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Original Article

Traditional Chinese herbal medicine *Astragalus Radix* and its effects on intestinal absorption of aconite alkaloids in rats

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ABSTRACT

Objective: *Astragalus Radix* (AR, Huangqi in Chinese) has been widely used as a *qi* (energy) restoring herb that is thought to act through reinvigorating the spleen and lung. Aconite is used to rebalance the body temperature during illness and played an irreplaceable role in disease control since ancient times, but it is limited by its strong neuro and cardiotoxicity. Since the Song Dynasty (1227), the two herbs have been commonly used as herbal pairs including in the famous Qifu Decotion, from the "*Wei's Family Prescription*". However, many ancient texts also record that they are not compatible using together, suggesting they can have negative outcomes when mixed. This study investigated whether *Astragali Radix* had either positive or negative effects on absorption of six different active alkaloids derived from aconite.
Methods: Single intestinal perfusion model was used to study the effects of *Astragali Radix* on aconite alkaloids absorption. Response of ABC transporters and distribution of three tight junction proteins on the surface of intestinal enothelium were assessed by Reverse Transcription-Polymerase Chain Reaction (RT-PCR), Western blot and immunofluorescence microscopy, respectively.
Results: The results showed that aconite alkaloids absorption could be inhibited, and different concentrations of *Astragali Radix* considerably increased the expression levels of the ABC transporters and tight junction proteins with *Astragali Radix* treatment.
Conclusion: These results suggest that *Astragali Radix* can block absorption of aconite alkaloids through the upregulation expression of ATP-binding cassette transporters (ABC transporters) and tight junction proteins. It demonstrates that co-administration of *Astragali Radix* with other drugs might change the absorption profile of the second drug which is important to know in clinic therapy.

1. Introduction

Astragalus Radix (AR, Huangqi in Chinese) is often used as a *qi*-invigorating herb in traditional Chinese medicine (TCM). It is thought to exert function through invigorating the spleen and lung which has an extensive and a long history clinical application (Chen et al., 2014). In TCM, the spleen is considered an important immune organ with the functions of nourishing and sustaining life. At the same time, it also has the functions of defending the body against diseases, the external environment and maintaining core body processes at equilibrium (Bronte and Pittet, 2013; de Matos Filho and Petroianu, 2015; Lewis et al., 2019). Modern medicine believes that the small intestine is the first barrier to defend the body against ingested harmful substance (Grant et al., 2015; Margarete, 2011). Since the spleen has a key role in maintaining the body equilibrium, we considered the possibility that the spleen is involved in supporting the small intestine to block absorption of harmful substance.

Aconite from *Aconiti Lateralis Radix Praeparata* (Fuzi in Chinese, FZ), has been known as the first drug to revive the *yang* for resuscitation and has played an important role in disease control since ancient times. FZ treatment leads to effects of expanding blood vessels, reducing inflammation as well as having anti-tumor properties (Wu et al., 2018), leading to its widespread use in the treatment of cardiovascular and digestive diseases. As an example, there are 113 prescriptions in the book "*Treaties of Febrile and Miscellaneous Diseases*" (Zhong-jing Zhang, Eastern Han Dynasty) and

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21 prescriptions contained aconite, despite use of the herb being known to cause side-effects. Along with the widespread use of FZ, it is regularly used in combination with other TCMs and/or modern drugs (Peter et al., 2013), increasing the chance of negative drug interactions and changed drug absorption properties in combination (Zhang et al., 2016; Zhu et al., 2014). AR and FZ are commonly used as a herbal pair, such as in the Qifu Decotion, a famous preparation in TCM first mentioned in "Wei's Family Prescription" (Song Dynasty). However, texts such as "The explanation of medicinal processing of Leigong" (Shi-cai Li, Ming Dynasty) and the book of "*Medica Truth*" (Qing Dynasty) indicate that the efficacy of FZ is reduced in the presence of AR. Supporting these texts, modern research shows that AR can reduce the toxicity of FZ (Zhang et al., 2018). But the mechanism by which AR limits FZ activity is not known. We hypothesized that AR, through its activation of splenic function, could impact on the absorption of FZ and thus reduce its potency. At present, the compatibility mechanism of FZ and AR has not been reported, and intestinal absorption is the key link of curative effect of TCM. Therefore, to fully understand the absorption characteristics of FZ and the influence mechanism of AR on FZ absorption can provide theoretical basis for guiding clinical medication of TCM, enrich the TCM theory of "spleen dominating *wei*", and explain that the biological essence of AR in FZ intestinal absorption will promote the clinical application of TCM.

For an orally administered drug, there are many barriers for drug absorption, with drug efflux transporters and the tight junction that forms between cells in the small intestine considered to be key factors that determine the rate of drug absorption. Efflux drug transporters linked to determining the rate of drug absorption in the small intestine include ABC transporters P-gp, BCRP and MRP2 (Wu et al., 2018). All three transporters are expressed on the intestinal epithelial layer and they are thought to be active in the efflux of drugs and toxicity substances to the intestinal lumen. The tight junction of the small intestinal mucosa cells is made up of Occludin, claudin, ZO-1, ZO-2, ZO-3 and cytoskeletal proteins (Arias et al., 2014; Ali et al., 2014). The permeability of the small intestinal mucosa is known to affect the amount of drug in the blood, while the permeability of the intestinal mucosa can change under certain conditions. Here, using a rat intestinal perfusion model we investigated whether AR reduces the toxic effects of FZ through blocking its absorption and reducing the concentration of FZ in the blood. We investigated whether changes in absorption could be due to changes in efflux drug transporter activity and/or cell tight-junction formation in the intestinal epithelial cells with AR administration.

2. Materials and methods

2.1. Reagents and chemicals

Astragali Radix (AR) (batch:161213) and *Aconiti Lateralis Radix Praeparata* (batch:20150601) were all purchased from Jiangxi Jiangzhong Traditional Chinese Medicine Decoction Co., Ltd., and were identified by Professor Shou-wen Zhang of Jiangxi University of Traditional Chinese Medicine. Hypaconitine, new aconitine, aconitine, benzoylhypaconine, benzoylmesaconine, benzoylaconine and astragaloside IV were obtained from the National Institute for Food and Drug Control (Beijing, China).

Phosphate buffered saline (PBS), Coomassie brilliant blue G-250, bovine serum albumin (BSA), and SDS-PAGE Gel Preparation Kits were all purchased from Beyotime Biotechnology (Shanghai, China). 5X protein loading buffer and Western Transfer Buffer were purchased from Beyotime Biotechnology (Shanghai, China). Radioimmunoprecipitation assay (RIPA) lysis buffer, protease inhibitors, phosphatase inhibitors, the bicinchoninic acid (BCA) Protein Assay Reagent kit, and the enhanced chemiluminescence (ECL) kit were purchased from Beijing Com Win Biotech Co., Ltd. (Beijing, China). A rabbit monoclonal anti-P-gp (MDR) antibody, a mouse monoclonal anti-multidrug resistance-associated protein2 (MRP2) antibody, and a rabbit monoclonal anti-breast cancer resistance protein (BCRP) antibody were purchased from Abcam (Cambridge, United Kingdom). A rabbit monoclonal anti-Claudin 1 antibody, a rabbit monoclonal anti-Occludin antibody, and a rabbit monoclonal anti-ZO-1 antibody were all purchased from Boster Biological Technology Co., Ltd (Wuhan, China). A Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mouse monoclonal antibody was purchased from Shanghai Bioleaf Biotech Co., Ltd. (Shanghai, China). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and HRP-labeled goat anti-rabbit IgG (H + L) were purchased from Beyotime Biotechnology (Shanghai, China). Trizol RNA extraction kit was purchased from Ambion (Thermo Fisher Scientific, Beijing, China). The reverse transcription kit was purchased from Promega (Madison, WI, USA). Power SYBR Green PCR Mix was purchased from Life Technologies, Inc. PCR primers were synthesized by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). All other chemicals and solvents were of analytical grade or above and used as received.

Male Sprague-Dawley rats (200–220 g, body weight) for the intestinal absorption study were obtained from the animal center of Jiangxi University of Traditional Chinese Medicine (Nanchang, China). The animals were housed in a room that was well ventilated and had a regular 12:12 h light:dark cycle throughout the experimental period. During this period all mice were given a commercial diet and water *ad libitum*. All animal studies were done according to the approved protocols and guidelines of the Institutional Animal Ethical Care Committee (NIPER). All efforts were made to minimize the number of animals used and their suffering.

2.2. Preparation of AR and Aconiti Lateralis Radix Praeparata water extract

AR and *Aconiti Lateralis Radix Praeparata* (FZ) decoctions were all prepared according to a method which has been established previously (Kong et al., 2017). The purity of the main bioactive compounds in AR and FZ were determined via high performance liquid chromatography (HPLC). The content of pureonebio and astragaloside IV in AR extract was 0.034% and 0.225%, respectively. The content of monoester alkaloids and diester alkaloids in FZ extract was 4.56% and 0.40%, respectively. Both extracts were stored at −20 °C for later use.

2.3. Effect of AR on intestinal absorption of six alkaloids of FZ by single-pass perfusion model

Fasted (16–18 h) male Sprague-Dawley rats were anesthetized with 10% chloral hydrate (3.4 mL/kg by intraperitoneal injection). Single-pass intestinal perfusion studies were carried out using an experimental design similar to that described by ourself before. The rats were fixed on a plate and body temperature was maintained at 37 °C using an overhead work-light and a heating mat. The abdominal cavity was opened along the middle line of abdomen and the intestinal segment to be inspected was separated. The intestinal segment was kept moist throughout the experiment by gently applying buffer using cotton wool balls saturated with warm saline, and was washed cleanly using constant temperature physiological saline at a flow rate of 5 mL/min. Test solution (50 mL) was preheated to 37 °C and irrigated for 10 min at a flow rate of 1 mL/min before reducing the flow rate to 0.2 mL/min for 30 min. The perfusate samples were collected at 15, 30, 45, 60, 75, 75, 90, 105 and 120 min before analysis of the collected samples for drug content by liquid chromatograph mass spectrometer

(LC-MS). The detection method was described previously (Kong et al., 2017), and the chemical structures of tested compounds has been showed in Fig. S1. Blood samples were taken at 120 min from hepatic portalvein, centrifuged to separate the plasma which was then frozen until LC-MS analysis. The rat was then euthanized and the perfused intestinal segment removed and its length and internal diameter was measured.

The characterization of intestinal absorption of six alkaloids of FZ and the intestinal absorption effects of AR extract on them was studied by the experimental method above. Correction for water flux was performed in outgoing drug concentration by the method previously described by Ali et al. (Ali et al., 2014). The intestinal permeability (Papp) of drugs was determined using the following mathematical expression (Dixit et al., 2012):

$$P_{app,G} = \frac{-Q\ln\left(\frac{C_{out(corr)}}{C_{in}}\right)}{2\pi rl}$$

where r is the luminal radius of intestinal segment and l is the mean length of perfused duodenum segment. $C_{out(corr)}$ is the correct content of drug solution of exit, C_{out} is the content of drug solution of exit. C_{in} is the perfusion drug content of intestinal inlet (ug/mL). Q is the perfusion rate (mL/min).

2.4. Dosage regimen and intestinal tissue sample processing of normal rats

A total of 20 SD rats with weighing 180–220 g were randomly divided into four groups. They were normal group, low dose group (3.33 g AR crude drug/kg), medium dose group (10 g AR crude drug/kg) and high dose group (30 g AR crude drug/kg), respectively. Rats were administered AR continuously for 7 d before being euthanized at day 8. A segment of the duodenum (10 cm) was removed, washed clean with 4 °C PBS and cut segments stored at −80 °C. Similarly, 10 cm of ileum was removed, washed and placed in an embedding box after cutting into segments. The box was immersed in 4% paraformaldehyde solution and stored at 4 °C.

2.5. Effect of AR extract on expression of ABC transporters (MRP2, PGP, BCRP) and tight junction (Claudin 1, Occludin, and ZO-1) proteins

Small pieces of duodenal tissue were precisely weighed and disintegrated using RIPA lysis buffer for 30 min, before being centrifuged for 5 min at 12000 rpm. The protein content of the supernatant was determined using the BCA kit according to the manufacturer's instruction. Protein samples were mixed with 5× loading buffer and were denatured at 100 °C for 3 min. An equal amount of protein (60 μg) was separated by SDS-PAGE (5% stacking and 10% separating gels) and then subsequently transferred from the gel to a polyvinylidene fluoride (PVDF) membrane. After blocking for 2 h with skim milk, the corresponding antibodies were added at appropriate dilutions and incubated with the membrane at 4 °C overnight. The membrane was washed before incubation with the corresponding secondary antibody at the appropriate dilution in the same buffer for 1 h at room temperature. Western blot signals were determined using an ECL chemiluminescence detection agent. The relative intensity of each protein band was scanned and densitometry was undertaken using ImageJ software.

2.6. Real-time PCR analysis

Total RNA of small pieces of duodenal tissue was extracted by using a Trizol kit according to the manufacturer's protocol. RNA yield was determined by measuring the optical density (OD) of the extracted RNA preparation at a wavelength of 280 nm using a micro-plate reader. Total RNA was stained with 0.5 g/mL ethidium bromide and analysed on a 1.0% agarose gel by gel electrophoresis to confirm RNA integrity, where three intact bands of 28S, 18S, 5S rRNA were observed under ultraviolet light. Total RNA (5 μg) was reverse transcribed into cDNA according using the reverse transcription kit according to the manufacturer's recommendations. SYBR Green real-time PCR amplification and detection were performed using an AB17500 fast system. All primer sequences were listed in Table 1. The real-time PCR amplification conditions were shown in Table 2. Relative mRNA levels were normalized against GAPDH mRNA levels, the method of $2^{-\triangle\triangle Ct}$ was adopted to calculate the relative expression of P-gp, MRP2, BCRP, Claudin 1, Occludin and ZO-1.

2.7. Measuring effect of AR extract on tight junction using immunofluorescence confocal microscopy

Ileum segments of rats were embedded with paraffin and cut into 5 μm slices, dew-axed and restored in a 96 °C water bath. Sealing was performed for 1 h at room temperature with 5% rabbit serum. Primary antibodies were added overnight at 4 °C, before secondary antibodies with FITC-labeled sheep anti-rabbit were added and incubated for 1 h (avoid light) , washed three times with 0.01 mol/L PBS and 5 min for one time. Samples were sealed with anti-fade mounting medium and observed using a laser co-aggregation microscope. Semi-quantitative analysis methods were used to analyse the immunofluorescence image using an image pro plus. In microscopy images, the positive expression marker green fluorescence and the nucleus was blue fluorescence.

ImageJ software was used for semi-quantitative analysis of immunofluorescence images. The calculation formula is as follows (L Zhang et al., 2015):

$$AOD = \frac{intDen}{Area} \quad (1)$$

where int*Den* is the integrated optical density (IOD) of the images, *Area* is the fluorescence region of the images, *AOD* is the average optical density.

The following formula is used to calculate AOD (%) of tight junction protein:

$$Relative\ AOD\ (\%) = \frac{AOD\ sample}{AOD\ control} \times 100 \quad (2)$$

2.8. Data analysis

All data presented in this study is the mean ± standard deviation (SD) from at least three independent experiments. Significant differences were analyzed using one-way ANOVA, followed by the

Table 1
Primers of GAPDH, P-gp, MRP2, BCRP, Claudin 1, Occludin, and ZO-1 for real-time PCR analysis.

Names of primer	Primer sequences (5′–3′)
GAPDH	F:TGGGTTTCCCGTTGATGA
	R:AGGGCTGCCTTCTCTTGT
P-gp	F:GCAGGTTGGCTGGACAGATT
	R:GGAGCGCAATTCCATGGATA
MRP2	F:TGATCGGTTTCGTGAAGAGCT
	R:ACGCACATTCCCAACACAAA
BCRP	F:GTTTGGACTCAAGCACAGCA
	R:TGAGTTTCCCAGAAGCCAGT
Claudin-1	F:CTCACAGAGAGGGGTCGTTG
	R:ACTGTTAGCGGCAGTTTGGT
Occludin	F:GGGGTGATTCGGATCCTGTC
	R:TCCTCCAAAGATGCCCGTTC
ZO-1	F:CCCTTACCTTTCGCCTGAAAC
	R:CCTTCGTCTCTGAGCATCGT

Table 2
Reaction conditions of real-time PCR.

Reaction phases	Temperatures/°C	Time	Cycle times
DNA dissociation	95	10 min	1
Annealing	95	5 s	
	60	10 s	45
extension	72	15 s	1

GraghPad 7.0 software. Statistical differences were considered significant at $P < 0.05$.

3. Results

3.1. Effect of AR on intestinal absorption of six alkaloids of FZ

We first looked at the effect of AR extract on the intestinal absorption of FZ alkaloids by assessing the remaining alkaloid concentration in the perfusate. Results from the single-pass intestinal perfusion experiments showed that AR can significantly reduce the intestinal absorption of six alkaloids of FZ in a dose dependent manner (Table 3). We next checked whether AR could interfere with absorption to the extent that blood plasma concentrations of the alkaloids would be reduced. The plasma concentration of all three alkaloids tested was significantly lower than that of the non-AR treated models (Table 4), confirming that the presence of AR does indeed inhibit the absorption of FZ alkaloids into the blood. Here only the plasma concentrations of three monoester alkaloids were detected since the diester-type alkaloids only occur at low concentrations in FZ, and were thus below our ability to detect in plasma.

3.2. Effect of AR on expression and mRNA levels of ABC transporters (P-gp, MRP2, BCRP) of normal rats

Since ABC transporters are key mechanisms by which the small intestine regulates the uptake of toxins, we tested whether AR could alter the expression levels of these transporters. We first analysed the relative mRNA levels of P-gp, MRP2 and BCRP in duodenal tissue in the presence of AR (Fig. 1). AR was found to significantly increase expression of P-gp and MRP2 in a dose-dependent manner ($P < 0.05$, $P < 0.01$). However, BCRP mRNA expression peaked at the lowest concentration of AR tested and then dropped away quickly.

We next quantitated the level of protein expression for the three ABC efflux transporters (P-gp, MRP2, BCRP) in the duodenal tissue of normal. Levels of P-gp, MRP2, BCRP protein increased significantly with higher AR concentrations compared to the non AR treated control group ($P < 0.05$ or $P < 0.01$). While there was a trend towards increasing expression of P-gp and MRP2 with higher doses of AR, BCRP expression peaked at the lower doses of AR and then dropped away significantly at higher doses, similar to the early expression profile seen with the mRNA expression which combined suggest that higher concentrations of AR may be antagonistic for BCRP expression. The results of the protein expression profiling support the mRNA expression data all three transporters and suggest that AR can upregulate the expression of these ABC transporters. Given that one of the functions of the ABC transporters is to efflux potential toxins, the increased expression of these proteins upon treatment with AR may contribute to reduced perfusion of FZ alkaloids into the blood plasma.

3.3. Regulation of protein and mRNA levels of proteins involved in formation tissue tight junctions (Claudin, Occludin-1, ZO-1) by AR

Cell boundaries in the intestinal tissue are a barrier for perfusion of toxins into the blood plasma. Here, we assessed the relative mRNA levels of the tight junction proteins Claudin-1, Occludin and ZO-1 in the duodenal tissue after treatment with AR. Compared with control group, AR was found to significantly ($P < 0.05$, $P < 0.01$) increase the mRNA levels of Occludin and ZO-1 in a dose-dependent manner (Fig. 2A). mRNA expression levels of Claudin-1, however, reached peak levels at the lowest AR concentration tested and maintained these levels at higher doses, suggesting that maximal expression levels of Claudin-1 are reached at low AR concentrations. We next examined changes in Claudin 1, Occludin and ZO-1 in the duodenal tissue of normal after oral administration of AR. Compared with the control group (without oral administration of AR), administration of AR led to a dose dependent increase in the protein levels of Occludin and ZO-1 ($P < 0.05$ or $P < 0.01$) (Fig. 2B and 2C). Mirroring changes seen in mRNA levels, levels of Claudin 1 protein rapidly increased to peak levels at the lowest dose and then maintained that level at the higher concentrations, with no true dose-dependent relationship evident. An important function of these tight junction proteins is to regulate the permeability of the boundary between cells in the intestinal epithelium tissue. An increase in levels of these proteins after AR administration could decrease the permeability along these boundaries and intestinal absorption, limiting the absorption of compounds across this barrier.

3.4. Immunofluorescence assessment of expression and localization of tight junction proteins Claudin-1, Occludin and ZO-1 after AR treatment

In order to assess where the increased expression of tight junction proteins might be occurring in rat duodenal tissue after AR treatment, we fixed and stained the tissue with antibodies against Claudin-1, Occludin and ZO-1 and detected the expression of these proteins using a FITC secondary antibody and a confocal microscope. Tissue was imaged using a 40× objective and DAPI was used to detect nuclear DNA of the cells. For non-AR treated control tissues, Claudin-1, Occludin and ZO-1 typically located on the cytoplasm and membranes of monolayer columnar epithelial cells, while the cytoplasm of goblet cells did not stain with antibodies to these tight junction proteins (Fig. 3A). The 4–6 μm monolayer columnar epithelial cells are closely arranged and the gap between cells can be seen faintly in non-AR treated control samples. After

Table 3
Effect of AR on intestinal absorption of six ester alkaloids on FZ (mean ± SD, $n = 5$).

Groups	Drug concentrations/ ($mg \cdot kg^{-1}$)	Papp/($\times 10^{-5}$cm × s^{-1})					
		benzoylhypaconine	benzoylmesaconine	benzoyl aconitine	hypaconitine	mesaconine	aconitine
FZ Alkaloid	360	7.77 ± 2.19	7.56 ± 2.16	7.63 ± 2.12	6.60 ± 1.69	7.37 ± 1.72	8.36 ± 2.26
FZ Alkaloid +AR extract	360 + 120	3.21 ± 0.54**	3.21 ± 0.50**	3.15 ± 0.60**	4.32 ± 1.69	4.42 ± 1.38*	5.20 ± 0.48*
	360 + 360	3.07 ± 1.01**	3.20 ± 1.10*	3.26 ± 8.25**	2.98 ± 1.18*	3.17 ± 1.15**	5.02 ± 1.15*
	360 + 1080	2.83 ± 0.29**	3.09 ± 0.70**	2.93 ± 0.38**	3.14 ± 0.82*	3.51 ± 0.82**	4.54 ± 0.72*

*$P < 0.05$, **$P < 0.01$ *vs* FZ group.

Table 4
Effects of AR on plasma concentrations of three monoester alkaloids in FZ in normal rats (mean ± SD, n = 5).

Groups	Crude drug concentrations/(mg·kg^{-1})	Plasma concentrations / (ng·mL^{-1})		
		Benzoylhypaconine	Benzoylmesaconine	Benzoyl aconitine
FZ Alkaloid	360	3.741 ± 1.144	9.670 ± 2.833	2.042 ± 0.562
FZ Alkaloid:AR extract	360 + 120	1.789 ± 0.923*	4.856 ± 2.300*	1.067 ± 0.455*
	360 + 360	2.034 ± 0.693*	5.241 ± 1.909*	1.124 ± 0.380*
	360 + 1080	2.010 ± 0.533*	5.251 ± 1.493*	1.162 ± 0.289*

*P < 0.05 *vs* FZ group.

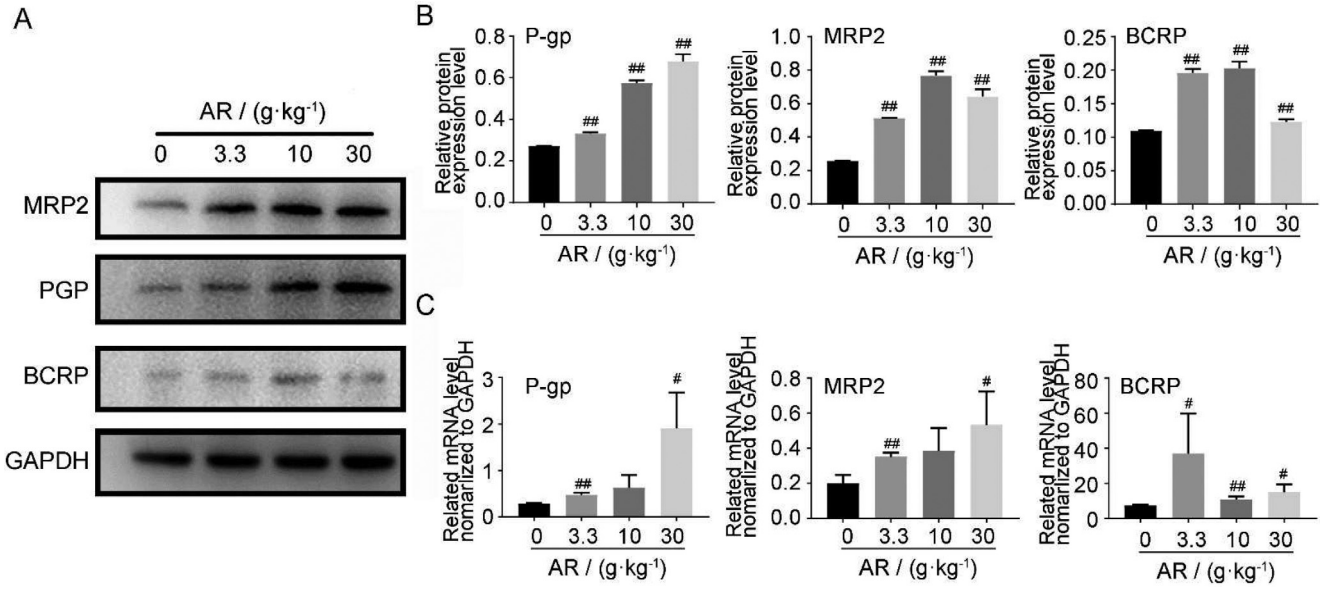

Fig. 1. Effect of AR on expression and mRNA levels of ABC transporters (P-gp, MRP2, BCRP) of normal rats. The protein levels of P-gp, MRP2, BCRP by Western blotting analysis (A); The semi-quantitative results protein levels of P-gp, MRP2, BCRP of Western blot analysis (B); mRNA levels of P-gp, MRP2 and BCRP (C). $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01 *vs* control group (without AR).

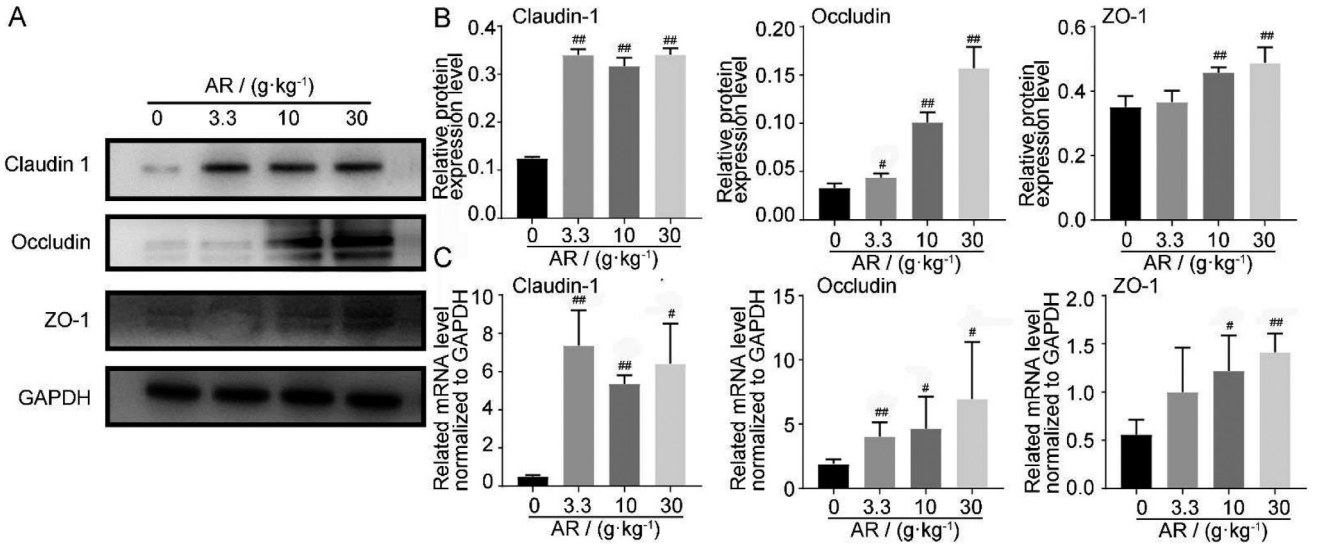

Fig. 2. Effect of AR on mRNA and protein levels of tight junction proteins Claudin, Occludin-1 and ZO-1 of in duodenal tissue of normal rats treated with AR. mRNA levels of Claudin, Occludin-1, ZO-1 (A); Protein levels of Claudin, Occludin-1, ZO-1 represented by Western blot analysis (B) and densitometry of Western blot signal for Claudin, Occludin-1 and ZO-1 (C). $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01 *vs* control group (without AR).

oral administration of AR, green fluorescence staining of Claudin-1, Occludin and ZO-1 in the intestinal villi of rats treated with AR was found to increase, with increased but weak staining of goblet cells was also evident. Increased staining for the tight junction proteins was evident in low, medium and high AR dose groups for Claudin, Occludin and ZO-1. Of note, areas of increased peripheral Occludin staining were evident for some cells at a medium concentration of AR. Quantification of fluorescence signal confirmed a dose-

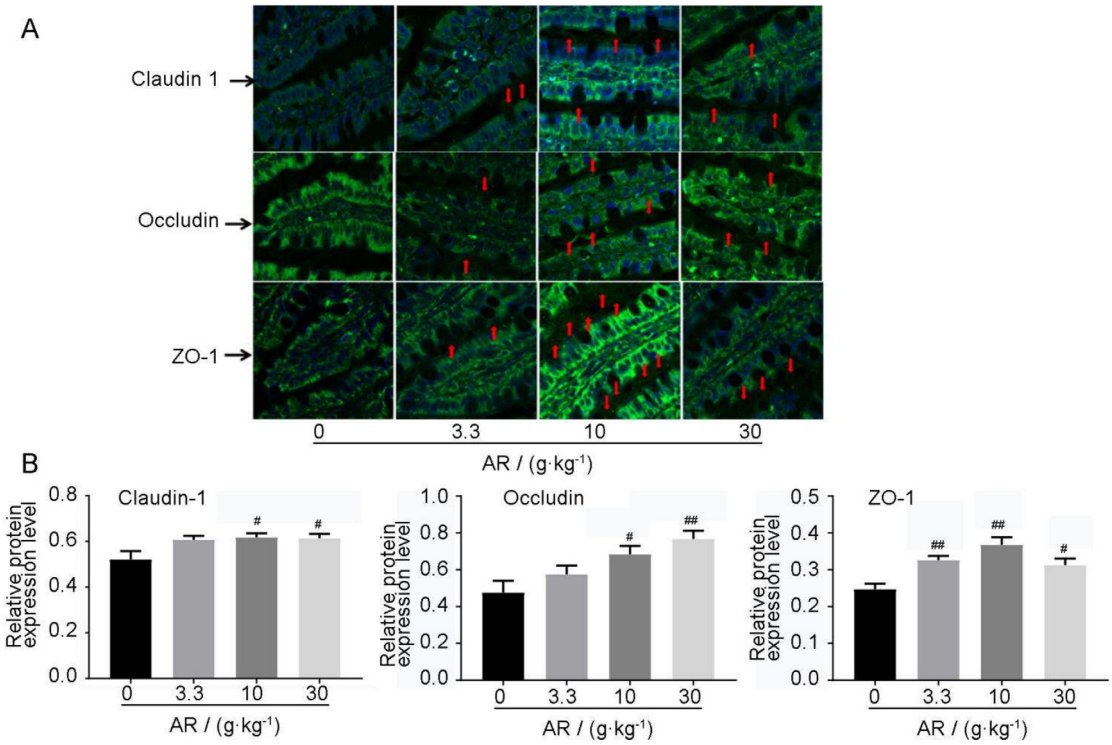

Fig. 3. Immunofluorescence analysis of effects of AR on tight junction proteins Claudin 1, Occludin and ZO-1 in rat duodenal tissue. Localization of three tight junction (Claudin-1, Occludin and ZO-1) proteins by AR in the rat duodenal. Red arrows refer to secretory protein (A). The bar graph illustrates the change in the expression of tight junction proteins with different doses of AR (B). Results are representative of three independent experiments. Cell nuclei were stained by DAPI and are shown in blue. Claudin-1, ZO-1 and Occludin are shown in green and pointed out by red arrow heads. Original magnification: 400×. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* control group (without AR).

dependent increase in levels of Occludin and ZO-1 (Fig. 3B). As seen for both mRNA and Western blot assessment of protein expression, the fluorescence signal for Claudin-1 peaked from high levels at the lowest dose of AR and maintained these levels at higher doses. Also of interest is that a general thickening of the mucus layer along the epithelial surface visibly thickened. In combination, the apparent thickening of the mucosal layer and increased expression of the tight junction protein Claudin-1, Occludin and ZO-1 with AR treatment could contribute to reduced intestinal absorption of other drugs.

4. Discussion

The theory of compatibility of traditional Chinese medicines refers to considering the combination of two or more herbs for treatment according to the need of illness and properties of herbs (Bi et al., 2014; Zhou et al., 2017). To be effective, there must be some positive interactions occurring between herbs when a combination therapy of Chinese herbs is applied. Positive interactions to be considered when trying to maximise benefits when treating with a combination of herbs include the whether the efficacy of the treatment increases or decreases (Guan et al., 2014; Zhou et al., 2018) and whether a combination of herbs can inhibit or eliminate toxic side effects (Xiu et al., 2015). More recently, combinations between traditional Chinese herbal medicines and modern drugs has become increasingly popular (Shi et al., 2019). Consequently, potential drug interactions that could impact on treatment efficacy when combining traditional and modern medicines should not be overlooked. Modern research indicates that common traditional Chinese medicines believed to nourish *qi* and invigorate the spleen such as *Ginseng Radix* et *Rhizoma*, *Atractylodis Macrocephalae Rhizoma* and *Glycyrhizae Preparata Radix* can inhibit the absorption of diester alkaloids and reduce the strong toxicity of aconite (You et al., 2015; Zhang et al., 2013). Research indicates that ginseng increases the excretion of diester toxic alkaloids of FZ by inducing the expression of P-gp (You et al., 2015) and *Atractylodis Macrocephalae Rhizoma* can reduce the toxicity of FZ by the means of inducing CYP3A to improve the metabolism of FZ. In addition, a decoction of Four Mild Drugs was found to inhibit the absorption of endotoxin (Ji et al., 2016). Inhibition of absorption of harmful substances is a function of the intestinal barrier which provides mechanical, microbial, chemical and immune barriers to drug absorption. These barriers could inhibit the absorption of harmful substances through means such as mechanical interception, reduced permeability, killing and immune clearance of foreign organisms amongst other means. In recent years a number of studies have reported that traditional Chinese herbal medicines believed to nourish *qi* and invigorate the spleen have the positive function of helping repair the mucosal barrier structure (Ji et al., 2016), chemical barrier (Zhang et al., 2014), and balancing the microbiome of splenectomized animals (Ji et al., 2016), with evidence that these medicines decrease the mucosal permeability of harmful substances. All of this research supports the concept that the "Spleen is the master and defender" and herbal medicines believed to improve splenic function can also impact on the efficacy of intestinal barriers (Ji et al., 2016; Zhang et al., 2014).

AR has the reputation of "invigorating *qi*" and is commonly used to provide *qi* to the spleen and lung. A major finding of this study is that AR could inhibit the intestinal absorption of six alkaloids of FZ. We found that treatment with AR up-regulated mRNA and protein expression of the ABC transporters P-gp, MRP2 and BCRP. ABC transporters commonly act as drug efflux transporters. In tumor tissue, the over expression and activity of ABC transporters increases tumor cell resistance to drugs (Breier et al., 2005; Zhang et al., 2015). A second major finding of this study is that

AR significantly increased the expression of proteins involved in formation of the tight junction which plays an important role at the boundary between apical and basolateral plasma membrane domains to prevent foreign bodies crossing the intestinal membrane between cells (Bachinger et al., 2019). Tight junctions are made up of transmembrane proteins (including Claudin and Occludin) and cytoplasmic proteins (such as ZO-1, ZO-2 and ZO-3) (Ortega-Olvera et al., 2018). Claudin-1 is a small transmembrane protein responsible for regulating the structure and function of the tight junction which is critical for epithelial barrier integrity (Wang et al., 2012). Occludin is a four transmembrane-helix integral membrane constituent protein of tight junction-strands (Cupri et al., 2015). ZO-1 is a key cytoplasmic protein assembled at tigh junctions that binds to Claudin and Occludin as well as to the actin cytoskeleton (Ding et al., 2017). Passive diffusion through tight intercellular junctions exists in the transport of most small molecules, and alkaloids such as FZ are no exception. AR increases the levels of mRNA and protein expression of the tight junction proteins Claudin-1, Occludin and ZO-1, potentially acting as a primary barrier against diffusion of solutes through the paracellular pathway (Bachinger et al., 2019).

Except the upregulation effects of ABC transporters and tight junction proteins we found that administration with AR could induce goblet cells to synthesize and secrete tight junction structural proteins and a thick layer of mucus was increasingly deposited on the surface of epithelium of the intestinal cavity (Fig. 3). The intestinal surface is covered with a protective mucus layer composed of mucoproteins which form a gel network structure. The composition and thickness of this mucosal layer determines the permeability of the mucus barrier to solutes and its protective efficacy against pathogens (Kamphuis, Mercier-Bonin, Eutamène, & Theodorou, 2017). Since the amount of mucus was observed to significantly increasing with AR administration, it is likely that the permeability of this barrier would reduce and its efficacy in preventing solute absorption would increase. Thus, our finding implicate treatment with AR as upregulating three physiological mechanisms that could impact on drug absorption, ABC transporters, the tight junction between intestinal epithelial cells and the gut epithelial mucosa.

Combined, the results of this study suggest that AR leads to physiological changes in the rat intestinal environment that could result in reduced absorption of drug like molecules, as indicated by inhibition of absorption for the six alkaloids tested in this study. Our findings provide evidence for a possible mechanism by which AR protects against FZ toxicity when used in traditional Chinese medicine as a herbal pair, such as in the Qifu Decotion (Zhang et al., 2018). A broader implication of these findings is that the uptake of a biologically active molecule, such as a modern anticancer drug, may be inhibited when used in combination with AR. However, further research needs to be undertaken to identify whether this property of AR is specific to compounds such as the alkaloids tested in this study or relevant to traditional or modern drugs in general.

Many ancient physicians have proposed that the ‘‘spleen is the master of the defense”, meaning that the spleen plays an important role in maintaining the bodies equilibrium and limiting negative interactions with the external environment. AR, a traditional medicine that has long been linked to healthy splenic function, appears to have more general properties in other organs that defend the body against external insult such as the small intestine. How might these traditional beliefs in Chinese medicine impact on modern medicine? Yan Li of the Ming Dynasty pointed out that ‘‘the spleen communicates with the small intestine” in his book of *Introduction to Medicine*. The results of this study support that traditional Chinese medicines promote activity of the spleen also modify the function of the small intestine. The small intestine is not only an important organ for digestion and absorption of nutrients, but also the first barrier for the body to resist endogenous and exogenous harmful substances (Greenwood-Van Meerveld et al., 2017). Thus, the spleens role as defender of the body in TCM and our understanding of the protective function of the small intestine in modern medicine could be linked, with the activity of TCM such as AR acting as conduits to promote both functions.

5. Conclusion

The present study has revealed that treatment with AR as upregulating three physiological mechanisms that could impact on drug absorption, ABC transporters, the tight junction between intestinal epithelial cells and the gut epithelial mucosa at least partly. It can also be found that the intestinal absorption mechanism of FZ may involve facilitated passive diffusion associated with the efflux transporters P-gp, MRP2 and BCRP. As a whole, FZ may be the substrate of them. In conjunction with results from previous studies along the direction of AR and FZ, these results provide updated information concerning the intestinal absorption process and the possible mechanism of these compounds.

Author contributions

XL contributed in collecting materials, design of the experiment and analysis of the data and drafted the paper. MJ contributed the experiment and instigation work, LC contributed to do the pharmacokinetics studies and critical reading of the manuscript, YL contributed in the identification of biological materials and running the part of laboratory work. XX and XL contributed to chromatographic analysis and critical reading of the manuscript. ZL contributed to the design of the experiments and DW contributed to the edition of the manuscript. All the authors have read the final manuscript and approved the submission.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References

Ali, M. A., Arnold, C. A., Singhi, A. D., & Voltaggio, L. (2014). Clues to uncommon and easily overlooked infectious diagnoses affecting the GI tract and distinction from their clinicopathologic mimics. *Gastrointestinal Endoscopy, 80*, 689–706.

Arias, A., Rigalli, J. P., Villanueva, S. S. M., Ruiz, M. L., Luquita, M. G., Perdomo, V. G., Vore, M., Catania, V. A., & Mottino, A. D. (2014). Regulation of expression and activity of multidrug resistance proteins MRP2 and MDR1 by estrogenic compounds in Caco-2 cells. Role in prevention of xenobiotic-induced cytotoxicity. *Toxicology, 320*, 46–55. https://doi.org/10.1016/j.tox.2014.03.007.

Bachinger, D., Mayer, E., Kaschubek, T., Schieder, C., Konig, J., & Teichmann, K. (2019). Influence of phytogenics on recovery of the barrier function of intestinal porcine epithelial cells after a calcium switch. *Journal of Animal Physiology and Animal Nutrition (Berl), 103*, 210–220.

Bi, X., Gong, M., & Di, L. (2014). Review on prescription compatibility of Shaoyao Gancao decoction and reflection on pharmacokinetic compatibility mechanism of traditional Chinese medicine prescription based on *in vivo* drug interaction of main efficacious components. *Evidence-Based Complementary and Alternative Medicine: eCAM, 2014* 208129.

Breier, A., Barancik, M., Sulova, Z., & Uhrik, B. (2005). P-glycoprotein–implications of metabolism of neoplastic cells and cancer therapy. *Current Cancer Drug Targets, 5*, 457–468.

Bronte, V., & Pittet, M. J. (2013). The spleen in local and systemic regulation of immunity. *Immunity, 39*, 806–818.

Chen, S. M., Tsai, Y. S., Lee, S. W., Liu, Y. H., Liao, S. K., Chang, W. W., & Tsai, P. J. (2014). Astragalus membranaceus modulates Th1/2 immune balance and activates PPARgamma in a murine asthma model. *Biochemistry and Cell Biology, 92*, 397–405.

Cupri, S., Graziano, A. C., Cardile, V., Skwarczynski, M., Toth, I., & Pignatello, R. (2015). A study on the encapsulation of an Occludin lipophilic derivative in liposomal carriers. *Journal of Liposome Research, 25*, 287–293.

de Matos Filho, A. S., & Petroianu, A. (2015). Spleen function after preservation in a physiological solution. *Journal of Surgical Research, 199*(2), 586–591. https://doi.org/10.1016/j.jss.2015.05.058.

Ding, C., Cong, X., Zhang, X. M., Li, S. L., Wu, L. L., & Yu, G. Y. (2017). Decreased interaction between ZO-1 and Occludin is involved in alteration of tight junctions in transplanted epiphora submandibular glands. *Journal of Molecular Histology, 48*, 225–234.

Dixit, P., Jain, D. K., & Dumbwani, J. (2012). Standardization of an *ex vivo* method for determination of intestinal permeability of drugs using everted rat intestine apparatus. *Journal of Pharmacological and Toxicological Methods, 65*, 13–17.

Grant, C. N., Mojica, S. G., Sala, F. G., Hill, J. R., Levin, D. E., Speer, A. L., et al. (2015). Human and mouse tissue-engineered small intestine both demonstrate digestive and absorptive function. American Journal of Physiology. *Gastrointestinal and Liver Physiology, 308*, G664–G677.

Greenwood-Van Meerveld, B., Johnson, A. C., & Grundy, D. (2017). Gastrointestinal Physiology and Function. *Handbook of Experimental Pharmacology Hazard, 239*, 1–16.

Guan, Y.-G., Liao, J.-B., Li, K.-Y., Li, Y.-C., Song, Y., Ling, J., & Su, Z.-R. (2014). Potential mechanisms of an antiadenomyosis chinese herbal formula Shaoyao-Gancao decoction in primary cell culture model. *Evidence-Based Complementary and Alternative Medicine, 2014*, 1–11. https://doi.org/10.1155/2014/982913.

Ji, Y. F., Wang, R. J., & Li, X. B. (2016). Research progress on chemical constituents and pharmacological effects of Sijunzi Decoction. *Chinese Traditional and Herbal Drugs*, 47.837-843.

Kamphuis, J. B. J., Mercier-Bonin, M., Eutamène, H., & Theodorou, V. (2017). Mucus organisation is shaped by colonic content: A new view. *Scientific Reports, 7*, 8527–8539.

Kong, X. Q., Jiang, Q. Y., Luo, Y., Liang, X. L., Huang, G. Y., Ji, M. M., et al. (2017). Effect of *Radix Astragali* and *Aconiti Lateralis Radix Preparata* on intestinal absorption of six kinds of alkaloids in *Aconiti Lateralis Radix Preparata*. *Chinese Traditional and Herbal Drugs, 48*, 4928–4934.

Lewis, S. M., Williams, A., & Eisenbarth, S. C. (2019). Structure and function of the immune system in the spleen. *Science Immunology, 4*, aau6085.

Margarete, S., & D.M. (2011). *The Digestive System*. Peking University Medical Publishing House.

Ortega-Olvera, J. M., Winkler, R., Quintanilla-Vega, B., Shibayama, M., Chavez-Munguia, B., Martin-Tapia, D., et al. (2018). The organophosphate pesticide methamidophos opens the blood-testis barrier and covalently binds to ZO-2 in mice. *Toxicology and Applied Pharmacology, 360*, 257–272.

Peter, K., Schinnerl, J., Felsinger, S., Brecker, L., Bauer, R., Breiteneder, H., et al. (2013). A novel concept for detoxification: Complexation between aconitine and liquiritin in a Chinese herbal formula ('Sini Tang'). *Journal of Ethnopharmacology, 149*, 562–569.

Shi, W. L., Zhao, J., Yuan, R., Lu, Y., Xin, Q. Q., Liu, Y., et al. (2019). Combination of *Ligusticum chuanxiong* and *Radix Paeonia* promotes angiogenesis in ischemic myocardium through Notch signalling and mobilization of stem cells. *Evidence-Based Complementary and Alternative Medicine: eCAM, 2019*, 7912402.

Wang, Hong-Bo, Wang, Peng-Yuan, Wang, Xin, Wan, Yuan-Lian, & Liu, Yu-Cun (2012). Butyrate enhances intestinal epithelial barrier function via up-regulation of tight junction protein claudin-1 transcription. *Digestive Diseases and Sciences, 57*(12), 3126–3135.

Wu, J. J., Guo, Z. Z., Zhu, Y. F., Huang, Z. J., Gong, X., Li, Y. H., et al. (2018). A systematic review of pharmacokinetic studies on herbal drug Fuzi: Implications for Fuzi as personalized medicine. *Phytomedicine, 44*, 187–203. https://doi.org/10.1016/j.phymed.2018.03.001.

Xiu, L. J., Sun, D. Z., Jiao, J. P., Yan, B., Qin, Z. F., Liu, X., et al. (2015). Anticancer effects of traditional Chinese herbs with phlegm-eliminating properties – An overview. *Journal of Ethnopharmacology, 172*, 155–161.

You, Q., Ma, Z. C., Wang, Y. G., Hu, D. H., Liang, Q. D., Xiao, C. R., et al. (2015). *Panax ginseng* inhibits intestinal absorption of toxic *Aconitum carmichaeli* alkaloids *in vitro*. *Chinese Journal of Integrative Medicine*. PMID: 26272549.

Zhang, B., Dou, Y. C., & Wang, C. J. (2014). Effect of Sijunzi decoction on TFF3 protein expression and TFF3 mRNA expression in intestinal mucosa of spleen deficiency rats. *Chinese Journal of Laboratory Diagnosis, 5*, 715–717.

Zhang, Guiyu, Ou, Rilan, Li, Fangyuan, Wu, Jinjun, Zheng, Liang, Tong, Yunli, & Lu, Linlin (2016). Regulation of drug-metabolizing enzymes and efflux transporters by *Astragali Radix* decoction and its main bioactive compounds: Implication for clinical drug–drug interactions. *Journal of Ethnopharmacology, 180*, 104–113. https://doi.org/10.1016/j.jep.2016.01.031.

Zhang, J. M., Liao, W., He, Y. X., He, Y., Yan, D., & Fu, C. M. (2013). Study on intestinal absorption and pharmacokinetic characterization of diester diterpenoid alkaloids in precipitation derived from Fuzi–Gancao herb-pair decoction for its potential interaction mechanism investigation. *Journal of Ethnopharmacology, 147*(1), 128–135. https://doi.org/10.1016/j.jep.2013.02.019.

Zhang, L., Du, S., Lu, Y., Liu, C., Wu, H., Yang, B., et al. (2015). Influence of puerarin, paeoniflorin, and menthol on structure and barrier function of tight junctions in MDCK and MDCK-MDR1 Cells. *Journal of Traditional Chinese Medicine, 2*, 111–119.

Zhang, T., Yu, H. H., Lin, Y., Li, X., Tan, L., Song, H., & P, Peng, Q. H., Wang, W., Liu, L., Chen, C., Cai, X. (2018). Combinative approaches of chemistry, pharmacology and toxicology for the optimal pharmaceutical preparation of an anti-arthritic Chinese medicine formulation QFJBT. *Digital Chinese Medicine, 1*, 289–301.

Zhang, Y. K., Wang, Y. J., Gupta, P., & Chen, Z. S. (2015). Multidrug resistance proteins (MRPs) and cancer therapy. *The AAPS Journal, 17*, 802–812.

Zhou, L., Zhou, C., Feng, Z., Liu, Z., Zhu, H., & Zhou, X. (2018). Triptolide-induced hepatotoxicity can be alleviated when combined with *Panax notoginseng* saponins and Catapol. *Journal of Ethnopharmacology, 214*, 232–239.

Zhou, M., Hong, Y., Lin, X., Shen, L., & Feng, Y. (2017). Recent pharmaceutical evidence on the compatibility rationality of traditional Chinese medicine. *Journal of Ethnopharmacology, 206*, 363–375.

Zhu, Q., Liu, Z., Li, P., & Cheng, Z. (2014). Drug interaction studies reveal that simotinib upregulates intestinal absorption by increasing the paracellular permeability of intestinal epithelial cells. *Drug Metabolism and Pharmacokinetics, 29*, 317–324.