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7-*O*-geranylquercetin contributes to reverse P-gp-mediated adriamycin resistance in breast cancer

Enxia Zhang[a,1], Jiasi Liu[a,1], Lei Shi[a], Xin Guo[a], Ze Liang[a], Jiaxin Zuo[a], Hong Xu[b], Huaxin Wang[b], Xiaohong Shu[a], Shanshan Huang[a], Shubiao Zhang[c], Xiaohui Kang[a,d,**], Yuhong Zhen[a,*]

[a] College of Pharmacy, Dalian Medical University, Dalian, 116044, China
[b] College of Basic Medical Sciences, Dalian Medical University, Dalian, 116044, China
[c] Key Laboratory of Biotechnology and Bioresources Utilization of Ministry of Education, Dalian Minzu University, Dalian, 116600, China
[d] State Key Laboratory of Fine Chemicals, Dalian University of Technology, Dalian, 116024, China

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ABSTRACT

Aims: To investigate the effect of 7-*O*-geranylquercetin (GQ), a derivative of quercetin (Q), on reversing drug resistance in breast cancer MCF-7/ADR cells and reveal the mechanisms related to P-glycoprotein (P-gp).
Main methods: Cell viability was determined by MTT assay. Accumulation of adriamycin (ADR) in cells was determined by confocal fluorescence microscope and microplate reader while that of rhodamine (Rh) was measured by flow cytometry. Expression levels of P-gp and MDR1 gene in cells were detected by western blot and Real-Time PCR, respectively. Molecular docking of GQ and Q with P-gp was conducted using AutoDock program. Xenograft model was established by inoculating MCF-7/ADR cells in BALB/c-nude mice. Tumor bearing mice were administered with ADR via tail vein injection and/or GQ (Q) by gavage. Expression levels of P-gp in tissues were detected by western blot and immunohistochemistry.
Key findings: GQ could reverse drug resistance of MCF-7/ADR cells to ADR. GQ inhibited the efflux of ADR by down-regulating the expression of P-gp protein and its encoding gene MDR1 in MCF-7/ADR cells. Molecular modeling showed that GQ matched with P-gp better than Q. GQ enhanced the antitumor effects of ADR and decreased the expression of P-gp in mice and its activities were higher than that of Q. GQ could reverse drug resistance of MCF-7/ADR cells by down-regulating the expression of P-gp in vitro and in vivo.
Significances: The reversal effect of GQ on P-gp-mediated drug resistance indicates its potential as a reversal agent for drug resistance in cancer chemotherapy.

1. Introduction

The efficiency of tumor chemotherapy has been gravely attenuated by the emergence of multidrug resistance (MDR) cancer cells [1,2]. P-glycoprotein (P-gp) is one of the most studied transporters that have been found to be associated with MDR. The over-expression of P-gp in tumor cells often leads to chemotherapy failure [3,4]. Therefore, a series of P-gp inhibitors have been developed to reverse MDR. The first generation inhibitors were not specifically designed for inhibiting MDR and had other pharmacological activities, for example verapamil and cyclosporine A. Because of their low affinity to P-gp, the first generation inhibitors were usually administrated at high doses to reverse MDR in vivo, which led to serious and life-threatening toxicities to the body [4]. The second generation inhibitors including valspodar and biricodar were designed to eliminate non-MDR reverse effects of the first generation. But their affinity towards P-gp was still too low to produce significant inhibition in vivo at tolerable doses [5,6]. Meanwhile, the first and second generation inhibitors interfered with CYP enzymes and affected other transporters to cause drug-drug interactions, resulting in increased concentration levels of the antitumor drugs in the plasma [7]. For instance, in phase I study of co-administration of P-gp inhibitor PSC833 with antitumor drug paclitaxel, PSC 833 decreased paclitaxel clearance by 50%, which could be detrimental to normal tissue [8,9]. The third generation of P-gp inhibitors exhibited an increased specificity to P-gp at concentrations of nanomolar range, and avoided effects on drug metabolic enzymes [6]. However, the phase III clinical trials of

* Corresponding author. College of Pharmacy, Dalian Medical University, No.9 Lvshun Southern Road, Lvshunkou District, Dalian, China.
** Corresponding author. College of Pharmacy, Dalian Medical University, Dalian, 116044, China.
E-mail addresses: kangxh@dmu.edu.cn (X. Kang), zhenyhwaner@aliyun.com (Y. Zhen).
[1] These authors contributed equally to this work.

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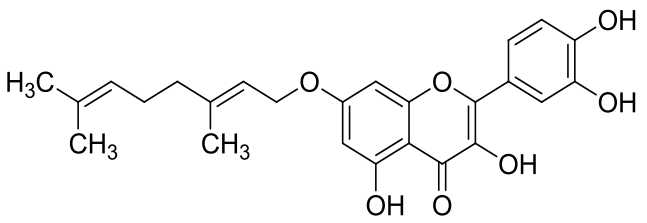

Fig. 1. Structure of GQ.

some third generation inhibitors, such as tariquidar and zosuquidar, failed due to their toxicity and lack of reversing MDR activities [10]. The current dilemma stressed the requirement to develop new MDR reversal agents.

Quercetin (Q) is a flavonoid existing in many edible fruits and vegetables such as tea, grape, tomato and apple. Q has multiple bioactivities including antioxidant, antiinflammatory, anticancer, antiviral activities [10]. Meanwhile, it can inhibit the expression of P-gp and its coding gene MDR1, and therefore act as a chemo-sensitizer to enhance the cytotoxic effects of chemotherapeutic drugs on MDR cells [11]. Nevertheless, the poor solubility severely restricted its application in clinical setting [12,13]. 7-*O*-Geranylquercetin (GQ) (Fig. 1) designed and synthesized by our group is a novel alkylated derivative of Q with better lipid solubility and higher antitumor activity in comparison with Q [14]. We have found that the accumulation of GQ in MCF-7 cells was much higher than that of Q [14], which might be the result of inhibition to efflux transporters [15]. However, whether it can reverse the drug resistance of MDR tumor cells still need to be proved.

In this study, we investigated the reversal effect of GQ on the drug resistance of breast cancer cells MCF-7/ADR and explored the possible mechanisms related to P-gp. Our study will provide reference for the research of high-efficient and low-toxic reversal agents against drug resistance in tumor chemotherapy.

2. Materials and methods

2.1. Reagents

MDR1 siRNA and PCR primers were designed and synthesized by GenePharma (Shanghai, China). GQ with the purity over 98% was synthesized in our lab according the reported methods [16]. Q was purchased from the Aladdin Industrial Corporation (Shanghai, China). ADR was purchased from Haizheng Pharmaceutical Co., Ltd (Zhejiang, China). RPMI-1640 and fetal bovine serum (FBS) were purchased from Gibco BRL (Gaithersburg, MD, USA). Rhodamine 123 (Rh) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Corporation (St. Louis, MO, USA). Hoechst was purchased from Solarbio (Beijing, China). BeyoECL Plus kit, phenylmethanesulfonyl fluoride (PMSF), penicillin, streptomycin and haematoxylin were purchased from Beyotime Institute of Biotechnology (Haimen, China). Lipofectamine 2000 was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). TransZol Up, EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix and TransStart Top Green qPCR SuperMix Kit were purchased from TransGen Biotech (Beijin, China). Antibody against P-gp was purchased from Beijing Bioss Biotechnology Co. Ltd (Beijin, China). β-actin, horseradish peroxidase-conjugated secondary antibody were purchased from Proteintech Group, Inc (Wuhan, China). SPlink detection kit was purchased from Zsbio Biotechnology Co., Ltd (Beijin, China). All other chemicals were of the highest purity available.

2.2. Cell line and cell culture

Human breast cancer cell line MCF-7 was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), drug resistant cell line MCF-7/ADR was obtained from School of Life Science and Biology, Dalian University of Technology (Dalian, China). All cells were cultured in the RPMI-1640 medium containing 10% FBS and 1% penicillin-streptomycin at 37 °C under a humidified atmosphere of 5% CO_2.

2.3. Animals

Female BALB/c-nude mice (4–6 weeks old) were provided by the Model Animal Research Center of Nanjing University, China (animal certificate SCXK: 2018-0008) and fed under a specific pathogen-free (SPF) condition. All the experiments were performed according to the Experimental Animal Management Law of China and approved by the Animal Ethics Committee of Dalian Medical University.

2.4. Cell viability assay

Cell viability was determined by MTT assay. GQ and Q were dissolved in dimethyl sulfoxide (DMSO) and diluted to the desired concentrations, and the concentration of DMSO was kept below 0.1% in treatment groups. MCF-7 and MCF-7/ADR cells (1.0×10^4 per well) were seeded in 96-well plates and cultured at 37 °C under 5% CO_2 for 24 h. Then the cells were treated with Q (10 μM) or various concentrations of GQ (2.5, 5, 10 and 20 μM) for 48 h using DMSO (0.1%) as a blank control. Cells were cultured with MTT solution (100 μL, 0.5 mg/mL) for 4 h at 37 °C and the insoluble formazan crystals were dissolved in DMSO (200 μL per well). Absorbance was measured using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 570 nm [16]. Subsequently, MCF-7 and MCF-7/ADR cells were treated with ADR (172, 86, 43, 21.5, 10.75, 5.33, 2.67, 1.33, 0.67 and 0 μM), ADR combining with various concentrations of GQ (2.5, 5 and 10 μM) or Q (10 μM) for 12, 24 and 48 h, respectively. Cell viability was measured by MTT assay and the half inhibition concentration (IC_{50}) values of ADR to MCF-7 and MCF-7/ADR cells were calculated.

2.5. Transfection of MDR1 siRNA

MDR1 is the encoding gene of P-gp. P-gp-silenced MCF-7/ADR (MCF-7/ADR-siRNA) cells were built by transfecting MDR1 siRNA into MCF-7/ADR cells. The sequences of MDR1 siRNA were as follows: sense, 5′-GACCAGGUAUGCCUAUUAUTT-3'; antisense, 5′-AUAAUAGG CAUACCUGGUCTT-3'. MCF-7/ADR cells (3×10^5 per well) were seeded in 6-well plates and cultured at 37 °C for 24 h until cell density reached 80–90%. MDR1 siRNA (1.5 μg) was mixed with lipofectamine 2000 at a weight ratio of 1:3. The complex with a volume of 500 μL was transfected into MCF-7/ADR cells at 37 °C for 6 h. Then, the MCF-7/ADR-siRNA cells were cultured with serum-free RPMI-1640 medium (2 mL per well) overnight and subjected to subsequent treatments.

2.6. Fluorescence microscopy assay for ADR uptake in cells

Uptake of ADR in MCF-7, MCF-7/ADR and MCF-7/ADR-siRNA cells was detected by confocal fluorescence microscope. MCF-7, MCF-7/ADR and MCF-7/ADR-siRNA cells (4×10^5 cells per well) were seeded in glass bottom culture dishes for about 24 h and then treated with ADR (5 μM) combining with various concentrations of GQ (2.5, 5 and 10 μM) or Q (10 μM) for 24 h. DMSO (0.1%) was used as a blank control. After being washed with PBS, the cells were stained with Hoechst (500 μL/per) for 30 min at room temperature. The cells were washed three times with PBS and then viewed and imaged by confocal fluorescence microscope (Olympus, Hataya, Japan).

2.7. Microplate reader assay for ADR accumulation in cells

Accumulation of ADR in MCF-7/ADR cells was quantitatively detected using a microplate reader. ADR was dissolved in 3 M ethanol

hydrochloride solution to the concentrations of 0, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 μM, respectively. The fluorescence intensity of the solutions was detected by a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) with an excitation wavelength of 530 nm and an emission wavelength of 570 nm. The standard curve was prepared, the linear regression equation and correlation coefficient were calculated. MCF-7/ADR cells (4×10^5 cells per well) were seeded in 6-well plates and cultured for 24 h and then treated with ADR (5 μM) combining with various concentrations of GQ (2.5, 5 and 10 μM) or Q (10 μM) for 24 h. DMSO (0.1%) was used as a blank control. After that, the cells were trypsinized and centrifuged at 1200 g for 5 min. Then, the cells were re-suspended in 3 M ethanol hydrochloride solution and kept overnight. The fluorescence intensity of ADR was measured by a microplate reader as described above and the concentration was calculated according to the standard curve.

2.8. Flow cytometry assay for Rh accumulation in cells

Flow cytometry was performed to detect Rh accumulation in MCF-7/ADR cells. MCF-7/ADR cells (4×10^5 cells per well) were cultured in 6-well plates for 24 h and treated with various concentrations of GQ (2.5, 5 and 10 μM) or Q (10 μM) for 24 h. After incubation with Rh (5 μM) for 1 h in the dark, the cells were trypsinized, centrifuged at 1200 g for 5 min and then re-suspended in PBS (1 mL). The intracellular fluorescence intensity was analyzed using FACS-Calibur flow cytometer (BD, Franklin, NJ, USA).

2.9. Real-Time PCR assay

MDR1 gene expression levels in MCF-7/ADR cells were detected by Real-Time PCR assay. MCF-7/ADR cells (4×10^5 per well) were incubated in 6-well plates overnight and then treated with various concentrations of GQ (2.5, 5 and 10 μM) or Q (10 μM) for 48 h. DMSO (0.1%) was used as a blank control. After medium was removed, the cells were lysed with TransZol Up (1 mL per well) for 5 min. Cell lysates were treated with chloroform (0.2 mL per well) at room temperature for 3 min and centrifuged for 15 min at 12000 g to get supernatant. The supernatants were mixed with equal volume of isopropanol to precipitate RNA. The RNA sediments were washed with 75% ethanol (1 mL) and then dissolved with RNase-free water. The concentrations of RNA solutions were calculated from the absorbance at 260 nm, and the purity was determined by the ratio of A_{260}/A_{280}. Reverse transcription reaction was done by mixing the RNA with EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix at 42 °C for 15 min. Then the solution was heated at 85 °C for 5 s and mixed with TransStart Top Green qPCR SuperMix for PCR amplification. According to the manufacturer's recommendations, the amplification conditions were as follows: pre-denaturation at 94 °C for 30 s, 40 cycles of denaturation at 94 °C for 5 s, annealing at 60 °C for 15 s, and extension at 72 °C for 10 s. The sequences of MDR1 primers were as follows: sense, 5′-GAGCCTA CTTGGTGGCACATAAA-3'; antisense, 3′-TGATGATGTGGGCTGCTGAT-5'.

2.10. Western blot assay

The expression levels of P-gp in MCF-7/ADR and MCF-7/ADR-siRNA cells were detected by western blot assay. The cells (4×10^5 per well) were incubated in 6-well plates overnight and then treated with various concentrations of GQ (2.5, 5 and 10 μM) or Q (10 μM) for 48 h. DMSO (0.1%) was used as a blank control. The cell lysates were prepared in an ice-cold lysis buffer containing PMSF (100 μL per well) and centrifuged at 12000 g for 10 min. Supernatants containing total cellular proteins were collected. The protein samples (30 μg per lane) were subjected to SDS-PAGE, and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked in TBST (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 g/L Tween-20) with 5% (w/v) nonfat milk powder and then incubated with P-gp primary antibody overnight at 4 °C. After washing three times with TBST, the membranes were exposed to horseradish peroxidase-conjugated secondary antibody at room temperature for 2 h. Protein bands were visualized by BeyoECL Plus reagents and imaged using a BioSpectrum Gel Imaging System (UVP, Upland, CA, USA). Densitometry of the signal bands was analyzed using LabWorks software (UVP) [17,18].

2.11. Molecular docking analysis

The structures of GQ and Q were optimized with the Gaussian 09 program [19] on the level of B3LYP/6-31 + G** [20,21], and later the optimized structures for ligand molecules were created in PBD format. The protein P-gp (PDB: 4XWK) was taken from the Protein Data Bank [22]. The original ligands in protein were removed and then polar hydrogen atoms were added. Both ligands (GQ and Q) and target protein (P-gp) were prepared with AutoDockTools-1.5.6, and all molecular docking studies for ligand-protein complexes were conducted using AutoDock Vina program [23]. Geometric optimizations of ligand-protein complexes were performed using a genetic algorithm, and only the best-scoring ligand-protein complexes were kept for discussions [24]. The 2D-structures displayed in Fig. 5were drawn by using Discovery Studio version 4.5 (Accelrys Inc., San Diego, CA, USA).

2.12. Tumor inhibition in mice

Antitumor effect of GQ combining with ADR was investigated in tumor-bearing mice. MCF-7/ADR cells were mixed with matrigel at a weight ratio of 3:1 and subcutaneously inoculated in the right armpit of female BALB/c nude mice (5×10^7 cells/mL, 200 μL) to form xenograft. Tumor volumes were measured with vernier calipers and calculated by the equation: Volume = $0.5 \times L \times W^2$ (L and W represented the length and the width of the tumor, respectively). When the tumors reached about 150 mm^3, the mice were randomly divided into six groups (n = 3) and treated with normal saline (control), Q, GQ, ADR, ADR combining with Q or GQ, respectively. Normal saline or ADR (3 mg/kg) were administered by tail intravenous injection, Q and GQ (18 mg/kg) were administered by gavage at an interval of 3 days. Tumor volume and status of the mice were recorded termly. The mice were sacrificed at day 21 after administration for 7 times. Tumors were removed from bodies, photographed and kept at −20 °C for western blot and Immunohistochemical (IHC) assay. Western blot was performed as described in 2.7.

2.13. IHC assay

IHC assay was conducted to detect the expression levels of P-gp in tumor tissues. Tumor tissues were fixed in formalin fixative for 12–16 h at room temperature and penetrated in paraffin. Sections were mounted on glass slides and dried in oven at 55 °C for 2–3 h. Subsequently, sections were soaked in xylene for 15 min and then in gradient ethanol for 5 min. For antigen retrieval, sections were immersed in citrate buffer (0.01 M, pH = 6.8) for 5 min. Then the endogenous peroxidase was inactivated by soaking the sections in 0.3% hydrogen peroxidase (H_2O_2). After being blocked with goat serum for 15 min, the sections were incubated with primary antibody at 4 °C overnight, then incubated orderly with biotinylated secondary antibody at 37 °C for 15 min and streptavidin HRP conjugate at 37 °C for 15 min. The sections were treated with freshly prepared diaminobenzidine (DAB) for 5 s, followed by counterstain with haematoxylin and seal with neutral balsam. Finally, the sections were imaged under a microscope and quantitatively analyzed using Image-Pro Plus (Media Cybernetics, USA).

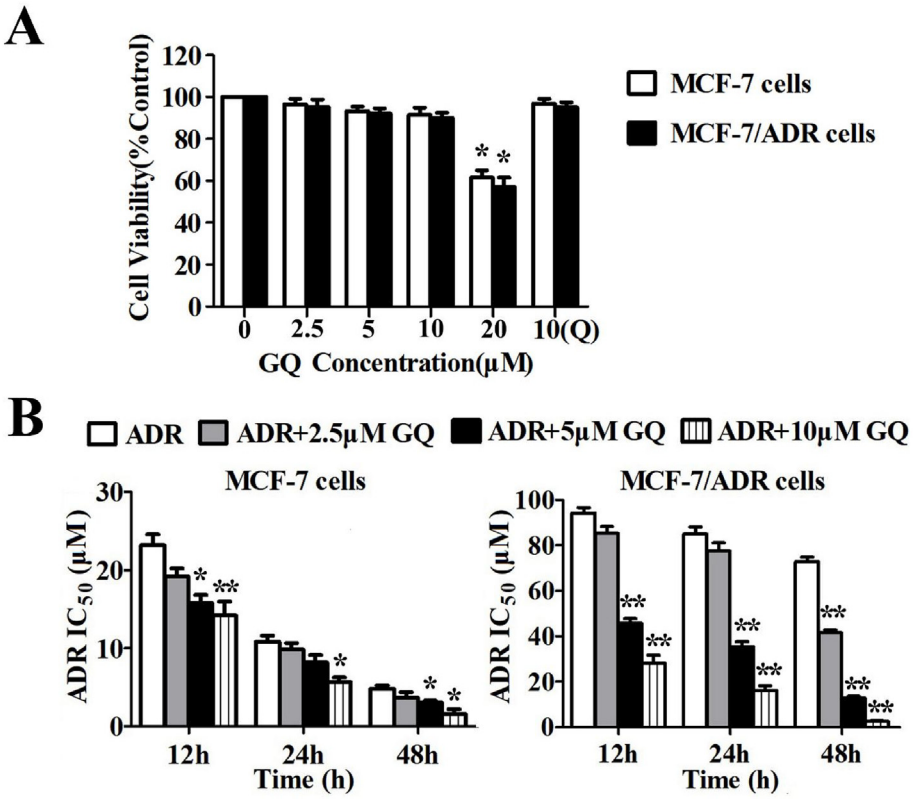

Fig. 2. GQ reversed drug resistance of MCF-7/ADR cells to ADR. (A) MCF-7 and MCF-7/ADR cells were treated with 10 μM Q and various concentrations of GQ for 48 h. (B) MCF-7 and MCF-7/ADR cells were treated with ADR or ADR combining with various concentrations of GQ for 12, 24 and 48 h. Cell viability was measured using MTT assay and the IC_{50} values were calculated. All data represent the means ± SD of three independent experiments. $*p < 0.05$, $**p < 0.01$, comparing with control group.

2.14. Statistical analysis

Each experiment was performed for at least three times and data were presented as mean ± standard deviation (SD). Data were analyzed using SPSS 21.0 (SPSS, Chicago, USA) by ANOVA test and Tukey's multiple comparison tests. $p < 0.05$ was considered statistically significant.

3. Results

3.1. GQ reversed the resistance of MCF-7/ADR cells to ADR

The cytotoxic activity of GQ to MCF-7 and MCF-7/ADR cells was measured by MTT assay. When MCF-7 and MCF-7/ADR cells were treated with Q or GQ at concentrations no more than 10 μM for 48 h, the cell proliferation rates were all above 90%. The proliferation rates of MCF-7 and MCF-7/ADR cells respectively dropped to 61.59% and 57.16% with the treatment of 20 μM GQ (Fig. 2 A). These results indicated that GQ did not affect cell proliferation at low concentrations (≤10 μM) while it inhibited cell growth significantly at 20 μM. We then evaluated the effect of GQ on the cytotoxicity of ADR. It was found that the IC_{50} values of ADR to MCF-7 cells were much lower than those to MCF-7/ADR cells and the values decreased with the treating time extension. GQ reduced the IC_{50} of ADR to both cell lines, but its effect on MCF-7/ADR cells was much stronger than that on MCF-7 cells (Fig. 2 B). For example, by combining with 0, 2.5, 5 and 10 μM GQ, the IC_{50} values of ADR to MCF-7 cells at 48 h were 4.8, 3.67, 3.03 and 1.56 μM, respectively, while those to MCF-7/ADR cells were 72.73, 41.48, 12.78 and 2.52 μM, respectively. As shown in Table 1, the IC_{50} values of ADR to MCF-7/ADR and MCF-7 cells were 72.73 μM and 1.21 μM, respectively, indicating a resistance fold of 60. When the concentrations of GQ were 2.5, 5 and 10 μM, the corresponding reversal folds were 1.8, 5.7 and 30, respectively, while the reversal fold of 10 μM Q was just 1.36. So, GQ could reverse drug resistance of MCF-7/ADR cells at a dose-dependent manner within a certain concentration range (≤10 μM) and its reversing effect was stronger than that of Q at the same dosage.

3.2. GQ increased the uptake of ADR in MCF-7/ADR cells by inhibiting P-gp

To investigate the effect of GQ on P-gp, we firstly compared the uptake of ADR in MCF-7, MCF-7/ADR and MCF-7/ADR-siRNA cells by fluorescence confocal microscope. The red and blue fluorescence indicated ADR and cell nuclei, respectively. The image showed that lots of ADR accumulated in MCF-7 and MCF-7/ADR-siRNA cells, and GQ did not affect the accumulation of ADR in these two kinds of cells. On the contrary, ADR was hardly taken into MCF-7/ADR cells. But GQ increased the accumulation of ADR in MCF-7/ADR cells at a dose dependent manner, and the effect of GQ was more obvious than that of Q at the sane concentration (Fig. 3 A). Similar result was obtained in quantitative detection by microplate reader (Fig. 3 B). The concentrations of ADR in MCF-7/ADR cells treated with 5 and 10 μM GQ (0.698 and 0.964 μM) were significantly higher than those in the cells treated with 10 μM Q (0.453 μM) or in the control cells (0.45 μM). The results demonstrated that GQ increased the accumulation of ADR in MCF-7/ADR cells, and its effect was stronger than that of Q.

Rh known as a P-gp substrate can be expelled from cells by P-gp. To investigate whether the accumulation enhancement of GQ to ADR is related to P-gp, the intercellular Rh concentration in GQ-treated cells was detected by flow cytometry (Fig. 3C and D). It was shown that the concentrations of Rh in the cells treated with 2.5, 5 and 10 μM of GQ increased significantly compared with those in control cells and the cells treated with 10 μM Q. These results proved that GQ could inhibit the drug efflux mediated by P-gp and its activity was higher than that of Q.

3.3. GQ inhibited the expression of MDR1 and P-gp

Effects of GQ on the expression of MDR1 gene and P-gp protein were investigated to reveal the mechanism of GQ reversing drug resistance. After MCF-7/ADR cells were treated with 2.5, 5 and 10 μM of GQ, the

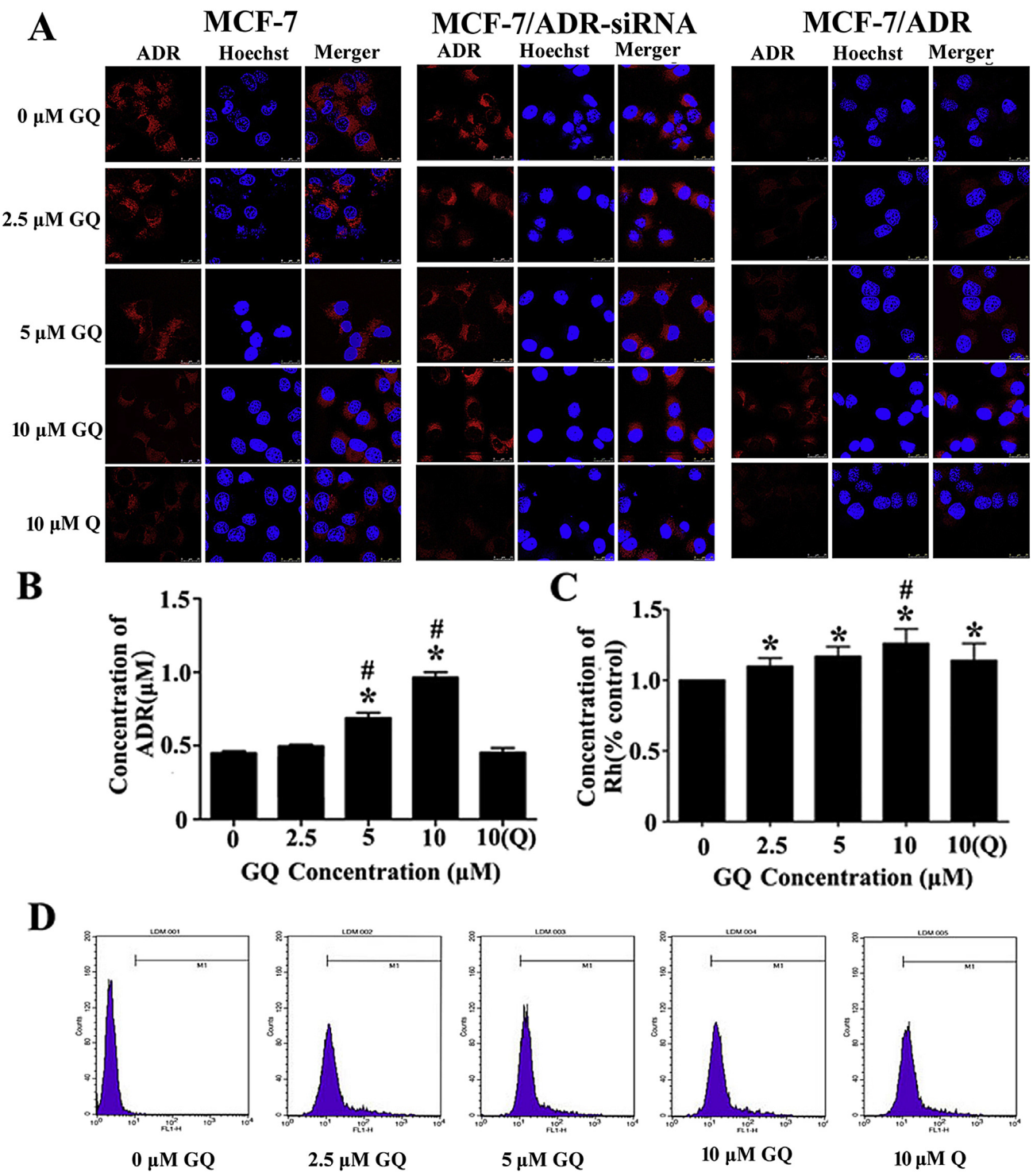

Fig. 3. Effect of GQ on the accumulation of ADR and Rh in MCF-7/ADR cells. (A) MCF-7, MCF-7/ADR and MCF-7/ADR-siRNA cells were treated with ADR (5 μM) combining 10 μM Q or various concentrations of GQ for 24 h. Then the cells were stained with Hoechst for 30 min, and accumulation of ADR in the cells was observed by confocal fluorescence microscope (scale bar = 25 μm). (B) MCF-7/ADR cells were treated with ADR (5 μM) combining 10 μM Q or various concentrations of GQ for 24 h, accumulation of ADR in cells was detected by microplate reader. (C) The quantitative analysis for D. (D) MCF-7/ADR cells (4×10^5 cells per well) were treated with various concentrations of GQ or Q (10 μM) for 24 h. Then the cells were incubated with Rh (5 μM) for 1 h. Accumulation of Rh in cells was detected by a flow cytometry. All data represent the means ± SD of three independent experiments. $*p < 0.05$, comparing with control group; $^{\#}p < 0.05$, comparing with Q treated group.

levels of MDR1 gene (Fig.4A) and P-gp protein (Fig. 4B) in the cells decreased significantly compared with those in control group. But there was a slight increase ($p < 0.05$) in P-gp level after the cells were treated with 10 μM Q (Fig. 4B). P-gp levels in MCF-7/ADR-siRNA cells, in which MDR1 gene was silenced by siRNA, were much lower than those in MCF-7/ADR cells. GQ (≤10 μM) did not obviously affect the expression of P-gp in MCF-7/ADR-siRNA cells (Fig. 4C). This indicated that GQ inhibited the expression of P-gp by down-regulating MDR1 gene in MCF-7/ADR cells in a dose-depend manner and the inhibitory effect was stronger than that of Q.

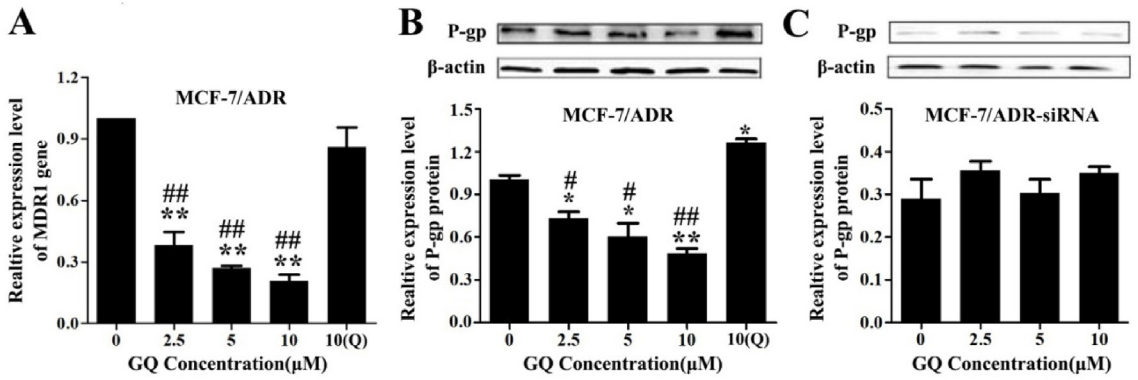

Fig. 4. GQ inhibited the expression of MDR1 gene and P-gp protein. (A) Real-Time PCR was conducted to detect the expression of MDR1 gene in MCF-7/ADR cells treated with 10 μM Q or various concentrations of GQ for 48 h. (B, C) Western blot was conducted to detect the expression of P-gp in MCF-7/ADR cells and MCF-7/ADR-siRNA cells treated with 10 μM Q and various concentrations of GQ for 48 h. All data represent the means ± SD of three independent experiments. $*p < 0.05$ and $**p < 0.01$ comparing with control group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ comparing with Q treated group.

3.4. Molecular docking of GQ with P-gp

Molecules GQ and Q were docked into the protein P-gp (PDB: 4XWK) to clarify the mechanism of GQ on the function of P-gp. The docking results showed that three amino acids, viz., Ser752, Ser725 and Tyr303 in P-gp protein interacted with GQ through hydrogen bonds with the binding energy of −10.8 kcal/mol. In addition, the long alkyl chain of GQ reached into the hydrophobic pocket that consists of the side chains of Phe331, Phe332, Leu335 and Phe728 (Fig. 5A). In the interaction of P-gp and Q, just two hydrogen bonds (Ser752 and Ser725) were formed with the binding energy of −9.3 kcal/mol (Fig. 5B). Compared with molecule Q, the long alkyl chain could make GQ match better with the active pocket in P-gp.

3.5. GQ enhanced the inhibitory effect of ADR on tumor growth

Influence of GQ on the antitumor efficacy of ADR was evaluated via a MCF-7/ADR xenograft model in mice. The tumor-bearing mice were treated with ADR, GQ, Q or their combination for 21 days. The tumor volumes in the groups treated only with ADR, GQ or Q continuely increased during therapy, but the growth speed in ADR or GQ treatment groups were slower than that in Q treatment group and control group. By combination with GQ or Q, the inhibition of ADR to tumor growth increased significantly, the tumor volumes at 21 d were obviously lower than those initially. The synergetic effect of GQ with ADR was more remarkable than that of Q at the same dosage (Fig. 6). These results indicated that GQ enhanced the antitumor effect of ADR in vivo, and its activity was stronger compared with Q.

3.6. GQ inhibited the expression of P-gp in tumor tissues

The expression levels of P-gp in tumor tissues were investigated by IHC and western blot assays (Fig.7). It was shown that the inhibitory effect of GQ on P-gp expression in tumor tissue was stronger than that of Q, and the effect of GQ combined with ADR was stronger than that of Q combined with ADR. These results suggested that GQ reversed the resistance of MCF-7/ADR to ADR by decreasing the expression of P-gp, and its effect was stronger than that of Q at the same dosage.

4. Discussion

Flavonoids have attracted considerable attention as P-gp inhibitors, since they shared inhibitory activities on P-gp and physiological safety as modulators of MDR [6,25]. Q has been demonstrated to reverse MDR [1] and be safe in human body via a phase I clinical trial [26]. Our previous studies showed that the accumulation of GQ was significantly higher than that of Q in MCF-7 cells, which suggested that GQ might have stronger ability to reverse MDR than Q [14]. The present study tried to investigate the effects of GQ on reversing MDR of breast cancer cells to ADR and reveal the possible mechanisms.

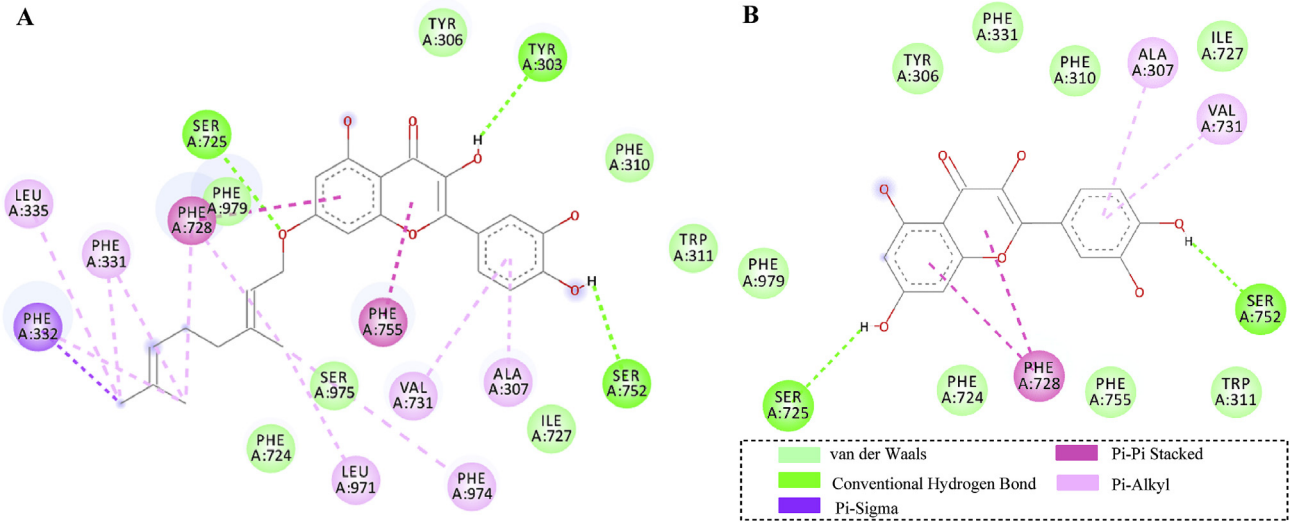

Fig. 5. Molecular docking of GQ (A) and Q (B) with P-gp.

Table 1
Reversal effect of GQ on the drug resistance of MCF-7/ADR cells to ADR.

	MCF-7	MCF-7/ADR	MCF-7/ADR			
			2.5 μM GQ	5 μM GQ	10 μM GQ	10 μM Q
ADR IC_{50} (μM)	1.2 ± 0.09	72.73 ± 2.11	41.48 ± 1.2	12.78 ± 0.81	2.52 ± 0.32	52.47 ± 3.22
Resistance fold	—	60	34.6	10	2	44.2
Reversal fold	—	—	1.8	5.7	30	1.36

MCF-7 and MCF-7/ADR cells were treated with ADR or ADR combining with GQ (Q). Cell viability was detected by MTT assay at 48 h, and the IC_{50} values were calculated. All data represent the means ± SD of three independent experiments.

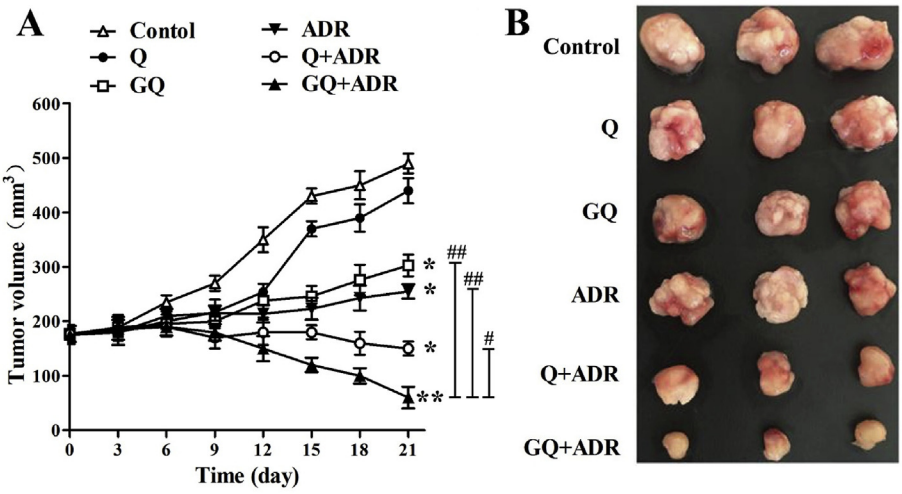

Fig. 6. GQ enhanced the inhibitory effect of ADR on tumor growth. Tumor-bearing mice were treated with ADR (3 mg/kg) via tail vein injection or treated with Q or GQ (18 mg/kg) by gavage for 7 times at an interval of 3 days. (A) Volumes of tumor in mice during the treatment. (B) Tumors removed from the mice after treatment. All data represent the means ± SD of three independent experiments. $*p < 0.05$, $**p < 0.01$ comparing with control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ comparing with GQ + ADR group.

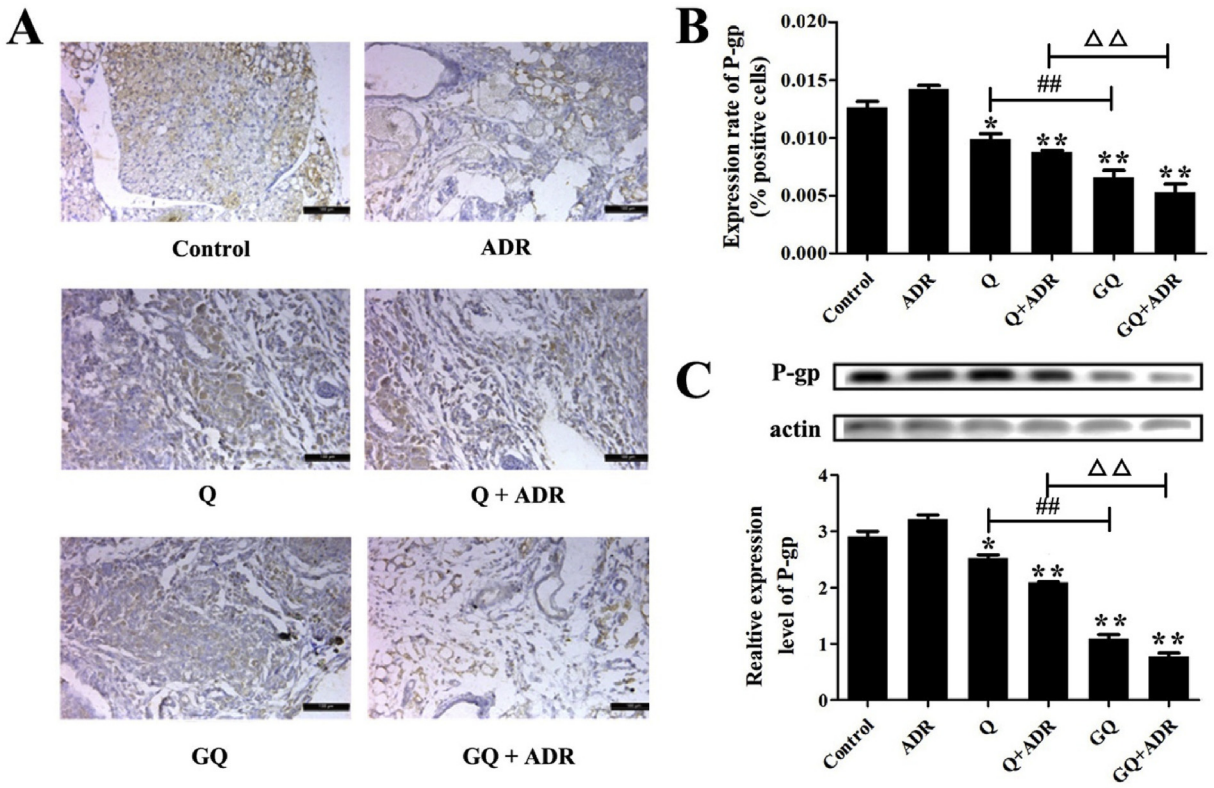

Fig. 7. Effect of GQ on the expression of P-gp in tumor tissues. (A,B) P-gp expression levels in tumor tissues was detected by IHC assay, the bar in (A) indicated 100 μm; (C) P-gp expression levels in tumor tissues was detected by western blot. All data represent the means ± SD of three independent experiments. $*p < 0.05$, $**p < 0.01$ comparing with control group. $^{\#}{}^{\#}p < 0.01$ comparing with Q treated group, $^{\triangle\triangle}p < 0.01$ comparing with Q + ADR group.

It was reported that Q had not only reverse activity against MDR but also antitumor effect [6]. In this study, we also found that GQ at higher concentrations (> 10 μM) could inhibit the growth of tumor cells. Therefore, in the MDR reversing study, GQ was used at low concentrations (≤10 μM) to avoid the interference of its anti-tumor activity. The reversal fold of GQ was about 22 times as that of Q at the same concentration in MCF-7/ADR cells, which preliminary indicated GQ was a superior MDR reverser.

Though we found that GQ increased the accumulation of ADR in MCF-7/ADR cells, whether it was mediated by P-gp should be illuminated. Rh was widely used as a probe substrate of P-gp [6]. This study demonstrated that GQ could raise the concentration of Rh in MCF-7/ADR cells, which indirectly proved that GQ enhanced ADR accumulation in MCF-7/ADR cells by inhibiting P-gp-mediated drug efflux. Our further exploration revealed that GQ could inhibit P-gp's function by down-regulating its expression.

Molecular docking studies had confirmed that flavonoids had strong binding affinity to P-gp [27,28]. The interaction mechanisms of flavonoids with P-gp have not yet been clearly defined. It was proposed that flavonoids might interact with P-gp at the ATP-binding site, steroid binding site [29] or substrate binding site of P-gp [30]. We found that GQ bound with P-gp via more hydrogen bonds and matched better in the active pocket compared with Q, which might be one of the reasons for the strong inhibition effect of GQ on P-gp. But this assumption needs further experimental confirmation.

Flavonoids could inhibit expression of P-gp and drug efflux mediated by it. Zhu et al. found that oroxylin A reduced P-gp-mediated drug efflux and expression level of MDR1 in MCF-7/ADR cells [31]. Borska et al. also reported that Q could inhibit P-gp expression and its function in cells of human pancreatic carcinoma line resistant to daunorubicin [15]. In our study, IHC and western blot assays showed that GQ could decrease the expression of P-gp both in tumor tissues and in cells, and Real-Time PCR assay indicated that GQ could down-regulate the expression of MDR1 gene. After silencing MDR1 gene by siRNA, the effect of GQ on P-gp expression did not appear. These evidences confirmed that GQ could inhibit the expression of P-gp by down-regulating MDR1 gene in MCF-7/ADR cells.

At the same time, we found that Q at the concentration of 10 μM increased the level of P-gp in MCF-7/ADR cells prominently, which seems to be inconsistent with the function of down-regulating P-gp expression [1]. Lohner K et al. reported that the expression of P-gp and the corresponding mRNA increased with the treatment of 13 kinds of flavonoids at 10 μM. He thought the increase in P-gp expression caused by flavonoids might serve as an adaptation and defense mechanism limiting the entry of lipophilic xenobiotics into the organism [32]. At the same concentration (10 μM), GQ did not increase the expression of P-gp, suggesting that GQ was more ideal for MDR reversal than Q.

Because of low solubility and poor bioavailability, the using of Q on reversal of MDR was severely limited in animal models and clinical trials [33,34]. Efforts have been made mainly on two aspects to overcome the obstacle. On one hand, the co-delivery systems of Q and chemotherapeutics were developed for increasing its solubility and reverse efficiency. Zhang et al. constructed a liposome loading ADR and Q, in which Q increased anti-tumor effect of ADR by down-regulating P-gp expression in tumor tissues [33]. On the other hand, derivatives of Q have been synthesized to improve solubility and MDR reversal activity [10,35]. Although these derivatives showed good P-gp inhibitory effects in vitro, the in vivo activity of them was not further reported. In this study, GQ, the alkyl derivative of Q, showed obvious reversal activity against MDR in mice with MCF-7/ADR xenograft and the effect was more significant than that of Q.

In addition, we found that GQ alone at a higher concentration (20 μM) could inhibit the growth of MCF-7/ADR cells. Our previous studies showed that GQ had stronger anti-tumor activity and better solubility than Q, and indicated that several mitochondria pathways were involved in GQ-induced apoptosis in tumor cells. For example, GQ induced apoptosis of MCF-7 cells via a caspase-independent Endo G-mediated mitochondria pathway [14], induced apoptosis and autophagy of lung cancer A549 and NCI–H1975 cells via ROS generation [36], and induced apoptosis of gastric cancer SGC-7901 and MGC-803 cells via ROS-MAPK mediated mitochondrial signaling pathway [37]. Therefore, GQ was more excellent in comparison with other P-gp inhibitors because of its inherent anti-tumor activity.

5. Conclusion

The present study demonstrated that GQ reversed the drug resistance of MCF-7/ADR to ADR by down-regulating the expression of P-gp protein as well as its encoding gene MDR1. In addition, inherent anti-tumor effect of GQ made it more advantageous than other P-gp inhibitors. This indicated that GQ was a potential reversal agent for drug resistance in cancer therapy.

Author contributions

Yuhong Zhen and Xiaohui Kang designed research; Enxia Zhang, Lei Shi, Xin Guo and Ze Liang peformed research; Xiaohui Kang, Hong Xu and Huaxin Wang contributed new analytical tools and reagents; Xiaohong Shu and Shanshan Huang analyzed the data; Jiasi Liu, Jiaxin Zuo and Yuhong Zhen wrote the paper.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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