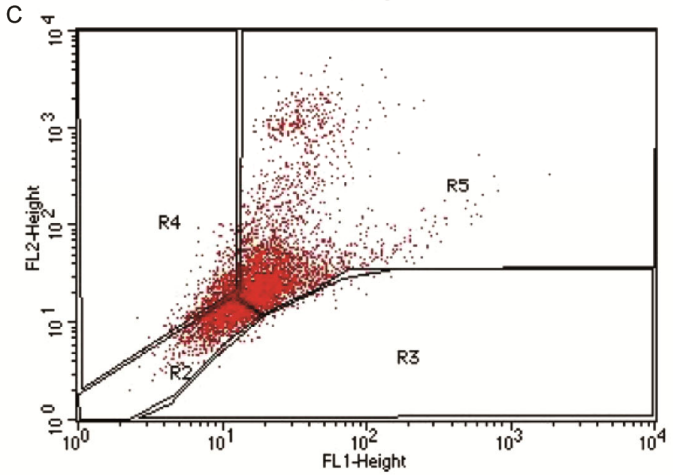
Gate: G1

Quad	Events	% Gated	% Total
LL	636	96.8	57.3
LR	2	0.3	0.18
UL	13	1.98	1.17
UR	2	0.3	0.18



Gate: G1

Quad	Events	% Gated	% Total
LL	967	17.43	9.67
LR	13	0.23	0.13
UL	2829	50.98	28.29
UR	1198	21.59	11.98



Gate: G1

Quad	Events	% Gated	% Total
LL	1530	27.05	15.3
LR	59	1.04	0.59
UL	438	7.74	4.38
UR	3276	57.92	32.76

Fig. 2 — Apoptosis analysis in quercetin and doxorubicin treated cells and comparison with control group. (A) Control; (B) Doxorubicin; and (C) Quercetin

in quercetin-treated cells (7.74%). This shows that doxorubicin inhibits cell proliferation preferably by inducing necrosis. These results showed that quercetin has apoptotic effects against human MDA-MB-231 cells and the efficiency was higher than doxorubicin.

Cell invasion assay

Tumor cell dissemination from the primary tumor to different organs is an essential event of metastasis. The cancer cell invasive ability with or without anti-cancer drug treatment was examined by membrane transwell inhibition assay. Transwell invasion assay showed that doxorubicin and quercetin significantly weakened the invasion capacity of MDA-MB231 cells (Fig. 4). These results suggest that doxorubicin and quercetin played important roles in inhibiting the migration and invasion potential of human TNBC cells.

GPR116 expression was decreased in TNBC at the mRNA and protein level

GPR116 is an orphan receptor known well for its important role in cancer cell metastasis *via* the Gαq-p63 RhoGEF-Rho GTPase pathway. GPR116 is an adhesion receptor, it promotes metastasis by enhancing cell adhesion and migration. Knockdown of GPR116 in highly metastatic TNBC cells (MDA-MB 231) was found to reduce cell dissemination and invasion[22]. The cell invasion assay found that both quercetin and doxorubicin inhibit the migration of cells and reduce invasion. Hence, the impact of quercetin and doxorubicin on the expression of GPR116 using qRT-PCR was performed (Fig 5). Relative quantification results found that both doxorubicin and quercetin down-regulate the expression of GPR116. Inhibition of GPR116 expression by quercetin is relatively 2-fold higher than in doxorubicin treated cells (Fig. 5A & 5B). Electrophoresis of the amplified product showed that the expression of GPR116 was relatively lower than the doxorubicin treated and untreated cells (Fig. 5C).

Western blotting was done to analyze the expression of GPR116 in after the treatment with the selected ligand candidates. Protein level expression of GPR116 was reduced much after the treatment with quercetin (Fig. 5D). Doxorubicin also showed a significant difference in the reduction of GPR116 expression than the control.

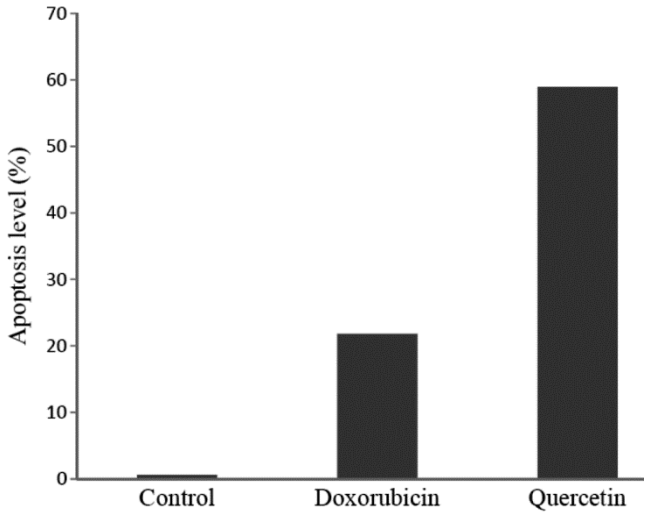


Fig. 3 — Flow cytometry analysis comparing apoptosis levels of quercetin and doxorubicin treated cells with untreated cells (control)

Discussion

Hormone therapy and immunotherapy are ineffective in TNBC because ER, PgR, and HER2 are not expressed. So, the treatment options are very narrow and it is mostly restricted to chemotherapy. There are several chemotherapeutic drugs available for the treatment of TNBC; however, important disadvantages of many chemotherapeutic agents are: that they cause drug resistance; lack specificity and could affect normal fast-dividing cells[43]. Doxorubicin is widely used for chemotherapy because it inhibits rapidly proliferating cancer cells by targeting topoisomerase II. However long-term anti-cancer drug treatment causes acquired drug resistance. Resistant cells showed decreased doxorubicin

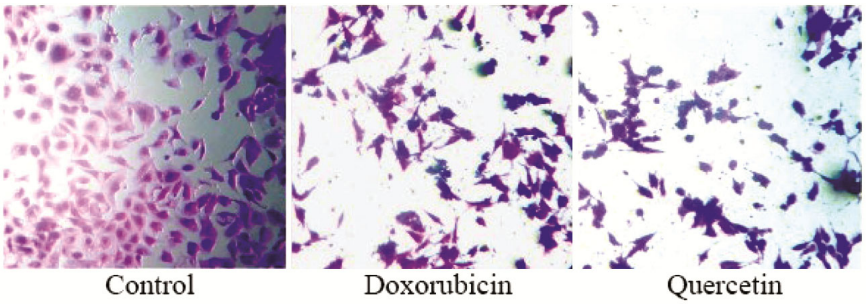


Fig. 4 — Quercetin and doxorubicin inhibit the migration of MDA-MB 231 cells after 24 h treatment

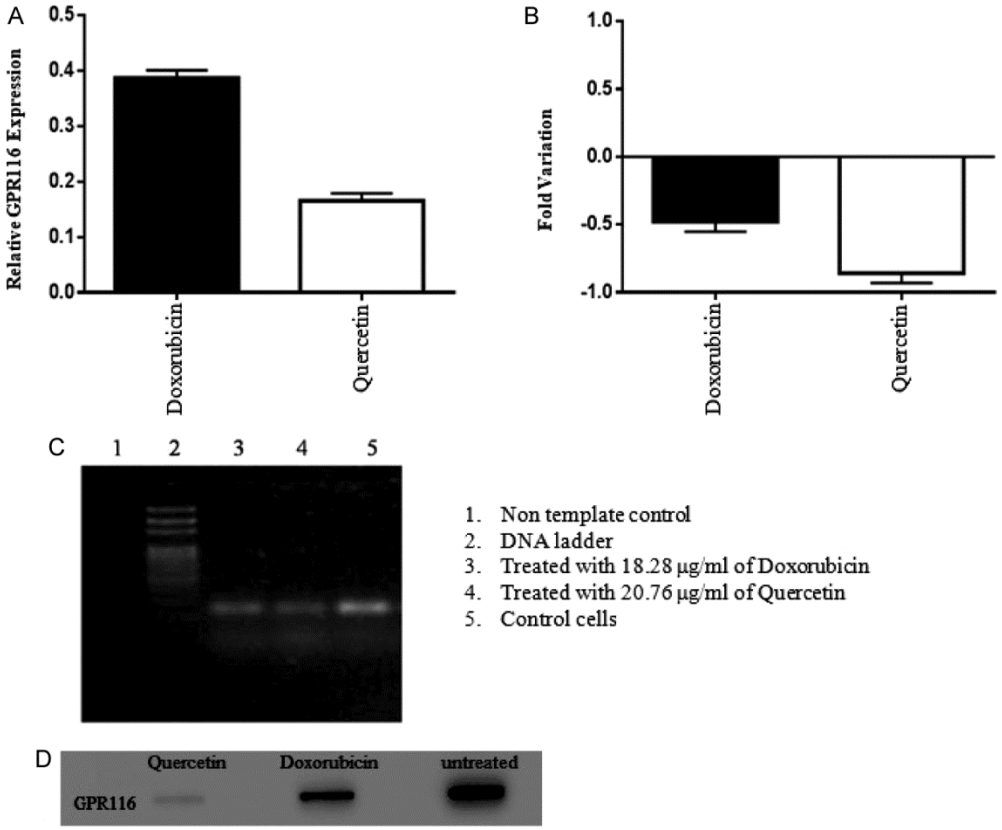


Fig. 5 — G-protein-coupled receptor 116 (GPR116) expression was decreased in triple-negative breast cancer (TNBC) cells at the mRNA and protein levels

sensitivity, decreased doubling time, and a high doxorubicin-efflux rate[44]. A natural compound can overcome the side effects caused by synthetic drugs and only affect cancer cells without harming normal cells. Hence, a natural drug having equivalent efficiency with standard FDA-approved drugs could be a good alternative. Concerning this, the current study provides evidence that quercetin also has an equivalent cytotoxic effect similar to doxorubicin. The dose-response study showed that quercetin responds better than doxorubicin (Fig. 1).

Most anti-cancer drugs trigger cancer cell death by inducing apoptosis. Quercetin was also known to induce apoptosis in different cancers[32,45]. Till now, only a few studies have been carried out about the anti-cancer and apoptotic effect of quercetin on TNBC. Our current research also proves that quercetin inhibits cell proliferation through inducing apoptosis. Efficiency was two-fold higher than doxorubicin (Figs 2 and 3). From the morphological analysis, it was observed that the cell morphology of doxorubicin and quercetin-treated MDA-MB-231 cells began to change with 20 µM. Significant morphological changes and more apoptotic cells were observed in quercetin-treated cells than in doxorubicin-treated cells. However, the finding of molecular targets to predict response to specific chemotherapy is essential for further improvement of treatment strategy.

Previous studies have shown that quercetin mediated the apoptosis in TNBC through modulation or downregulation of protein expressions such as FAS receptor, p53, Bcl-2, FoXo3a, lipogenic enzyme (fatty acid synthase), and ß-catenin. But till now no research has been conducted on the interaction of chemotherapeutic drugs with GPCR of TNBC cells. Though these GPCRs are not functionally mutated in cancer, they regulate important cellular events like growth/proliferation, metabolism, death/apoptosis, ion and nutrient transport, and migration[19]. One such GPCR is GPR116, an orphan adhesion GPCR indeed involved in cell-cell adhesion and migration, thus it promotes metastasis. GPR116 promotes cell motility through activation of the Gαq-p63RhoGEF-RhoA/Rac1 pathway. A previous study has shown that the knockdown of GPR116 in MDA-MB-231

results in a significant decrease in cell migration and invasion[20]. Hence, our current research studied the effect of quercetin and doxorubicin on the expression of GPR116 by qRT-PCR. Expression of GPR116 at mRNA and protein levels was highly reduced in quercetin-treated cells than in doxorubicin-treated cells (Fig. 5). A 2-fold reduced expression protein was observed in quercetin-treated cells. The correlation of qRT-PCR results with cytotoxicity assay and cell invasion assay confirmed that there is a connection between the reduction of GPR116 expression and reduced cell invasion and cell death. Hence, quercetin showed more efficient inhibition in cell invasion and cancer cell death than doxorubicin.

Conclusion

This study demonstrates that both quercetin and doxorubicin inhibit the expression of GPR116 in triple-negative breast cancer cell line MDA-MB-231. Results showed that quercetin and doxorubicin effectively reduce cancer cell proliferation and invasion of cells. However, the effect of quercetin was significantly higher than doxorubicin. We therefore conclude that quercetin and doxorubicin exhibit a tumor-suppressor function *via* suppressing proliferation, and invasions, and promoting apoptosis by targeting GPR116. These experimental (pre-clinical) findings may bring a novel approach in clinical settings for targeting GPR116 interaction as new therapeutic applications for triple negative breast cancer patients.

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Conflict of interest

All authors declare no conflicts of interest.

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