Revista Brasileira de Farmacognosia 27 (2017) 776–779

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Short communication

Impact of chrysosplenetin, per se or in combination with artemisinin, on breast cancer resistance protein (Bcrp)/ABCG2 mRNA expression levels in mice small intestine

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ARTICLE INFO

Article history:
Received 22 February 2017
Accepted 14 June 2017
Available online 16 November 2017

Keywords:
Chrysosplenetin
Artemisinin
Bcrp/ABCG2 mRNA expression
Western blot
RT-qPCR

ABSTRACT

Our previous work revealed that chrysosplenetin in combination with artemisinin inhibited *in vivo* P-glycoprotein (P-gp, one of classic multi-drug resistance proteins) mediated digoxin transportation activity by reversing the upregulated P-gp/Mdr1 mRNA expression levels by artemisinin. Therefore, chrysosplenetin might be a potential artemisinin-resistance reversal agent as a P-gp inhibitor. But it still remains unknown if chrysosplenetin has an impact on another pivotal multi-drug resistance protein, breast cancer resistance protein (Bcrp), which is co-expressed with P-gp in apical membrane of intestinal epithelial cell and overlaps some of the substrates and inhibitors. This study, therefore, further addressed the impact of chrysosplenetin, per se or in combination with artemisin, on Bcrp/ABCG2 mRNA expression levels in mice small intestine determined by western blot and real time-quantitative polymerase chain reaction (RT-qPCR) assay. The drugs were intragastrically administrated once per day for 7 days. Novobiocin, a known Bcrp inhibitor, was observed to have no impact on Bcrp/ABCG2 levels with or without artemisinin versus vehicle. Interestingly, artemisinin alone attenuated Bcrp level while chrysosplenetin alone increased it ($p < 0.05$). Relative mRNA level was significantly decreased when co-used with artemisinin and chrysosplenetin in ratio of 1:2 ($p < 0.05$). The discrepant results for chrysosplenetin on Bcrp/ABCG2 mRNA expressions might be closely related to the transcriptional or posttranscriptional regulation.

Introduction

The human ATP-binding cassette (ABC) proteins belong to a large protein superfamily; thus far, 48 ABC transporters have been identified in humans and twelve of them have been recognized as putative drug transporters. Among them, P-glycoprotein (P-gp, gene symbol ABCB1 or Mdr1), the multidrug resistance protein 1 (MRP1, gene symbol ABCC1), and the breast cancer resistance protein (BCRP, gene symbol ABCG2) are the best distinguished and most paramount drug transporters of multi drug resistance (MDR) (Gillet and Gottesman, 2011). There is mounting evidence to support that human BCRP/rat Bcrp plays a pivotal role in drug disposition by expelling a broad range of structurally different metabolites out of cells. Bcrp substantially overlaps with substrates and inhibitors of P-gp or MRP1 and resembles P-gp in tissue distribution and expression. In this regard, a very active Bcrp transporter could probably diminish drug delivery to the target organs which leads to MDR, despite peripheral drug concentrations being within their therapeutic extent.

Bcrp is composed of 655 amino acids (72 kDa) and organized into six transmembrane α-helices, containing only one nucleotide-binding domain (NBD) near its *N*-terminal and one membrane-spanning domain (MSD) (Lecerf-Schmidt et al., 2013; Noguchi et al., 2014; Mao and Unadkat, 2015). Therefore, Bcrp per se is a half transporter that transforms to a functional efflux pump when a disulfide bridge at Cys 603 of two proteins is

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https://doi.org/10.1016/j.bjp.2017.06.005

homodimerized (Lecerf-Schmidt et al., 2013; Noguchi et al., 2014; Mao and Unadkat, 2015) and confers an atypical MDR phenotype (Lagas et al., 2009; Ni et al., 2010).

To date, artemisinin antimalarial drugs are still of the utmost importance in the worldwide combination therapy of resistant falciparum malaria (Tripathi et al., 2013). Artemisinin resistance, defined as a delayed clearance of parasites after clinical therapy, has been reported (Meshnick et al., 1996; White, 2004). The mechanism of artemisinin resistance remains unclear and probably dominated by multiple mechanisms, which involved numerous multidrug resistance proteins such as several members of the ABC transporter super-family (Alcantara et al., 2013). This causes a low bioavailability and blood concentration for terminal drugs (Meng et al., 2014).

Numerous polymethoxylated flavonoids are discovered to modulate the activity of drug metabolizing enzymes and ABC transporters, which raises the potential for alterations in the pharmacokinetics of substrate drugs (Wesolowska, 2011; Yuan et al., 2012). Chrysosplenetin is one of the known polymethoxylated flavonoids in *Artemisia annua* L. and other several Chinese herbs (Numonov et al., 2015). In our previous work, chrysosplenetin was observed to improve the bioavailability and anti-malarial efficiency of artemisinin in combination ratio of 1:2 partially relying on its strong inhibition on rat CYP3A metabolic activity in an un-competitive manner (Wei et al., 2015) and on P-gp *in vivo* efflux efficacy (Yang et al., 2016) via reversing the up-regulated P-gp/Mdr1 mRNA expression levels by artemisinin (Ma et al., 2017). Hence, chrysosplenetin might be a dual inhibitor on CYP3A and P-gp. However, the function of chrysosplenetin on Bcrp/ABCG2 expression is still unknown, which is an obstacle for us to systematically evaluate the possibility of chrysosplenetin being as an inhibitor of artemisinin resistance.

Therefore, we here aimed to further investigate the impact of chrysosplenetin in the absence and presence of artemisinin on Bcrp/ABCG2 expression levels using by western blot and real time-quantitative polymerase chain reaction (RT-qPCR) methods.

Materials and methods

Artemisinin (white crystal) was purchased from Chongqing Huali Konggu Co., Ltd. (purity ≥99.0%, Chongqing, China). CHR (purity ≥98.0%) was purified in our lab from an acetone layer of artemisinin industrial waste materials using multiple column chromatography methods as described in the literature (Wei et al., 2015). The industrial wastes were kindly provided by Chongqing Huali Konggu Co., Ltd. and 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).

Healthy male ICR mice, weighing 18–22 g, were purchased from an animal centre of Ningxia Medical University (Ningxia, China). All animals were housed in polycarbonate cages and acclimated in an environmentally controlled room (23 ± 2 °C, with adequate ventilation and a 12-h light/dark cycle) prior to use and were provided with standard laboratory food and water before and during the experiments. The experimental protocol was approved by the University Ethics Committee (Ningxia Medical University, China; ethic approval: 2014-029). All procedures involving animals were in accordance with the Regulations of the Experimental Animal Administration, State Committee of Science and Technology. Animals were randomly divided into nine groups ($n = 6$ for each group) including negative control (0.5% sodium carboxymethylcellulose, CMC-Na), artemisinin alone (40 mg/kg), novobiocin (positive control, 100 mg/kg), novobiocin–artemisinin (positive control in combination, 1:1, 100:100 mg/kg), artemisinin–chrysosplenetin (1:0.1, 40:4 mg/kg), artemisinin–chrysosplenetin (1:1, 40:40 mg/kg), artemisinin–chrysosplenetin (1:2, 40:80 mg/kg), artemisinin–chrysosplenetin (1:4, 40:160 mg/kg), and chrysosplenetin alone (80 mg/kg). The drugs were intragastrically administrated once per day for consecutive seven days. Then the mice were euthanized and sacrificed by cervical vertebra dislocation. Small intestines were harvested and cleaned using normal saline at least three times. Total RNA was extracted from the mouse small intestine using E.Z.N.A.™ Total RNA Kit (OMEGA bio-tek, Norcross, GA, USA), in accordance with the manufacturer's instructions. RNA concentrations were measured with a microplate spectrophotometer (Bio-RAD, USA) at a wavelength of 260 nm. RNA quality was evaluated using electrophoresis in 1% agarose gels. Total RNA (3 μg) was reverse transcribed into first-strand complementary DNA (cDNA) using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Each cDNA sample (1 μl) was amplified with 12 μl of Thermo Scientific Maxima SYBR Green qPCR Master Mix (2X), ROX Solution (Thermo Scientific) and 1 μM of each primer. Amplification was performed in a RT-qPCR IQ5 System (Applied Biosystems, Foster City, USA) with the following parameters: denaturation at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 61 °C for 30 s and extension at 72 °C for 45 s. The sequences of the oligonucleotide primers used for this study were 5′-GCA TTC GCT GTG GTT GAG T-3′ (sense) and 5′-TAT CCG TGG CAT CTC TGG A-3′ (antisense) for ABCG2 (product size, 123 bp from Sangon Biotech (Shanghai) Co., Ltd.); and 5′-GGT GAA GGT CGG TGT GAA CG-3′ (sense) and 5′-CTC GCT CCT GGA AGA TGG TG-3′ (antisense) for GAPDH (product size, 233 bp, from Invitrogen Biotechnology Co., Ltd.). The relative expression levels of ABCG2 in each sample (normalized to that of GAPDH) were determined using $2^{-\Delta\Delta Ct}$ method. All RT-qPCR experiments were repeated three times.

The total proteins were harvested by KenGEN Whole Cell Lysis Assay Kit (KenGEN BioTECH, Nanjing, China), and the protein concentrations were determined using KeyGEN BCA Protein Quantitation Assay kit (Nanjing KeyGEN Biotech, China). An equal quantity of protein (80 μg) from total protein was resolved using 7.5% SDS-PAGE gel and subsequently transferred onto nitrocellulose membranes (Bio-Trace). After blocking the membrane with 5% non-fat milk in Tris-buffered saline (Biotopped) at room temperature for 1 h, the membrane was incubated at 4 °C for 12 h with rabbit polyclonal antibody against ABCG2 (1:100; sc-25822, Santa Cruz) and mouse monoclonal antibody against β-actin (1:150; sc-47778, Santa Cruz). The membranes were incubated with horseradish Peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG and Goat Anti-Mouse IgG (ZSGB-BIO, Beijing, China) for 2 h and signals were observed using Super Signal West Pico (Thermo Scientific). Western blotting bands intensity was quantified by densitometric analysis using ImageJ version 2x (NIH Image software, Bethesda, MA, USA).

Data was analyzed using the SPSS 18.0 software (IBM, USA) and submitted to a one-way analysis of variance (ANOVA) to detect significant differences between study groups. Turkey's test was applied to identify any difference between means using a significance level of $p < 0.05$.

Results and discussion

Bcrp expression level in mice small intestine was measured by western blot assay. As displayed in Fig. 1, artemisinin alone down-regulated Bcrp level while chrysosplenetin alone up-regulated it when compared with negative control ($p < 0.05$). Novobiocin, a known specific Bcrp inhibitor (Duan and You, 2009) was observed to have no effect on Bcrp expression level versus vehicle. It is

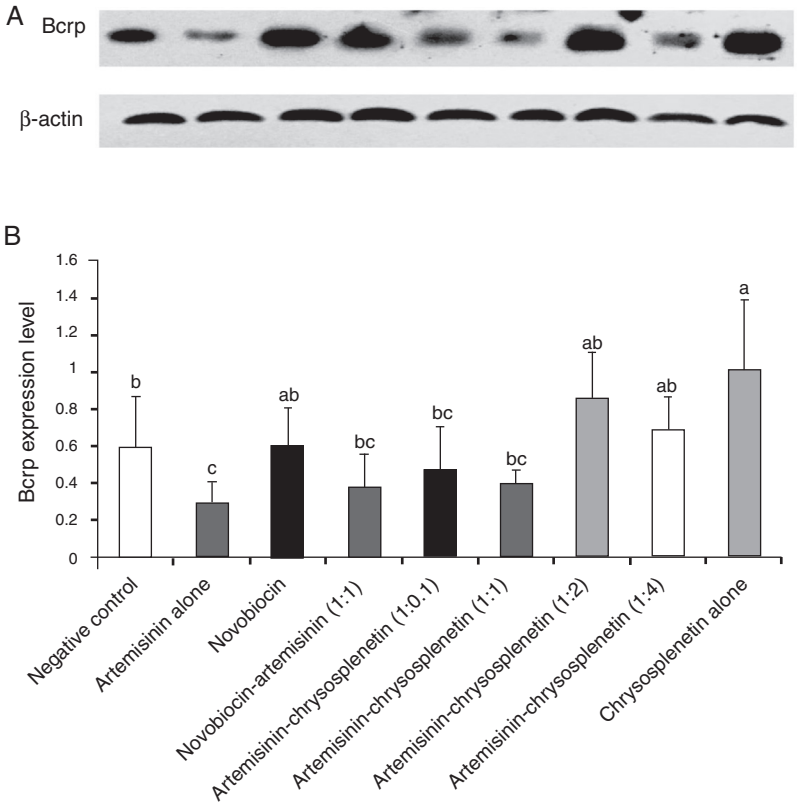

Fig. 1. Effect of chrysosplenetin per se or in combination with artemisinin on Bcrp expression level in mice small intestine. Data were expressed as mean values ± SD ($n = 6$) for each group. (A) Western blotting bands. (B) Quantification of Bcrp assessed by western blotting analysis was normalized to the expression level of β-actin antibody. Different lower-case letters indicate significant differences between different groups with a significance level of $p < 0.05$.

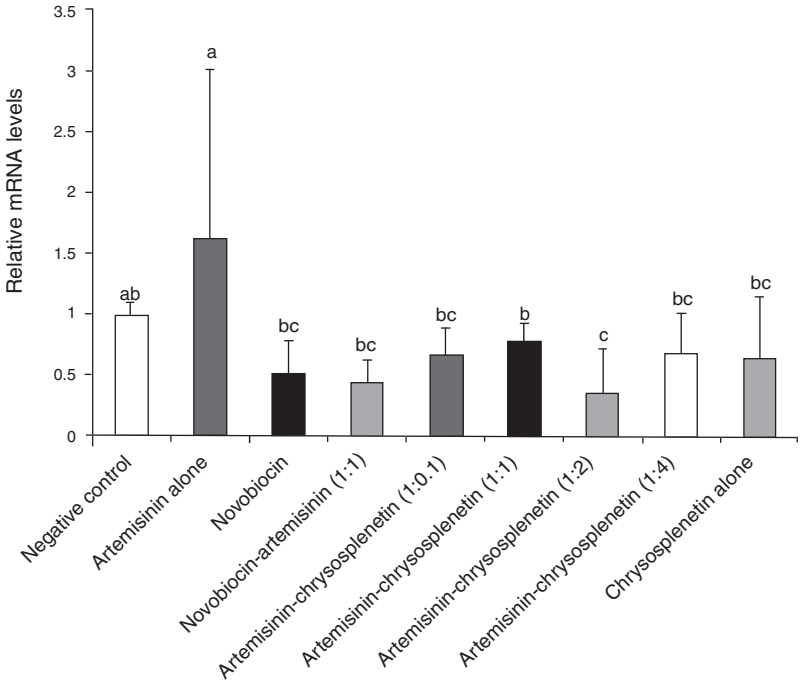

Fig. 2. Impact of chrysosplenetin per se or in combination with artemisinin on ABCG2 mRNA expression level in mice small intestine. Data were described as mean values ± SD ($n = 6$) for each group. Different lower-case letters indicate significant differences between different groups with a significance level of $p < 0.05$.

in accordance with the literature (Shiozawa et al., 2004) which reported that novobiocin is a competitive inhibitor of Bcrp. Therefore, the expression of Bcrp does not change. When compared with artemisinin alone, however, novobiocin alone, chrysosplenetin alone and artemisinin–chrysosplenetin combination groups in ratio of 1:2 and 1:4 remarkably elevated the levels ($p < 0.05$).

The identical small intestine was utilized to determine the gene expression level of ABCG2 by RT-qPCR. Data are described in

Fig. 2. Contrary to the results of Bcrp expression, artemisinin alone increased ABCG2 mRNA level while artemisinin–chrysosplenetin (1:2) significantly attenuated it versus negative control ($p < 0.05$). Moreover, all study groups reversed the upregulated relative mRNA levels by artemisinin ($p < 0.05$).

In this paper, contradicting data for chrysosplenetin in the presence or absence of artemisinin on the regulation of Bcrp/ABCG2 mRNA expression are observed. Artemisinin was found to downregulate Bcrp protein expression but upregulate ABCG2 mRNA level. It might be because transcriptional or posttranscriptional regulation for Bcrp protein plays a predominant role according to the literature (Mao and Unadkat, 2015). Artemisinin, therefore, might promote its resistance after a multi-dose oral administration from the view of P-gp based on our previous work. It indicates a risk of artemisinin resistance in its clinical therapy. But the possibility of artemisinin resistance seems to be weakened at Bcrp protein expression level. So this work will be of benefit to accurately evaluate the venture of artemisinin on its resistance from different aspects.

Secondly, chrysosplenetin showed a regulating function on P-gp/Mdr1 and Bcrp/ABCG2, totally opposite to artemisinin based on our previous and this work. The reason might be closely related to the competition of artemisinin and chrysosplenetin on the identical binding sites in P-gp or Bcrp protein. It needs further research to certify the potential mechanism in our future study.

In general, artemisinin and chrysosplenetin have the opposite effect on Bcrp/ABCG2 mRNA expression levels. When they are co-used in ratio of 1:2, ABCG2 mRNA level was remarkably down regulated. The results will be helpful to some extent understand the mechanism of chrysosplenetin as a potent inhibitor on artemisinin resistance at P-gp/Mdr1 mRNA and ABCG2 mRNA (encoding gene for Bcrp) levels. However, it is too early to reach an eventual conclusion whether chrysosplenetin could be clinically co-used with artemisinin to delay or reverse artemisinin resistance. The decisive factors in formation of artemisinin resistance and the regulation of chrysosplenetin on these factors deserve to study before the arrival of a final conclusion.

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

WM, YZ and YZ contributed in running the laboratory work. CZ and JW analyzed the data. LM and BY 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.

Acknowledgement

This work was financially supported by the National Natural Science Foundation of China (No. 81560580).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2017.06.005.

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