School of Pharmacy[1], Institute of Pharmaceutics[2], Anhui Academy of Chinese Medicine; Engineering Technology Research Center of Modernized Pharmaceutics[3], Education Office of Anhui Province, Hefei, PR China

Rosmarinic acid, the active component of *Salvia miltiorrhizae*, improves gliquidone transport by regulating the expression and function of P-gp and BCRP in Caco-2 cells

Mengying Li[1], Dengke Yin[1,2,3,*], Jiacheng Li[1], Fuping Shao[1], Qingqing Zhang[1], Qianqian Jiang[1], Mengmeng Zhang[1], Ye Yang[1,3,*]

Received August 31, 2019, accepted October 17, 2019

**Corresponding authors: Prof. Dr. Dengke Yin, Prof. Dr. Ye Yang, School of Pharmacy, Anhui University of Chinese Medicine, Hefei 230012, PR China.*
yindengke@ahtcm.edu.cn, gnayye@126.com

Pharmazie 75: 18-22 (2020) *doi: 10.1691/ph.2020.9754*

Salvia miltiorrhiza (Danshen) is typically used in the treatment of diabetic complications and is often co-prescribed with gliquidone in China. However, whether danshen affects the absorption of gliquidone has not been elucidated. In this study, the effects of an aqueous extract of danshen (danshen injection, DSI) and its primary compounds (danshensu, protocatechuic aldehyde, rosmarinic acid and salvianolic acid B) on gliquidone transport across Caco-2 monolayer cells was investigated. DSI enhanced the transport of gliquidone in Caco-2 cell monolayers from the apical (AP) to basolateral (BL) sides and from the BL to AP sides. Rosmarinic acid (RA) also significantly increased the Papp (AP-BL) value for gliquidone transport. Verapamil (a P-gp inhibitor) and Ko143 (a BCRP inhibitor) inhibited the BL-AP transport of gliquidone and promoted the AP-BL transport of gliquidone, whereas MK571 (an MRP1 inhibitor), probenecid (an MRP2 inhibitor), and benzbromarone (an MRP3 inhibitor) had no effect on gliquidone transport. RA also enhanced the intracellular accumulation of Rho123 and Hoechst 33342. The expression of P-gp and BCRP was significantly downregulated, and P-gp ATPase activity was promoted by RA in a dose-dependent manner. These results indicate that an aqueous extract of danshen can increase the transport of gliquidone in Caco-2 cell monolayers and that RA may be the primary compound associated with this activity, which is in agreement with RA simultaneously suppressing the function and expression of P-gp and BCRP.

1. Introduction

Diabetes mellitus is a syndrome of altered energy homeostasis that is caused by the abnormal metabolism of carbohydrates, proteins and fats and has become a serious global public health concern. As of 2015, 8.3% of adults were estimated to have diabetes worldwide, with T2DM accounting for approximately 90% of these cases (Einarson et al. 2018). The pharmacological treatment of hyperglycemia in T2DM patients is typically initiated with an oral antidiabetic drug, and among oral hypoglycemic agents, sulfonylureas is a particularly suitable first-line drug. Gliquidone is a second-generation sulfonylurea-type oral hypoglycemic agent approved by the US Food and Drug Administration for the treatment of T2DM. However, sulfonylureas cause much more hypoglycemia than has been recognized, which may be relevant to its pharmacokinetic profile (Confederat et al. 2015).

Danshen, the dried root of the plant *Salvia miltiorrhizae* Bunge, is used to promote blood flow and treat vascular disease in China. Two hundred one compounds have been reported from danshen, including lipophilic diterpenoids, water-soluble phenolic acids, and other constituents that have exhibited various pharmacological activities, such as anti-inflammation, anti-oxidation, anti-tumor, anti-atherogenesis, and anti-diabetes activities (Mei et al. 2019). T2DM is associated with various comorbidities, such as diabetic nephropathy (Alnaggar et al. 2019), diabetic cardiopathy (Zhang et al. 2019), and diabetic hypertension (Viswanathan and Smina 2019). The co-administration of danshen and gliquidone is a common therapeutic schedule for patients with diabetes mellitus and diabetic complications in clinical settings in China. However, whether danshen affects the absorption of gliquidone and its mechanism of action have yet to be elucidated.

Nowadays, more and more patients are taking herb-drug combination therapies to cure diabetes, especially in China. However, this activity increases the probability of herb-drug interactions due to the complex ingredients in herbs potentially acting upon the same metabolic enzyme and transport proteins as chemical medicines (Obach 2000; Weber et al. 2004). A number of clinical studies and

Abbreviations:
BCRP, breast cancer resistance protein; DDIs, drug-drug interactions; DSI, danshen injection; DSS, danshensu; ER, efflux rate; Ho33342, Hoechst 33342; MRP1, multidrug resistance-associated protein 1; MRP2, multidrug resistance-associated protein 2; MRP3, multidrug resistance-associated protein 3; PA, protocatechuic aldehyde; Papp, apparent permeability coefficient; P-gp, P-glycoprotein; RA, rosmarinic acid; Rho123, Rhodamine 123; SAB, salvianolic acid B; T2DM, type 2 diabetes

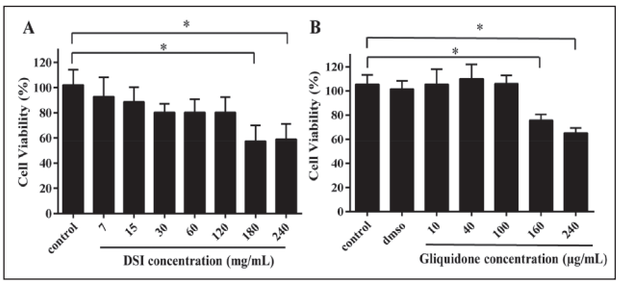

Fig. 1: Cytotoxicity of DSI (A) and gliquidone (B) measured by MTT (n = 6). *p < 0.05

case reports have indicated some incompatibilities between TCM (Traditional Chinese Medicine) and conventional drugs (Meisel and Johne 2003, Yuan et al. 2004). Thus, research on interactions between TCM and conventional drugs will be useful for guidance of their combined use in clinical settings.

In this study, the intestinal transport profile of gliquidone in Caco-2 cells by co-administration of danshen aqueous extracts and its active ingredients were investigated to provide beneficial information for the clinical application of danshen and gliquidone.

2. Investigations and results

2.1. Cytotoxicity of the DSI and gliquidone

The cytotoxicities of DSI and gliquidone were calculated using the MTT assay. As shown in Fig. 1A, after incubating for 12 h, the DSI (7-120 mg/mL) demonstrated low cytotoxicity toward Caco-2 cells. At concentrations of 160 and 240 μg/mL, gliquidone showed cytotoxicity toward Caco-2 cells (Fig. 1B), while no significant difference in cell viability was observed compared with the control group when the concentration was less than or equal to 100 μg/mL.

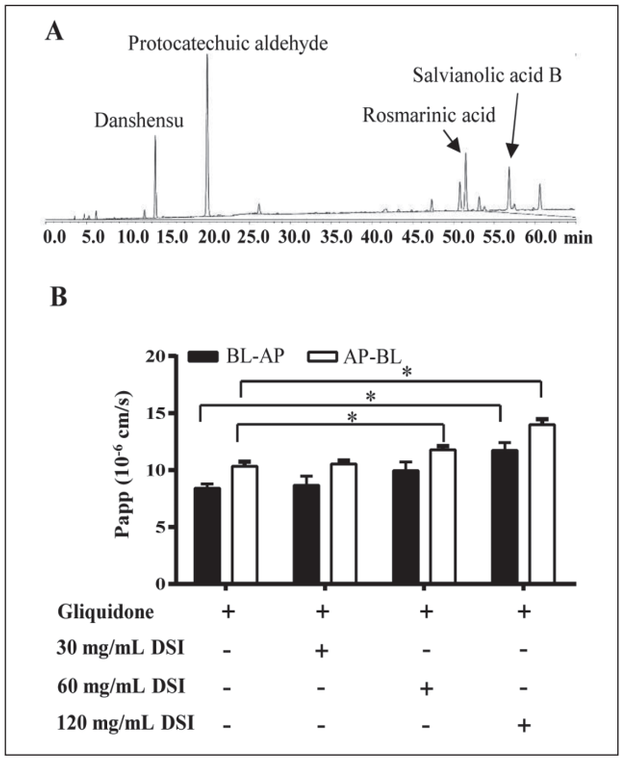

Fig. 2: (A) HPLC chromatogram of DSI. (B) Effect of DSI on the transport of gliquidone across Caco-2 monolayer cells (n=3). *p < 0.05

2.2. Influence of DSI on the transport profile of gliquidone

The primary ingredients in DSI are DSS, PA, RA, and SAB (Fig. 2A) at concentrations of 925.55±9.42, 243.55±2.78, 352.98±4.62, and 322.12±5.15 μg/mL, respectively. The effect of DSI on the absorption of gliquidone was evaluated using Caco-2 cell monolayers (Fig. 2B), the results of which showed that DSI (120 mg/mL) could significantly increase the Papp values of gliquidone from the AP to BL and the BL to AP sides in a dose-dependent manner. Similarly, DSI (60 mg/mL) also could significantly increase the Papp values of gliquidone from the AP to the BL side.

2.3. Effect of the primary compounds of DSI on transport of gliquidone

The inhibitory effects of DSS, PA, SAB and RA of DSI on the bi-directional transport of gliquidone across Caco-2 monolayer cells were evaluated. These compounds, with concentrations in DSI of 120 mg/mL, showed no cytotoxicity toward Caco-2 cells during the transport experiments as determined by MTT assays of DSI. As shown in Fig. 3A, RA significantly increased the Papp (A-B) value of gliquidone transport. The structure of RA is shown in Fig. 3B.

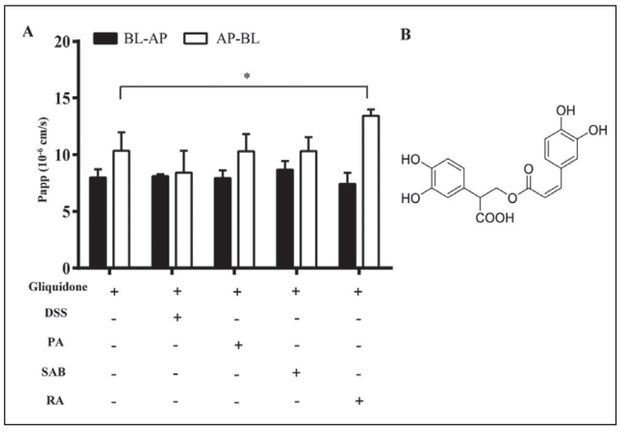

Fig. 3: (A) Effect of the primary active compounds of DSI on the transport of gliquidone across Caco-2 monolayer cells (n=3). *$P < 0.05$ (B) The structure of RA.

2.4. Effect of efflux transporter inhibitors on gliquidone transport

To determine which transporters are responsible for the efflux of gliquidone, we performed transport experiments in the presence of verapamil, MK571, probenecid, benzbromarone, and Ko143, the results of which are shown in Fig. 4. No significant differences in the bi-directional transport of gliquidone were observed when cells were co-incubated with MK571, probenecid or benzbromarone compared with that observed in the group treated with gliquidone alone, indicating that MRP1, MRP2, and MRP3 are not involved in the efflux of gliquidone ($P > 0.05$). In contrast, verapamil and Ko143 markedly decreased the BL to AP efflux of gliquidone by approximately 13-21% and increased the AP to BL influx of gliquidone by approximately 14-17 % ($P < 0.05$).

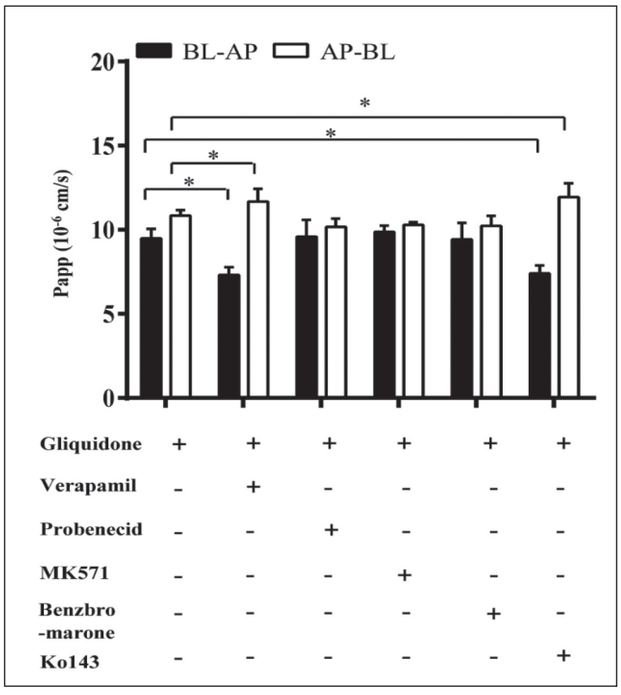

Fig. 4: Effect of verapamil, Ko143, MK571, probenecid and benzbromarone on the Papp values for gliquidone (n=3). *$P < 0.05$

2.5. *Effect of RA on the transport and intracellular accumulation of Rho123 and Hoechst 33342*

The effect of RA on the transport of Rho123 and Ho33342 was examined in Caco-2 cells, the results of which are shown in Fig. 5. The results indicated that RA increases the transport of Rho123 in the AP-BL direction (Fig. 5a) but decreases Rho123 transport in the BL-AP direction (Fig. 5b), with RA (30 μg/mL) exhibiting a similar inhibitory effect as verapamil (25 μg/mL). In addition, RA administration also significantly increased the transport of Ho33342 from the AP to the BL side (Fig. 5c) and decreased the efflux the BL to the AP side (Fig. 5d), and when the concentration of RA increased to 30 μg/mL, the inhibitory effect was comparable to Ko143 (5 μg/mL). RA also reduced the efflux rate (ER) of Rho123 (Fig. 6a) and Ho33342 (Fig. 6b). In addition, RA significantly enhanced the intracellular accumulation of Rho123 (Fig. 6c) and Ho33342 (Fig. 6d) in a dose-dependent manner.

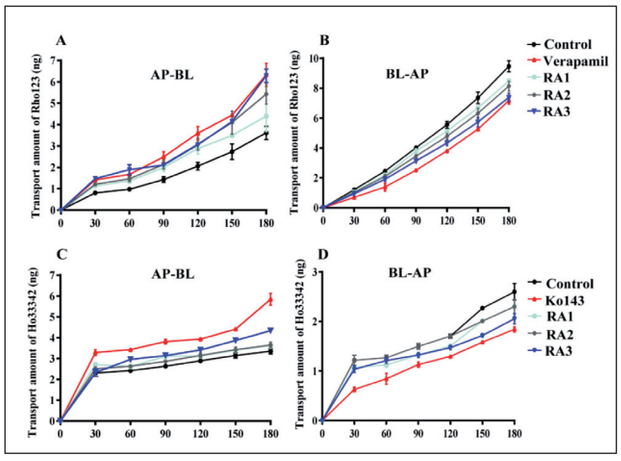

Fig. 5: Effect of RA on the transport of Rho123 from the AP to the BL side(A) or from the BL to the AP side (B) and of Ho33342 from the AP to the BL side (C) or from the BL to the AP side (D) in Caco-2 monolayer cells (n=3).

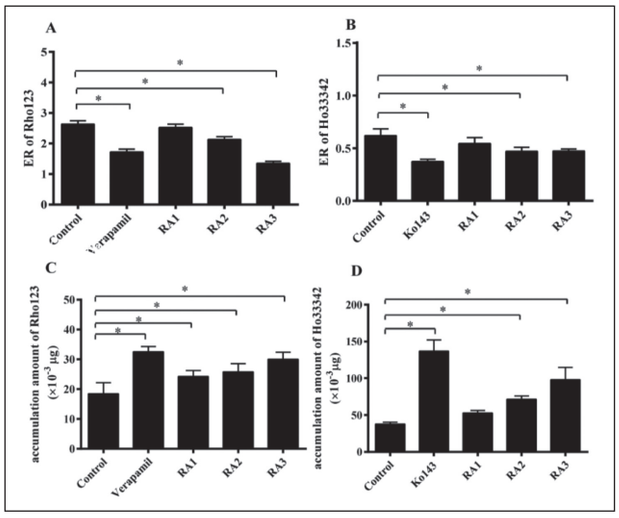

Fig. 6: Effect of RA on the efflux rate of Rho123 (A) and Ho33342 (B) and on the intracellular accumulation of Rho123 (C) and Ho33342 (D) (n=3). * $P < 0.05$

2.6. *RA inhibits the expression of P-gp and BCRP proteins in Caco-2 cells*

The P-gp and BCRP protein levels were downregulated by treatment with RA for 3 h, as determined by immunofluorescence (Fig. 7a) and Western blot analyses (Fig. 7c). As the concentration of RA increased, the expression of P-gp and BCRP decreased. RA (15 and 30 μg/mL) significantly inhibited P-gp and BCRP expression in both immunofluorescence (Fig. 7b) and Western blot analyses (Fig. 7d).

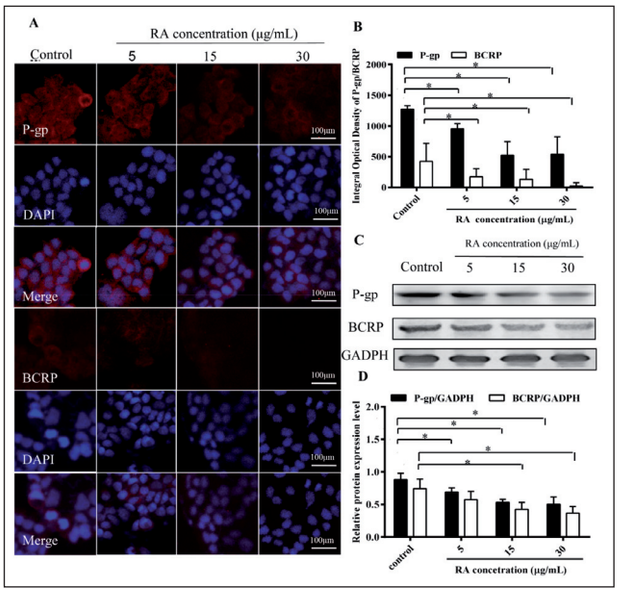

Fig. 7: Effect of RA on the expression of P-gp and BCRP in Caco-2 cells, as determined by immunofluorescence and Western blot analyses. Immunofluorescence image of P-gp and BCRP using fluorescence microscopy (A), and its integral optical density analyzed using ImageJ (B) (n=10). Western blots for P-gp and BCRP (C) and the corresponding densitometry results using GADPH as a control and presented as the percentage of the controls (D) (n=3). *$P < 0.05$

2.7. *Effect of RA on P-gp ATPase activity*

As shown in Fig. 8, the P-gp ATPase activity of cells treated with verapamil was higher than that observed in the basal group. Similarly, after treatment with RA (from 5 to 30 μg/mL), the P-gp ATPase activity increased from 1.86±0.33 to 2.51±0.37 times that of the basal group ($P < 0.05$).

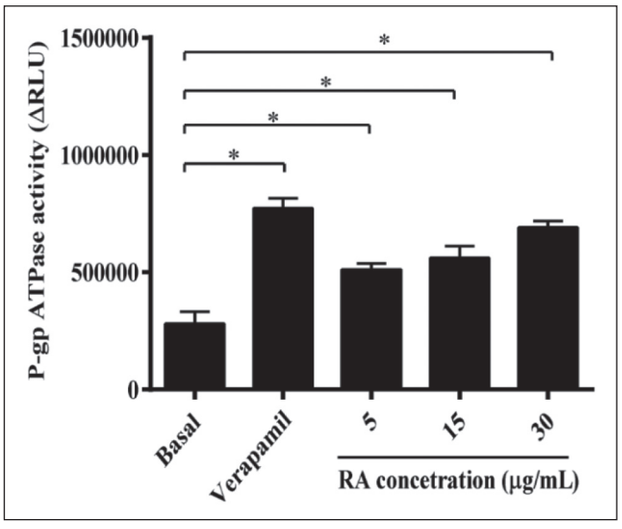

Fig. 8: The effect of RA on P-gp ATPase activity. Verapamil (0.5 mmol/L) was used as a positive control (n=3). *$P < 0.05$

3. Discussion

As a second-generation oral antidiabetic drug, gliquidone has kidney protection effects and is typically used in the treatment of diabetes with kidney disease and diabetic nephropathy (Rybka et al. 1988; Malaisse 2006). Compounds from danshen, including lipophilic diterpenoids, water-soluble phenolic acids, and other constituents have shown various pharmacological activities, such as anti-inflammation, anti-oxidation, anti-tumor, anti-atherogenesis, and anti-diabetes activities (Mei et al. 2019). Because of the

protective effects of danshen and gliquidone toward hypoglycemic and diabetic complications, the co-administration of danshen and gliquidone is a common therapeutic schedule for patients with diabetes mellitus and diabetic nephropathy in China.

Human efflux transporters, primarily P-glycoprotein (P-gp), multidrug resistance-associated protein (MRPs), and breast cancer resistance protein (BCRP), are widely present in the small intestine villus epithelial cells (Alqahtani et al. 2018), renal tubular epithelial cells, hepatocytes and biliary epithelial cells. Efflux transporters act as efflux pumps and have activity that is directly dependent on ATP hydrolysis and can transport compounds from inside the cell back into the intestinal lumen, preventing their absorption into the blood (Hunter et al. 1993; Fromm et al. 2000). Interestingly, P-gp has been identified as being important for the underlying mechanism of drug interactions in humans (Zakeri-Milani and Valizadeh 2014). Unfortunately, these efflux transporters are easily affected by xenobiotic drugs. Changes in the expression and/or activity of efflux transporters can cause DDIs between efflux transporters substrates, resulting in changes in their pharmacokinetics, bioavailability, toxicity, and therapeutic response (Verschraagen et al. 1999). Thus, the regulation of commonly used drugs with activity toward efflux transporters should be investigated to avoid adverse interactions in clinical practice. However, there have been no reports on efflux transporters involved in the absorption of gliquidone. Verapamil and Ko143 were observed to significantly promote the AP-BL transport of gliquidone and inhibit the BL-AP transport, indicating that P-gp and BCRP may be involved in the efflux of gliquidone (Fig. 4) and providing a rationale for future guidance on rational drug use in the clinic.

There are many reports on the effects of danshen and its active ingredients on transporters. It was previously shown that tanshinone I, an active triterpenoid in danshen, is an inhibitor of P-gp (Li et al. 2008). In addition, tanshinone IIA can promote the expression of efflux ABC transporters, including P-gp, BCRP and MRP1 (Li and Lai 2017). Our results suggested that DSI, comprising the water-soluble components of danshen, can enhance the AP-BL transport of gliquidone in Caco-2 cell monolayers (Fig. 2b). The primary ingredient of DSI is DSS, PA, RA and SAB (Fig 2a), the effects of which on gliquidone transport in Caco-2 cell monolayers was determined such that the strength of the interaction of each compound could be independently quantified rather than the combined effects of a mixture containing all of the compounds. Our results indicated that RA could significantly increase the Papp (AP-BL) value of gliquidone transport (Fig. 3a). DSI affected the transport of gliquidone in both directions. However, the active ingredient RA only affected the transport of gliquidone from the AP to the BL side. There are many components in DSI that have multiple effects on the transport of gliquidone. Wang and Sweet (2012) demonstrated that multiple danshen components, such as lithospermic acid, protocatechuic acid, rosmarinic acid, and salvianolic acid A are competitive inhibitors of mOat1 and mOat3, and these ingredients may alter the transport of gliquidone in both directions by other means.

RA is the major active compound in danshen and has anti-oxidative and anti-inflammatory activities (Adomako-Bonsu et al. 2017; Rahbardar et al. 2018). To further elucidate the mechanism by which RA enhances the transport of gliquidone, we examined the effect of different concentrations of RA on the transport of Rho123 (a P-gp substrate) and Ho33342 (a BCRP substrate). RA reduced the efflux rate of Rho123 (Fig. 6a) and Ho33342 (Fig. 6b) and enhanced the transport of Rho123 and Ho33342 from the AP to the BL side and reduced their transport from the BL to the AP side in a dose-dependent manner (Fig. 5). RA also significantly enhanced the intracellular accumulation of Rho123 (Fig. 6c) and Ho33342 (Fig. 6d) in a dose-dependent manner. The levels of P-gp and BCRP protein expression were significantly downregulated by RA in Caco-2 cells (Fig. 7). These results demonstrated, for the first time, that RA has an inhibitory effect on the functions and expression of P-gp and BCRP in Caco-2 cells. It was previously reported that RA could decrease the expression of P-gp in SGC7901/Adr (Li et al. 2013) and SGC7901/5-Fu (Yu et al. 2019) cells. However, it was also reported that RA increased the expression of P-gp and BCRP in HepG2 cells (Wu et al. 2016). Thus, the effect of RA on the expression of P-gp in cells from different sources may be different.

The drug efflux function of P-gp is coupled to ATP hydrolysis, which can be stimulated in the presence of P-gp substrates. Verapamil can stimulate the P-gp ATPase activity and promote the consumption of ATP while stabilizing the "intermediate" P-gp conformation and prevent ATP from driving P-gp to the "closed" conformation to reduce verapamil transport (Ledwitch et al. 2016). Our results indicate that RA can promote P-gp ATPase activity, similar to verapamil (Fig. 8), since as the consumption of ATP increased, the transport capacity of P-gp was inhibited.

In summary, our results showed that gliquidone is the substrates of P-gp and BCRP. DSI could increase the transport of gliquidone in Caco-2 cell monolayers and RA may be the primary compound for this action. In addition, RA could simultaneously inhibit the function and expression of P-gp and BCRP, while activating P-gp ATPase activity. Further clinical studies should be considered to avoid potential risks after the co-administration of RA and gliquidone.

4. Experimental

4.1. Chemicals and reagents

DSI (Lot.1805083), a sterile aqueous extract preparation from danshen, was purchased from the Shanghai Zhongxi Pharmaceutical Co., Ltd. (Shanghai, China). One milliliter of DSI is equal to 1.5 g of crude drug. Gliquidone (purity ≥ 98.0%) was obtained from Yuanye Bio-Technology Co., Ltd. (Shanghai, China). HPLC grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). DSS (purify ≥98.0%), PA (purify ≥98.0%), RA (purify ≥98.0%), and SAB (purify ≥98.0%) were purchased from Yuanye Bio-Technology Co., Ltd. (Shanghai, China). DMEM and FBS were purchased from Hyclone (Thermo Fisher Scientific, New York, US) and non-essential amino acids were purchased from Solarbio (Beijing Solarbio Science & Technology Co., Ltd., China). Verapamil, Ko143, MK571, probenecid, and benzbromarone were purchased from MCE (Shanghai, China). All other reagents and solvents were of analytical grade or higher.

4.2. HPLC analysis of gliquidone and DSI

The HPLC analysis of gliquidone was performed on a Shimadzu LC-15C SPD-15 system (Shimadzu Scientific Instruments, Kyoto, Japan). Chromatography was performed using a Hypersil BDS C18 (4.6 mm × 200 mm, 5 μm) analytical column (Elite, China) at 30 °C. The mobile phase consisted of A (Acetonitrile)/B (0.09% H_3PO_4) (65:35, v/v) with 1.0 mL/min flow rate, and the detection wavelength was set at 294 nm. The HPLC equipment for DSI analysis was controlled using a U3000 pump (ThermoFisher Scientific, New York, US) using a ZORBAX SB-C18 column (4.6 × 250 mm, 5 μm) (Agilent Technologies, Beijing, China) at 40 °C. Elution was performed with the solvents (A) CH_3CN and (B) 0.05% (v/v) trifluoroacetic acid. Separation was initiated using 2% A and 98% B with a linear gradient to 30% A and 70% B over 65 min; 30% A and 70% B to 2% A and 98% B from 65 to 66 min; and 2% A to 98% B for 24 min (66 to 80 min). The flow rate was 0.8 mL/min and the detection wavelength was set at 288 nm.

4.3. Cell culture

Caco-2 cells were obtained from the Shanghai Institute of Cell Bank and were grown in cell culture flasks in DMEM supplemented with 10% (v/v) heat-inactivated FBS, 1% (v/v) non-essential amino acids solution, 1% (v/v) l-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37 °C under an atmosphere with 5% CO_2. Caco-2 cells were inoculated onto Millicell suspension cell culture inserts in 12-well plastic plates at a density of 1×10^5 cells/cm^2 for approximately 21 days. Transepithelial electrical resistance (TEER was performed to assess the integrity of the cell monolayer by using a Millicell ERS-2 instrument (Millipore, Shanghai, China). When the TEER value was above 350 Ω.cm^2, the cells were considered intact and suitable for transport studies (Chen et al. 2014).

4.4. Cytotoxicity test

The cytotoxic effects of the various solutions were evaluated using the MTT cytotoxicity method. DSI (1.5 g crude drug/mL) was diluted to 240, 180, 120, 60, 30, 15, and 7 mg crude drug/mL with DMEM, and 50 mg of gliquidone was dissolved in 1 mL of DMSO and then diluted to 240, 160, 100, 40, and 10 μg/mL with DMEM. Caco-2 cells were seeded into 96-well plates at a density of 10,000 cells/well. After 12 h, the medium was replaced with the different concentrations of DSI and gliquidone for 12 h. Then, MTT was added to each well, and the cells were cultured for 4 h. The OD value was measured by using a microplate reader (Molecular Devices, San Jose, USA) at a wavelength of 490 nm, and cell viability (%) was calculated as the percentage of control.

4.5. Transport experiments

After washing the inserts three times with warm HBSS buffer for 20 min, DSI (30, 60 or 120 mg crude drug/mL) was added at both the AP and BL sides of Caco-2 monolayer cells. HBSS containing gliquidone (100 μg/mL) was added either to the AP or BL compartment (0.75 or 1.75 mL respectively), and transport experiments were

performed at 37 °C under an atmosphere containing 5% CO_2. Samples (0.3 or 0.6 mL) were collected after 30, 60, 90, 120, 150 and 180 min from the receiver compartment, which were immediately replaced with an equal volume of fresh, pre-warmed HBSS. In experiments designed to examine the effect of the active ingredients in DSI on the transport of gliquidone, DSI was replaced with DSS (75 μg/mL), PA (20 μg/mL), RA (30 μg/mL), or SAB (25 μg/mL) in HBSS. These concentrations were equal to the concentration of these compounds in DSI (120 mg crude drug/mL).

To investigate the roles of P-gp, MRP1, MRP2, MRP3 and BCRP in the transport of gliquidone, verapamil (a selective P-gp inhibitor, 25 μg/mL), Ko143 (a BCRP inhibitor, 5 μg/mL), MK571 (an MRP1 inhibitor, 6 μg/mL), probenecid (an MRP2 inhibitor, 15 μg/mL), or benzbromarone (an MRP3 inhibitor, 200 μg/mL) were added at both the AP and BL sides of Caco-2 cell monolayers, with gliquidone (100 μg/mL) added to either the AP or BL side (0.75 or 1.75 mL respectively). As a control, gliquidone transport was also assessed in the absence of any inhibitor.

To study the mechanism by which RA affects the transport of gliquidone, RA (5, 15, or 30 μg/mL, equal to the concentrations of RA in DSI of 30, 60, 120 mg crude drug/mL) were added at both the AP and BL sides of Caco-2 cell monolayers. Rho123 (P-gp substrate) and Ho33342 (BCRP substrate) were added to either the AP or BL side (0.75 or 1.75 mL respectively), with HBSS buffer was added to the other side. As a control, the transport of Rho123 and Ho33342 was assessed in the absence of RA.

Samples were collected from both sides of Caco-2 cell monolayers and used for HPLC analysis. The Papp and ER values were calculated as previously reported (Liang et al. 2012).

4.6. Intracellular accumulation of Rho123 and Ho33342

For the intracellular accumulation analysis, Caco-2 cells attached to the surfaces of 24-well plates were treated with Rho123 or Ho33342 or combined with RA for 3 h. Subsequently, cells were washed with PBS, lysed in RIPA lysis buffer and the concentrations of Rho123 or Ho33342 were determined using a fluorescence spectrophotometer (Hitachi, Tokyo, Japan) at wavelengths of 488 nm (excitation)/530 nm (emission) for Rho 123 and 350 nm (excitation)/461 nm (emission) for Ho33342.

4.7. Immunofluorescence detection of P-gp and BCRP by fluorescence microscopy

Cells were harvested for immunofluorescence analysis following exposure to drugs for 3 h. Cells grown on coverslips were fixed with 4% (v/v) paraformaldehyde for 30 min at room temperature and then permeabilized with 0.5% (v/v) Triton-100 at 25 °C for 15 min. The cells were then covered with goat serum for 1 h at room temperature followed by incubation with diluted primary antibody against P-gp (Cusabio, Wuhan, China) and BCRP (Abbkine, Wuhan, China) at 4 °C overnight. Cells were then probed with a cyanine-3 conjugated secondary antibody (Abbkine, Wuhan, China) at room temperature for 2 h in the dark. Subsequently, cells were washed with PBS and incubated with DAPI for another 5 min. Images were taken using a DMi8 fluorescence microscope. Ten images per slide were quantitatively assessed using Image J (Media Cybernetics, USA), and the expression level of the target protein was evaluated by calculating the optical density value in the visual field and was defined as integral optical density (IOD).

4.8. Detection of P-gp and BCRP protein expression by Western blot analysis

Cells were harvested for Western blot analysis following exposure to drugs for 3 h. Equal amounts of protein were resolved by SDS-PAGE and transferred onto PVDF membranes. The membranes were then incubated at 4 °C overnight with an anti-P-gp (Cusabio, Wuhan, China) or an anti-BCRP antibody (Abbkine, Wuhan, China), with a GAPDH antibody (Abbkine, Wuhan, China) used as the loading control, and the protein bands were visualized by ECL (ThermoFisher Scientific, New York, US). Images were collected using an Amersham Imager 600 instrument (GE, Boston, USA), and the gray values were analyzed using ImageJ.

4.9. P-gp ATPase activity assay

The P-gp activity was assessed using a Pgp-Glo™ Assay System (Promega, Madison, USA), which included Pgp-Glo assay buffer, ATP detection substrate (lyophilized), recombinant human P-gp membranes, MgATP, ATP detection buffer, Na_3VO_4, and verapamil. A recombinant human P-gp membrane fraction was incubated in Pgp-Glo assay bu-er with MgATP (25 mmol/L) for 40 min at 37 °C. Untreated (NT) and Na_3VO_4- (0.25 mmol/L) treated control samples were included with the samples treated with verapamil- (0.5 mmol/L) and RA (5, 15, 30 μg/mL). The samples were measured using a microplate reader (Thermo Scientific, New York, US). The calculations of the luminescent signals were on the basis of the manufacturer's specifications, and the influence of the test drug on P-gp ATPase activity was then estimated.

4.10. Statistical analysis

Data were expressed as means±standard deviation (SD). Significant differences were determined using one-way analysis of variance (ANOVA), with the significance level set at 0.05.

Acknowledgements: This work was supported by the Natural Science Foundation of Anhui Province (1708085MH216) and National Natural Science Found of China (81874348, 81102682).

Conflict of interest: The authors declare that there is no conflict of interest regarding the publication of this paper.

References

Adomako-Bonsu AG, Chan SL, Pratten M, Fry JR (2017) Antioxidant activity of rosmarinic acid and its principal metabolites in chemical and cellular systems: Importance of physico-chemical characteristics. Toxicol in vitro 40: 248-255.

Alnaggar ARLR, Sayed M, El-Deena KE, Gomaa M, Hamed Y (2019) Evaluation of serum adiponectin levels in diabetic nephropathy. Diab Metabol Syndrome 13: 128-131.

Alqahtani S, Bukhari I, Albassam A, Alenazi M (2018) An update on the potential role of intestinal first-pass metabolism for the prediction of drug-drug interactions: the role of PBPK modeling. Expert Opin Drug Metab Toxicol 14: 625-634.

Chen Z, Ma T, Huang C, Zhang L, Zhong J, Han J, Hu T, Li J (2014) Efficiency of transcellular transport and efflux of flavonoids with different glycosidic units from flavonoids of Litsea coreana L in a MDCK epithelial cell monolayer model. Eur J Pharm Sci 53: 69-76.

Confederat L, Constantin S, Lupascu F, Panzariu A, Hanzianu M, Profire L (2015) Hypoglycemia induced by antidiabetic sulfonylureas. Rev Med Chir Soc Med Nat Iasi 119: 579584.

Einarson TR, Acs A, Ludwig C, Panton UH (2018) Economic burden of cardiovascular disease in type 2 diabetes: a systematic review. Value Health 21: 881-890.

Fromm MF, Kauffmann HM, Fritz P, Burk O, Kroemer HK, Warzok RW, Eichelbaum M, Siegmund W, Schrenk D (2000) The effect of rifampin treatment on intestinal expression of human MRP transporters. Am J Pathol 157: 1575-1580.

Hunter J, Hirst BH, Simmons NL (1993) Drug absorption limited by P-glycoprotein-mediated secretory drug transport in human intestinal epithelial Caco-2 cell layers. Pharm Res 10: 743-749.

Ledwitch KV, Gibbs ME, Barnes RW, Roberts AG (2016) Cooperativity between verapamil and ATP bound to the efflux transporter P-glycoprotein. Biochem Pharmacol 118: 96-108.

Li FR, Fu YY, Jiang DH, Wu Z, Zhou YJ, Guo L, Dong ZM, Wang ZZ (2013) Reversal effect of rosmarinic acid on multidrug resistance in SGC7901/Adr cell. J Asian Nat Prod Res 15: 276-285.

Li K, Lai H (2017) TanshinoneIIA enhances the chemosensitivity of breast cancer cells to doxorubicin through down-regulating the expression of MDR-related ABC transporters. Biomed Pharmacol 96: 371-377.

Li XX, Zhou ZW, Zhou SF (2008) Role of P-glycoprotein in the transport of tanshinone I, one active triterpenoid from Salvia miltiorrhiza. Drug Metab Lett 2: 223-230.

Liang XL, Zhao LJ, Liao ZG, Zhao GW, Zhang J, Chao YC, Yang M, Yin RL (2012) Transport properties of puerarin and effect of Radix Angelicae Dahuricae extract on the transport of puerarin in Caco-2 cell model. Journal Ethnopharmacol 144: 677-682.

Malaisse WJ (2006) Gliquidone contributes to improvement of type 2 diabetes mellitus management: a review of pharmacokinetic and clinical trial data. Drugs in R&D 7: 331-337.

Mei XD, Cao YF, Che YY, Li J, Shang ZP, Zhao WJ, Qiao YJ, Zhang JY (2019) Danshen: a phytochemical and pharmacological overview. Chin J Nat Med 17: 59-80.

Meisel C, Johne A, Roots I (2003) Fatal intracerebral mass bleeding associated with Ginkgo biloba and ibuprofen. Atherosclerosis 167: 367

Obach RS (2000) Inhibition of human cytochrome P450 enzymes by constituents of St John's Wort, an herbal preparation used in the treatment of depression. J Pharmacol Exp Ther 294: 88-95.

Rahbardar MG, Amin B, Mehri S, Mirnajafi-Zadeh SJ, Hosseinzadeh H (2018) Rosmarinic acid attenuates development and existing pain in a rat model of neuropathic pain: An evidence of anti-oxidative and anti-inflammatory effects. Phytomedicine 40: 59-67.

Rybka J, Gregorová A, Zmydlená A (1988) Clinical experience with Glurenorm in type 2 diabetics with renal insufficiency. Vnitřní lékařství 34: 786-792.

ViswanathanV, Smina TP (2019) Blood pressure control in diabetes-the Indian perspective. J Hum Hypertens 33: 588-593.

Verschraagen M, Koks CH, Schellens JH, Beijnen JH (1999) P-glycoprotein system as a determinant of drug interactions: the case of digoxin-verapamil. Pharmacol Res 40: 301-306.

Wang L, Sweet DH (2012) Active hydrophilic components of the medicinal herb Salvia miltiorrhiza (Danshen) potently inhibit organic anion transporters 1 (Slc22a6) and 3 (Slc22a8). Evid Based Complement Alternat Med 2012: 872458.

Weber CC, Kressmann S, Fricker G, Müller WE (2004) Modulation of P-glycoprotein function by St John's wort extract and its major constituents. Pharmacopsychiatry 37: 292-298

Wu J, Zhu Y, Li F, Zhang G, Shi J, Ou R, Tong Y, Liu Y, Liu L, Lu L, Liu Z (2016) Spica prunellae and its marker compound rosmarinic acid induced the expression of efflux transporters through activation of Nrf2-mediated signaling pathway in HepG2 cells. J Ethnopharmcol 193: 1-11

Yuan CS, Wei G, Dey L, Karrison T, Nahlik L, Maleckar S, Kasza K, Ang-Lee M, Moss J (2004) Brief communication: American ginseng reduces warfarin's effect in healthy patients: a randomized, controlled Trial. Ann Int Med 141: 23-27

Yu C, Chen DQ, Liu HX, Li WB, Lu JW, Feng JF (2019) Rosmarinic acid reduces the resistance of gastric carcinoma cells to 5-fluorouracil by downregulating FOXO4-targeting miR-6785-5p. Biomed Pharmacother 109: 2327-2334.

Zhang X, Yang L, Xu X, Tang F, Yi P, Qiu B, Hao Y (2019) A review of fibroblast growth factor 21 in diabetic cardiomyopathy. Heart failure reviews. DOI: 10.1007/s10741-019-09809-x

Zakeri-Milani P, Valizadeh H (2014) Intestinal transporters: enhanced absorption through P-glycoprotein-related drug interactions. Expert Opin Drug Metab Toxicol 10: 859-871.