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Short communication

Chrysosplenetin, in the absence and presence of artemsininin, alters breast cancer resistance protein-mediated transport activity in Caco-2 cell monolayers using aristolochic acid I as a specific probe substrate

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ABSTRACT

The present study describes the impact of chrysosplenetin, in the absence and presence of artemisinin, on *in vitro* breast cancer resistance protein-mediated transport activity in Caco-2 cell monolayers using aristolochic acid I as a specific probe substrate. We observed that novobiocin, a known breast cancer resistance protein active inhibitor, increased $P_{app\,(AP\text{-}BL)}$ of aristolochic acid I 3.13 fold ($p < 0.05$) but had no effect on $P_{app\,(BL\text{-}AP)}$. Efflux ratio (P_{BA}/P_{AB}) declined 4.44 fold ($p < 0.05$). Novobiocin, consequently, showed a direct facilitation on the uptake of AAI instead of its excretion. Oppositely, both artemisinin and chrysosplenetin alone at dose of 10 μM significantly decreased $P_{app\,(BL\text{-}AP)}$ instead of $P_{app\,(AP\text{-}BL)}$. Chrysosplenetin alone attenuated the efflux ratio, which was suggestive of being as a potential breast cancer resistance protein suppressant. Oddly, $P_{app\,(BL\text{-}AP)}$ as well as efflux ratio were respectively enhanced 2.52 and 2.58 fold ($p < 0.05$), when co-used with artemisinin and chrysosplenetin in ratio of 1:2. The potential reason remains unclear; it might be relative to binding sites competition between artemisinin and chrysosplenetin or the homodimer/oligomer formation of breast cancer resistance protein bridged by disulfide bonds, leading to an altered *in vitro* breast cancer resistance protein-mediated efflux transport function.

Introduction

Artemisinin resistance in *Plasmodium falciparum*, characterized by slow parasites clearance in patients receiving artemisinin or an artemisinin-based combination therapy (ACT), was first detected along the Thai–Cambodian border and has been spread across mainland Southeast Asia (Noedl et al., 2008; Dondorp et al., 2009; Amaratunga et al., 2012; Ashley et al., 2014; Imwong et al., 2017). The resistant mechanism to artemisinin is still ambiguous and many multidrug resistance proteins probably involved in, such as P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), bile salt export pump (BSEP), and multidrug resistance-associated proteins (MRP) 1–4 (Alcantara et al., 2013; Rijpma et al., 2014).

BCRP belongs to the ABC transporter family (Allikmets et al., 1998; Doyle and Ross, 2003) with a *C*-terminal transmembrance domain and an N-terminal ATP-binding domain (Litman et al., 2000), consisting of 655 amino acids (72-kDa). Therefore, BCRP is a half transporter that transforms to a functional efflux pump when homodimerized by a disulfide bridge at Cys 603 of two proteins (Lecerf-Schmidt et al., 2013; Noguchi et al., 2014; Mao and Unadkat, 2015) and confers an atypical MDR phenotype.

In our previous work, we found chrysosplenetin, a known polymethoxylated flavonoids in *Artemisia annua* L., in combination with artemisinin (2:1) decreased Bcrp/ABCG2 mRNA expression levels in mice small intestine (data not shown). Therefore, we here aimed to further investigate the effect of chrysosplenetin in the absence or presence of artemisinin on *in vitro* BCRP-mediated transport

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activity by using AAI as a specific probe substrate in Caco-2 cell monolayers.

Materials and methods

Artemisinin was purchased from Chongqing Huali Konggu Co., Ltd. (Chongqing, China) with purity ≥99.0%. Chrysosplenetin (purity ≥98.0%) was purified in our lab from an acetone layer of waste materials in artemisinin industrial production by using multiple column chromatography methods as described in the literature (Wei et al., 2015). The waste materials were kindly supplied by Chongqing Huali Konggu Co., Ltd. The voucher specimen (20100102) has been deposited with College of Pharmacy, Ningxia Medical University, for further references. Novobiocin was purchased from Hefei Bomei Biotechnology Co., Ltd. (CAS: 1476-53-5, purity ≥90%, China). Both aristolochic acid I (AAI, 110746-201510, purity ≥98%) and indomethacin (I.S., 100258-200904, purity ≥99%) were purchased from National Institutes for Food and Drug Control (China).

Methanol and acetonitrile (HPLC-grade) was purchased from Tedia (Ohio, USA). MTT (thiazolyl blue) and Lucifer yellow were purchased from Sigma–Aldrich Co. Ltd. (USA). HBSS (Hanks Balanced Salt Solution) was provided by Kangwei Shiji biotechnology Co. Ltd. (H1020, China).

An Agilent HPLC 1200 system was used for the determination of AAI. Samples were separated with a Zorbax SB-C18 column (4.6 × 250 mm, 5 μm, Agilent Technologies, USA). AAI concentration was analyzed by RP-HPLC-UV assay according to the reported method with some modification (Kimura et al., 2014; Ma et al., 2015). Mobile phase consisted of 45% of acetonitrile and 55% of 1% acetic acid in water. Column temperature was set at 30 °C and flow rate was set at 1.0 ml/min. AAI was detected at the wavelength of 250 nm. The injection volume was 20 μl.

Standard stock solutions of AAI and indometacin (I.S.) were individually prepared in methanol at concentration of 40 μM and 2.80 μM, and stored at 4 °C before use. Standard working solutions were prepared by diluting the stock solution in methanol to obtain a serial of desired concentrations. Chrysosplenetin and artemisinin were respectively dissolved in DMSO (dimethylsulfoxide) in the strength of 100 mM. All the solutions were stored at −20 °C and brought to room temperature before use.

The chromatographic method was validated for specificity, linearity, sensitivity, precision and accuracy. All validation runs were performed in five replicates on three consecutive days to assess inter-day and intra-day variation. Calibration curve was constructed for the range 0, 1, 2, 5, 10, 20, 50, 100 μM. Blank Caco-2 monolayer buffer samples ($n = 5$) were injected for specificity test. Precision and accuracy was assessed at three concentrations, *i.e.* low (LQC, 5 μM), medium (MQC, 20 μM) and high quality controls (HQC, 100 μM). It was further subdivided into intra-day and inter-day precision. The lowest limit of quantification (LLOQ) was determined by serial dilution of working standards.

Stability experiments were performed under different conditions by simulating conditions occurring during study sample analysis. Experiments were manipulated to determine the stability at 37 °C for 3 h, ambient temperature for 4 h, and −20 °C for 7 days.

Caco-2 cells (Fig. 1) were seeded in the transwell polycarbonate inserts at a density of 10^6 cells per well and were grown in a culture medium consisting of Dulbecco's modified Eagle's medium (DMEM/F-12) supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids, 1% L-glutamine, 100 U/ml penicillin-G and 100 μg/ml streptomycin. The culture medium was replaced every alternate day and the cells were maintained at 37 °C, 95% relative humidity and 5% CO_2. Permeability studies were conducted with the monolayers cultured for 19–21 days.

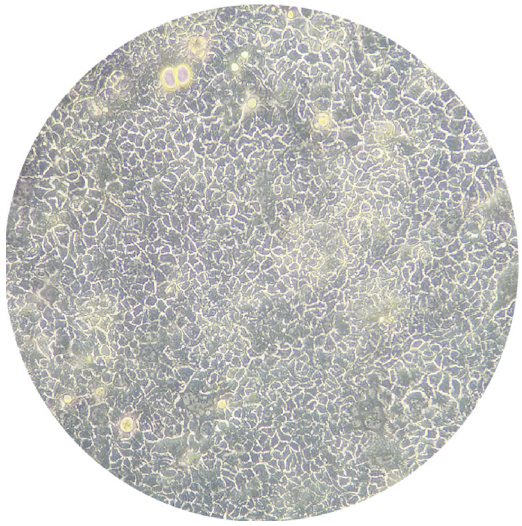

Fig. 1. Caco-2 cells growth situation.

To ensure the monolayer integrity throughout the course of transport experiment, transepithelial electrical resistance-values (TEER values) were measured with a Millicell-ERS Volt-ohmmeter. Apparent permeability coefficient (P_{app}) of Lucifer yellow, which always used as a marker of paracellular transport, was also determined. The integrity of monolayers was confirmed when the TEER values of Caco-2 cells exceeded 400 Ω/cm^2, and the P_{app} values of Lucifer yellow were less than 0.5×10^{-6} cm/s (Aspenström-Fagerlund et al., 2012).

To ensure the proper concentrations of AAI used in the uptake and transcellular transport study, the cytotoxicity effect was evaluated by MTT assay. Caco-2 cells were seeded into 96-well plate at a density of 1.0×10^4 cells/well. AAI were added at designated concentrations followed by 4 h incubation at 37 °C. After removing the medium, the serum-free medium containing 15 μl of MTT (5.0 mg/ml) was added and incubated for another 4 h. In the end, 200 μl of DMSO replaced the MTT medium to dissolve formazan, and then the absorbance at a wavelength of 490 nm was measured by SpectraMax M2 microplate reader.

Before the experiments, Caco-2 cells were divided into five groups including negative control (HBSS), artemisinin alone (10 μM), novobiocin (positive control, 100 μM), chrysosplenetin (10 μM), and artemisinin–chrysosplenetin (1:2). Cell monolayers were washed twice with warm HBSS and equilibrated with HBSS for 30 min at 37 °C. The transport studies were initiated by loading the individual HBSS solution of AAI (90 μM) onto the donor compartment. The other side was termed the receiver compartment, and the final volume in each of the chambers was 2.5 ml on the apical side (AP) and 2.5 ml on the basolateral side (BL). A 200 μl aliquot of samples was separately taken out from the donor and receiver chambers each 30 min till 2 h and fresh transport medium was immediately complemented. Transport experiments were conducted in an incubator maintained at 37 °C and shaken with a speed of 50 rpm. A 200 μl aliquot of cell lysates was used for extraction at 0, 30, 60, 90, and 120 min. Total 100 μl of the harvested sample was added into 1 ml ethyl acetate containing 2 μg/ml of indomethacin (I.S.), vortex-mixed for 3 min and then centrifuged at 13,800 × *g* for 10 min. And then 850 μl of supernatant was evaporated to dryness under vacuum and the residue was resuspended in 200 μl of mobile phase. After being vortex-mixed and centrifuged at 13,800 × *g* for 10 min, the supernatant was used to determine AAI concentration by an established RP-HPLC-UV method.

All data were analyzed using the SPSS 18.0 software (IBM, USA) and submitted to a one-way analysis of variance (ANOVA).

Table 1
Apparent permeability coefficient of Lucier yellow.

No.	Fluorescence intensity	Concentration (μg/ml)	P_{app} (cm/s)
1	103.26	0.012	4.04×10^{-7}
2	102.68	0.009	3.03×10^{-7}
3	101.97	0.005	1.68×10^{-7}

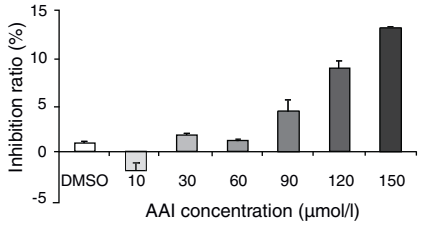

Fig. 2. The cytotoxicity effect of AAI on the viability of Caco-2 cells.

Significant differences were at $p < 0.05$ or $p < 0.01$ level between the groups. Turkey's test was used to identify any difference between means using a significance level of $p < 0.05$.

Results and discussion

The TEER values of Caco-2 cell lines were determined to be over $420\ \Omega \times cm^2$ (454, 430, and $476\ \Omega \times cm^2$). Apparent permeability coefficients of Lucier yellow displayed in Table 1 revealed a normal transport pathway in established Caco-2 cell monolayer mode, without a leakage of Lucier yellow. Meanwhile, MTT cytotoxicity test (showed in Fig. 2) indicated that no remarkable poisonous was observed under 90 μM of AAI within 3 h.

Fig. 3 shows the representative chromatograms of blank HBSS buffer, standard substances spiked in HBSS buffer, and sample harvested after 1 h. Injection of blank transport buffer onto the HPLC column showed no interference.

The calibration curve of AAI presented a well linearity in the range 0–100 μM when spiked in blank HBSS buffer. The regression equation for calibration curves was $Y = 1.01X$ ($r = 0.99996$). The intra- and inter-day precisions (%CV) were less than 3.10% and accuracies (%RE) were within -0.32% and 3.20% (Table 2). The LLOQ of AAI was found to be 1 μM. This indicated that the method was feasible for the analysis of AAI.

Stability results were described in Table 3. It showed that the analytes were stable after being placed at 37 °C for 3 h, ambient temperature for 4 h, and −20 °C stored for 7 days.

As shown in Table 4, the efflux ratio of AAI in Caco-2 cell monolayers in negative control group was 6.80, which indicated that efflux transporters might be involved in the transport of AAI. It is in accordance with the literature (Ma et al., 2015). $P_{app\ (AP\text{-}BL)}$ value of AAI significantly increased 3.13 folds when co-used with positive control novobiocin ($p < 0.05$). No significance was observed in $P_{app\ (BL\text{-}AP)}$ value ($p > 0.05$) but the efflux ratio (P_{BA}/P_{AB}) was drastically decreased 4.44 folds ($p < 0.05$). Novobiocin, therefore, mainly showed a direct promotion on the uptake of AAI instead of the inhibition of BCRP-mediated AAI efflux.

Both chrysosplenetin and artemisinin alone declined $P_{app\ (BL\text{-}AP)}$ while have no impact on $P_{app\ (AP\text{-}BL)}$ relative to negative control ($p < 0.05$). Moreover, chrysosplenetin significantly attenuated the efflux ratio. It implied that chrysosplenetin inhibited the *in vitro* BCRP-mediated efflux of AAI in Caco-2 cell monolayers when independently used. However, when combined with artemisinin and chrysosplenetin in ratio of 1:2, P_{app} (BL-AP) and efflux ratio were significantly increased 2.52- and 2.58-fold ($p < 0.05$) along with an unchanged P_{app} (AP-BL).

In conclusion, BCRP-mediated efflux of AAI was inhibited by chrysosplenetin alone while remarkably promoted when co-used with artemisinin. The potential reason has not been fully understood. BCRP possesses multiple drug binding sites in a large pocket formed by TM α-helices. Some inhibitors as BCRP substrates can act as competitive inhibitors. In this regard, it is possible that chrysosplenetin and artemisinin interact with BCRP on binding sites and induce conformational changes in the large binding pocket, and thus allosterically affect the efflux transport of AAI as a specific BCRP substrate. Secondly, BCRP is assumed to act as a functional homodimer bridged by disulfide bonds (Kage et al., 2002) or oligomer (Kage et al., 2005). This deserves a further work to investigate whether chrysosplenetin in the presence of artemisinin altered

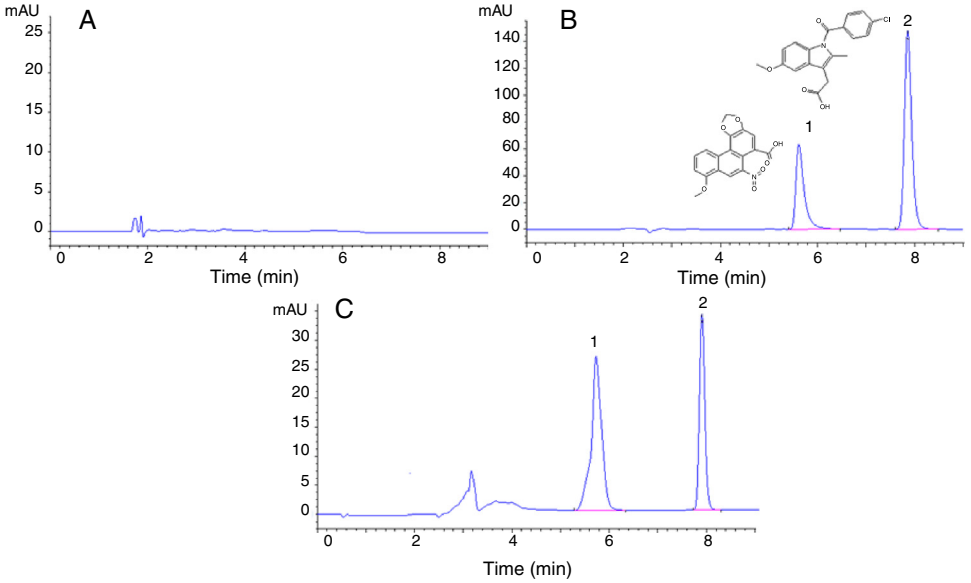

Fig. 3. Representative RP-HPLC-UV chromatograms of analytes: (A) blank HBSS buffer, (B) blank HBSS buffer spiked with AAI (1) and indometacin (2, IS), and (C) a study sample containing AAI (1) and indometacin (2, IS) after incubation for 1 h.

Table 2
Method validation for the analysis of AAI (Mean ± SD, $n = 5$).

Theoretical concentration[a] (μmol/l)	Accuracy and precision						Average extraction recoveries(%)
	Intra-day			Inter-day			
	Measured concentration (μg/l)	Precision (%CV)	Accuracy (%RE)	Measured concentration (μg/l)	Precision (%CV)	Accuracy (%RE)	
5	5.04 ± 0.03	0.60	0.80	5.16 ± 0.16	3.10	3.20	70.80 ± 0.32
20	20.13 ± 0.21	1.04	0.65	20.33 ± 0.43	2.12	1.65	72.40 ± 0.07
100	99.68 ± 0.90	0.90	−0.32	100.15 ± 1.90	1.90	1.50	78.30 ± 0.13

[a] Selected concentrations represent the low, medium and high (LQC, MQC, and HQC, respectively) concentrations.
Intra-day and inter day accuracy and precision were determined with $n = 5$ for each concentration.
%RE (% relative error) = [(Measured concentration − theoretical concentration)/theoretical concentration] × 100.
%CV (% coefficient of variation) = (SD/mean) × 100.

Table 3
Stability studies for AAI under different storage conditions ($n = 5$).

Condition	Quality control [a] (μM)	Measured concentration (ng/ml)	Precision (%CV)	Accuracy (%RE)
37 °C for 3 h	5	5.08 ± 0.24	4.72	1.60
	20	20.40 ± 1.35	6.62	2.00
	100	110.35 ± 3.56	3.23	10.35
Room temperature for 4 h	5	5.19 ± 0.32	6.17	3.80
	20	20.35 ± 2.02	9.93	1.75
	100	111.45 ± 4.15	3.72	11.45
−20 °C for 7 days	5	4.78 ± 0.22	4.60	−4.40
	20	18.43 ± 0.35	1.90	−7.85
	100	89.18 ± 3.23	3.62	−10.82

[a] Selected concentrations represent the low, medium and high (LQC, MQC, and HQC, respectively) concentrations.
Data were expressed as mean ± SD ($n = 5$).
%RE (% relative error) = [(Measured concentration − theoretical concentration)/theoretical concentration] × 100.
%CV (% coefficient of variation) = (SD/mean) × 100.

Table 4
Chrysosplenetin alters permeability of AAI across Caco-2 cell monolayers in the absence or presence of artemisinin (mean ± SD, $n = 3$).

Drugs	Dosage (μM)	P_{app} ($\times 10^{-5}$ cm/s)		P_{BA}/P_{AB} (efflux ratio)
		AP-BL	BL-AP	
Negative control	–	3.35 ± 0.11	22.78 ± 0.00	6.80 ± 0.21
Novobiocin (positive control)	100	10.5 ± 1.46[a]↑	16.6 ± 0.28	1.53 ± 0.19[a]↓
Artemisinin alone	10	2.49 ± 0.12	15.2 ± 1.41[a]↓	6.09 ± 0.87
Chrysosplenetin alone	10	3.08 ± 0.06	15.5 ± 0.28[a]↓	5.04 ± 0.01[a]↓
Artemisinin:Chrysosplenetin (1:2)	10:20	3.28 ± 0.03	57.5 ± 8.62[a]↑	17.52 ± 2.58[a]↑

[a] $p < 0.05$ *vs* negative control.

BCRP homodimer/oligomer levels which might lead to the adverse result in this study.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Author's contributions

YZ and CZ contributed in running the laboratory work. JC and LM analyzed the data. BY and JW revised the manuscript critically. XW and JC designed the study, supervised the laboratory work and contributed to modify the manuscript. All the authors have read the final manuscript and approved its submission.

Conflicts of interest

The authors declare no conflicts of interest.

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