ผลของสารสกัดจากกวาวเครือขาว (Pueraria candollei) และ miroestrol ต่ำการทำงานของ P-glycoprotein ในลำไส้หนู mice

บทคัดย่อ

ผลของสารสกัดจากกวาวเครือขาว (Pueraria candollei) และ miroestrol ต่ำการทำงานของ P-glycoprotein ในลำไส้หนู mice

บทนำ: กวาวเครือขาว Pueraria candollei Wall. ex Benth. var. mirifica (family Leguminosae) ได้ถูกนำมาใช้ประโยชน์ในทางแพทย์แผนไทยโดยจัดหน่ายในรูปผลิตภัณฑ์สุขภาพอย่างกว้างขวาง โดยเฉพาะสรรพคุณด้านการชะลอวัย จึงเป็นที่น่าสนใจในการศึกษาผลของสารกัด และสารสำคัญ miroestrol ที่ได้จากกวาวเครือขาวต่อการทำงานของ P-glycoprotein ซึ่งเป็น efflux transporter ที่สำคัญที่มีผลต่อประสิทธิภาพของยาหลายชนิด เพื่อเป็นข้อมูลการกิดอัลกิริยาระหว่างสมุนไพรกับยาในเชิงคลินิก วัตถุประสงค์การศึกษา: การศึกษาแมวตัวต้นเป็นตัวหลักของผลของสารสกัดจากกวาวเครือขาว และ miroestrol สู่การทำงานของ P-glycoprotein วัสดุและวิธีการทดลอง: โดยพิจารณาจากผลของสารสกัดกวาวเครือขาว และ miroestrol ต่อการทำงานของ Rhodamine 123 ซึ่งเป็น substrate ของ P-glycoprotein ผ่านลำไส้สั้น กลับด้าน ในหลอดทดลอง ผลการศึกษา: ผลการศึกษาพบว่าสารสกัดกัมพลังกวาวเครือขาว และ ส่วนสกัด ethyl acetate ของกวาวเครือขาว ไม่มีผลต่อการทำงานของ P-glycoprotein ส่วน miroestrol ซึ่งเป็นสารสำคัญที่พบในกวาวเครือขาวและมีฤทธิ์ต่ออัลกิริยาของยา สามารถยับยั้งการทำงานของ P-glycoprotein ในลำไส้หนูได้เล็กน้อย โดยพบว่ามีฤทธิ์ยับยั้ง P-glycoprotein ได้ที่ความเข้มข้นตั้งแต่ 100 μM ขึ้นไป และเมื่อให้ miroestrol ผ่านลำไส้สั้น พบว่ามีการเปลี่ยนการแสดงออกของ ABCB1A และ ABCB1B mRNA สรุปผล: miroestrol และสารสกัดจากกวาวเครือขาวมีผลต่อการทำงานของ P-glycoprotein น้อยมาก อย่างไรก็ตาม ควรจะมีการศึกษาเพิ่มเติมเกี่ยวกับการบริโภคกวาวเครือขาวในระยะยาว และฤทธิ์ของสารสำคัญชนิดต่างๆที่พบในกวาวเครือขาวต่อการทำงานของ protein transporter ชนิดอื่นๆ ต่อไป

คำสำคัญ: Pueraria candollei, miroestrol, P-glycoprotein, efflux protein transporter

1 อาจารย์, Ph.D., คณะเภสัชศาสตร์ มหาวิทยาลัยขอนแก่น อ.เมือง จ. ขอนแก่น 40002
2 อาจารย์, บ.ศ. วิทยาลัยแพทยศาสตร์และสาธารณสุข มหาวิทยาลัยขอนแก่น อ.เมือง จ. ขอนแก่น 40190
3 รองศาสตราจารย์, Ph. D., คณะเภสัชศาสตร์ มหาวิทยาลัยขอนแก่น อ.เมือง จ. ขอนแก่น 40002
4 ติดต่อผู้ contacto: เลนเน็ก พัฒนเศรษฐี, คณะเภสัชศาสตร์ มหาวิทยาลัยขอนแก่น อ.เมือง จ. ขอนแก่น 40002 โทรศัพท์ +6643 202378, โทรสาร +6643 202379, e-mail: denpat@kku.ac.th

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Abstract

Effects of Pueraria candollei extracts and miroestrol on intestinal P-glycoprotein function in mice

Denpong Patanasethanont¹, Latiporn Udomsuk², Waraporn Putalun³, Kanokwan Jarukamjorn³

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Introduction: Pueraria candollei Wall. ex Benth. var. mirifica (family Leguminosae), as known Kwao Kruea Khaw in Thai, has been widely used in Thai traditional medicine and is supplied in commercial health products for rejuvenation. It is interesting to investigate the potential effect of P. candollei and its component, miroestrol, on P-glycoprotein function to provide the important drug-herb interaction data for applying in clinical relevance. Objective: This present study is investigated the effect of P. candollei extract and miroestro, the potent phytoestrogen found in P. candollei on P-glycoprotein, the efflux transporter, function. Materials and Methods: Determine effect of substances on P-glycoprotein function by employing the in vitro transport of rhodamine 123, a substrate of P-glycoprotein, across everted intestinal sac was studied in mice. The ability of P. candollei tincture and the P. candollei ethyl acetate extract fraction to inhibit p-glycoprotein function were examined. Results: P. candollei extract has no effect on P-glycoprotein function. Miroestrol showed negligible inhibitory effect on P-glycoprotein function at the concentration at 100 μM and higher. For expression of P-glycoprotein, change in expression of ABCB1 and ABCB2 mRNAs in mice administered with miroestrol at 0.5 mg/kg/day subcutaneously for 7 days was not observed. Conclusion: Miroestrol, and P. candollei extracts have negligible effect on p-glycoprotein function. However, the effect of phytoestrogens contained in the tuberous root of P. candollei on P-glycoprotein and other protein transporters after long term of administration should be further investigated.

Keywords: Pueraria candollei, miroestrol, P-glycoprotein, efflux protein transporter

1 Lecturer, Ph. D., Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, 40002 Thailand
2 Lecturer, Ph.D., College of Medicine and Public Health, Ubonratchathani University, Warincharanrap, unorachathan, 34190 Thailand.
3 Associated Professor, Ph. D., Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, 40002 Thailand
* Corresponding author: Denpong Patanasethanont, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, Thailand, Tel: +6643 202378, Fax: +6643 202379, e-mail: denpat@kku.ac.ac.th

1. Introduction

One of the membrane transporter superfamily having the ATP-binding cassette (ABC) with well-preserved homology of the site where ATP binds is P-glycoprotein (P-gp), about 170 kDa in size, that is well known as the most typical efflux pump in the cell membrane. (Takano et al., 2006). P-glycoprotein involved in the transport of many substances including toxins from the liver, kidney, and gastrointestinal tract. P-glycoprotein and other efflux transporters limit permeation of toxins and xenobiotics to vital structure, such as the brain, placenta and testis (Leonard et al., 2003). Many in vitro and in vivo studies have demonstrated that P-glycoprotein plays a significant role in drug absorption and disposition, as its localization appears to have a greater impact on limiting cellular uptake of drugs from intestinal lumen into epithelial cells and from blood circulation into the brain than on enhancing the excretion of drugs out of hepatocytes and renal tu-
bules into the adjacent luminal space (Lin and Yamazaki, 2003). By the major role of this efflux transporter as described, limiting influx into and facilitating efflux from the enterocytes of their substrates, it could be able to serve as a determinant of oral bioavailability and intestinal efflux clearance for certain drugs, and also be related to drug–drug interactions, when multiple drugs that are substrates, inducers, or inhibitors for this transporter are administered together. In a clinical setting, interindividual and intraindividual variations and the modulation of expression and functional activity of p-glycoprotein intestinal efflux transporters are particularly important especially in patients who uses narrow therapeutic index drugs whose pharmacokinetic parameters would be affected.

Pueraria candollei Wall. ex Benth. var. mirifica (family Leguminosae), as known Kwao Kruea Khaw in Thai, has long been widely used in traditional medicine and supplied as commercial health products for rejuvenation. (Cherdshewasart and Sriwatcharakul, 2008). Miroestrol, one of the strong phytoestrogens (Chansakaow et al., 2000) among many of phytoestrogens found in tuberous root of P. candollei first demonstrated its estrogenic activity by induction of mammogenic effects in ovariectomized rats (Benson et al., 1961). It has been postulated to produce effects on reproductive organs, such as vaginal cornification and increased uterine weight in ovariectomized rats (Malaivijitnond et al., 2004), and the plant extract also increases the length of the follicular phase and total menstrual cycle and causes ovulation blockage in cynomolgus monkeys (Trisomboon et al., 2005). Estrogenic activity of miroestrol was estimated to have 0.25 times the estrogenic activity of 17β-estradiol using a vaginal cornification assay (Jones et al., 1961).

Recently, the effects of this plant and its compounds on the drug metabolizing enzymes, CYP2B9 and CYP1A2 were reported (Udomsuk et al., 2010). It is of interest investigate the potential effect of P. candollei and its component, miroestrol, on P-glycoprotein function to provide important drug-herb interaction data for applying in clinical relevance. The objective of this present study is to investigate the effect of P. candollei extract and miroestrol on p-glycoprotein function by employing in vitro transport of rhodamine 123 across everted intestinal sac study and the effect on expression of ABCB1 and ABCB2 mRNAs by using semi-quantitative reverse transcription-polymerase chain reaction technique.

2. Materials and Methods

2.1 Materials

Miroestrol was isolated from tuberous roots of P. candollei var. mirifica as described previously (Chansakaow et al., 2000) and NMR identification was performed and compared with authentic standards of miroestrol from Assoc. Prof. Dr. Chaiyo Chaichantipyuth, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. Rhodamine 123 and verapamil hydrochloride were supplied by Sigma-Aldrich (Germany). TRIZOL® reagent and dNTP mixture were supplied by Invitrogen® (Carlsbad, CA, USA). Random primers and RNase inhibitor were obtained from Takara Bio Inc. (Otsu, Shiga, Japan). Forward and reverse primers of ABCB1A, ABCB1B, and GAPDH genes were synthesized by Bio Basic, Inc. (Markham Ontario, Canada). Primer sequences were as follows; ABCB1A forward primer 5'-GCTTACAGCCAGCATTCTCC-3'; ABCB1A reverse primer 5'-CCAGCTCACATCCTGCTCA-3'; ABCB1B forward primer 5'-ACTCGGGAGCAGAAGTTTGA-3'; ABCB1B reverse primer 5'-GCACCAAAGACAACAAGA-3'; GAPDH forward primer 5'-TCC ACT CAC GGC AAA TTC AAC G-3'; GAPDH reverse primer 5'-TAG ACT CCA CGA CAT ACT CAG C-5'. All others chemicals used for the experiments were of the highest available purity from commercial suppliers.
2.2 Preparation of P. candollei tincture extract

The fresh tuberous roots were washed, dried in an oven at 45°C until the weight remained constant, and then powdered. The extract was prepared as P. candollei tincture. Powdered tuberous root of P. candollei (2.5 g) was extracted with 40% (v/v) ethanol (62.5 mL) at room temperature for 7 days. Then, the solvent was evaporated in vacuo and the extract fraction was dissolved in DMSO to obtain P. candollei tincture extract fraction.

2.3 Animal Experiments

Experiments with animals, animal handling, and the treatment protocol were approved by the Animal Ethics Committee of Khon Kaen University, Khon Kaen, Thailand (Approval document No. AEKKU42/2552). Mice in this study were obtained from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. At all times, mice were housed on wood chip bedding in stainless-steel cages with water and commercial mouse diet supplied ad libitum and acclimated for at least 7 days in housing with a 12-h dark light cycle under controlled temperature (22±2°C) before performing experiments.

2.4 In vitro transport of rhodamine 123 across everted intestinal sac

To examine the effects of P. candollei extract and miroestrol on P-gp function, serosal-to-mucosal transport of a substrate across intestinal everted sac was examined, as described previously (Takano et al., 2006; Yumoto et al., 1999). Briefly, male C57BL/6 mice at 6 weeks of age were fasted overnight with free access to water before the experiments. The whole small intestine was flushed with 50 mL of ice-cold saline. The mice were exsanguinated, and the small intestine was isolated and a segment of jejunum was everted, and a 3-cm-long everted ileum sac was prepared. Rhodamine 123, a p-glycoprotein substrate, was prepared at a concentration of 5 μM in pH 7.4 isotonic Dulbecco’s PBS containing 25 mM glucose and 0.4% DMSO. The rhodamine 123 solution (0.3 mL) was introduced into the everted sac (serosal side), and the both ends of the sac were ligated tightly. The sac containing rhodamine 123 was immersed into 3 mL of PBS, prewarmed at 37°C and pre-oxygenated with 5% CO₂/95% O₂. The bubbling of CO₂/O₂ gas was continued throughout the efflux study. In an inhibition study, verapamil, a typical inhibitor for P-gp, was added in the mucosal medium at the final concentration of 300 μM for verapamil. Similarly, the test compounds, miroestrol, P. candollei tincture, or P. candollei ethyl acetate extract were added in the mucosal medium at various final concentrations. The efflux of rhodamine123 across everted mice intestine following application to the serosal side was measured by sampling 250 μL of the mucosal medium periodically for 90 min and equal volume of fresh mucosal medium was re-supplied each time. After sampling, 250 μL acetonitrile (ACN) was added into samples, and then centrifuged at 10,000 rpm for 5 min. Concentrations of rhodamine 123 in samples were determined by using a microplate fluorometer (Molecular Devices, Sunnyvale, CA) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm because no metabolism of rhodamine 123 was observed under the present experimental conditions.

2.5 Semi-quantitative reverse transcription-polymerase chain reaction

Male C57BL/6 mice at 6 weeks of age were used in this study. Mice were subcutaneously administered with miroestrol in corn oil at a dose of 0.5 mg/kg/day once a day for 7 days. The control group was subcutaneously administrated with corn oil daily for 7 days. The mice were decapitated 24 h after the last treatment. Testes were immediately excised for preparing total RNAs as described elsewhere (Udomsuk et al., 2010).

Mouse ABCB1A, ABCB1B, and GAPDH mRNAs were semi-quantified by RT-PCR. Testicular total RNA was reverse-transcribed using ReverTraAce
reverse transcriptase (Toyobo Co., Ltd.), then cDNA was amplified under the conditions recommended by the supplier of Illustra Hot Start Master Mix (GE Healthcare, UK). The conditions of PCR cycle of ABCB1A and ABCB1B were 95°C 3 min; 39 cycles of 95°C 30 sec, 56°C 30 sec, 72°C 1 min; 72°C 3 min. The condition of GAPDH was 95°C 4 min; 32 cycles of 95°C 30 sec, 56°C 30 sec, 72°C 1 min; 72°C 5 min. After separation of the PCR products by 2% agarose gel electrophoresis, the target cDNA were detected under ultraviolet light in the presence of ethidium bromide and semi-quantified by Syngene gel documentation (Ingenius L, Cambridge, UK) and the GeneTools match program.

2.6 Data analysis

Statistically significant differences were determined by Student’s t-test, or one way analysis of variance (ANOVA) with the Tukey’s pos hoc or the Scheffe’s test for post-hoc analysis. A difference of P value less than 0.05 was considered statistically significant.

3. Results

3.1 Effect of P. candollei tincture extract on rhodamine 123 transport across everted mice ileum in vitro To test the effect of P. candollei tincture extract on transport of rhodamine 123 across everted ileum, P. candollei tincture extract was prepared to a final concentration of 30% of the original tincture preparation in mucosal medium. As shown in Figure 1, an inhibitory effect by 30% P. candollei tincture extract in the transport of rhodamine 123 from serosal to mucosal surface was observed but there was no statistically significant difference between the treatment group and the control group. The inhibitory potencies of both verapamil and 30% P. candollei tincture on rhodamine 123 transport were compared. It was revealed that the inhibitory effect of 30% P. candollei tincture was less potent than that of 300 μM verapamil, a typical P-gp inhibitor (22.7% and 72.7% inhibition in comparison with control, respectively).

3.2 Effect of P. candollei ethyl acetate extract on rhodamine 123 transport across everted mice ileum in vitro

As shown in Figure 2, it was revealed that the inhibitory effect on P-glycoprotein function of 30% ethyl acetate extract of P. candollei seemed to be higher than 30% P. candollei tincture extract. The inhibitory potency in the transportation of rhodamine 123 in the serosal side was 5 μM. Each value represents mean ± SE (n=6), * P<0.05, significantly different from control.
3.3 Effect of Miroestrol on rhodamine 123 transport across everted mice ileum in vitro

The effects of miroestrol, the strong phytoestrogen from tuberous roots of *P. candollei*, on P-glycoprotein function were investigated. Efflux of rhodamine 123 transport across ileum were inhibited 31.8, 18.2, and 0% by 500, 100, and 30 μM of miroestrol, respectively. The inhibitory effects of miroestrol were statistically significant (P<0.05) at the concentration of 100 μM or higher as shown in Figure 3 (A, B, C).

3.4 Expression of ABCB1 mRNA in mice administered miroestrol

Expression of ABCB1A and ABCB1B mRNA in mice, that were administered 0.5 mg/kg/day of miroestrol by subcutaneously administration for 7 days, was analyzed by semi-quantitative reverse transcription-polymerase chain reaction. When compared to the
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control group, only a small upregulated ABCB1A and ABCB1B mRNA expression was observed in the test group, there were no statistical significant changes of ABCB1A and ABCB1B mRNA expression (Figure 4).

Figure 4. Expression of ABCB1A and ABCB1B mRNAs in mice exposed to miroestrol by semi-quantitative reverse transcription-polymerase chain reaction. The dose of miroestrol was 0.5 mg/kg/day subcutaneously administration for 7 days. Each value represents mean ± SD, * P<0.05, significantly different from control.

4. Discussion and Conclusion

The tuberous root of *P. candollei*, known as “Kwao Krue R Khaow”, the rejuvenating herb, has often been used in Thai traditional medicine. Because of the wide use of phytoestrogens in many aspects of healthcare, they have recently gained much attention and can decrease the incidence of estrogen related cancer and cardiovascular disease, and even climacteric symptoms (Albertazzi *et al.*, 1999). However, the scientific evidence for clinical usefulness of this plant is still limited. From this reason, the drug-herb interaction should be of concern for safety in concurrent use with other drugs. In this study, the effects of *P. candollei* on P-glycoprotein function, an important efflux protein transporter, was investigated by employing in vitro transport study using rhodamine 123 transport across mice intestine.

From our results in Figure 1., verapamil, a well characterized P-gp inhibitor, significantly inhibited 72.7% transport of rhodamine 123, p-glycoprotein substrate, across everted rat ileum, similar to that was similar to that reported by Yumoto *et al.* (1999). This revealed that the expression of P-glycoprotein in mice intestine has been functionally confirmed.

*P. candollei* tincture was prepared by immersing the tuberous root powder in 40% ethanol for 7 days. The extract of *P. candollei* tincture inhibited the transport of rhodamine 123 across everted rat ileum by only 22.7% without significant difference from control. It can be assumed that the tincture of *P. candollei* has negligible or no effect on p-glycoprotein function in case of concurrent administration with drug that are substrates of p-glycoprotein, such as digoxin and cyclosporine, etc.

From data shown in Figure 2 and Figure 3, the ethyl acetate extract fraction had slightly more inhibitory effect on p-glycoprotein function, than that of *P. candollei* tincture (31.8% vs 22.7%).

Among phytoestrogens found in *P. candollei* ethyl acetate extract fraction (Chansakaow *et al.*, 2000), genistein and daidzein, have been reported for their ability to inhibit p-glycoprotein-mediated transport (Limtrakul *et al.*, 2005; Branda *et al.* 2006), we next examined the effect of this extract fraction on rhodamine 123 transport across everted mice ileum. Miroestrol, a potent phytoestrogen, also found in highest yield in the ethyl acetate extract fraction, are of interest to examine the effect of miroestrol on p-glycoprotein function. From the experiment we found that the minimum concentration required for miroestrol to produce a significant modulation of p-glycoprotein activity appeared to be, in general, 100 μM or higher (Figure 3).

In humans, two members of the P-glycoprotein gene family, ABCB1 and ABCB3 (MDR1 and MDR3)
exist, while three members of this family, abcb1a, abcb1b, and abcb2 (mdr1a, mdr1b and mdr2), are found in mice (Gottesman and Pastan, 1993; Schinke, 1997). The P-glycoprotein encoded by the human MDR1 and mouse mdr1a/1b genes functions as a drug efflux transporter, whereas human MDR3 P-glycoprotein and mouse mdr2 P-glycoprotein are believed to be functional in phospholipid transport (van Helvoort et al., 1996; Ruetz and Gros, 1994). To determine the effect of miroestrol on expression of p-glycoprotein in the intestine, we employed the semi-quantitative reverse transcription-polymerase chain reaction of ABCB1A and ABCB1B mRNAs. From our experiment, it was revealed that there was no significant change in expression of either ABCB1A and ABCB1B mRNA after administration of miroestrol at the dose of 0.5 mg/kg/day by subcutaneously administration for 7 days in mice. Competitive inhibition might be suspected to be the mechanism of miroestrol in inhibition of p-glycoprotein function.

As reviewed by Lin and Yamazaki (2003), the potential risk of P-glycoprotein-mediated drug interactions may be greatly underestimated if only plasma concentration is monitored. From animal studies, it is clear that P-glycoprotein inhibition always has a much greater impact on tissue distribution, particularly with regard to the brain, than on plasma concentrations. Therefore, the potential risk of P-glycoprotein mediated drug interactions should be assessed carefully. According to the transport data from this present study, and considering together with assuming human intestinal volume of 1.65 L/70 kg (Davies et al., 1993), for a single oral dose, it would need an intake around 60 mg of miroestrol to achieve the minimum effective concentration (100 μM) that can interact with intestinal p-glycoprotein. From our survey (data not shown), six of the various commercial products of P. candollei available in Thailand contain around 150-800 mg of P. candollei dried powder per capsule contains around 0.2-1 μg miroestrol per capsule, so the concentration of miroestrol in the intestine after a single oral dose is estimated to be only 0.34 -1.6 nM.

In conclusion, these findings indicate that miroestrol, the strong phytoestrogen contained in tuberous root of P. candollei has negligible effect on p-glycoprotein function and has no effect on expression of ABCB1 and ABCB2 mRNAs in mice. The minimum P-glycoprotein inhibitory effect concentration is 100 μM, therefore, systemic inhibition of P-glycoprotein by miroestrol may be insignificant after regular supplement of commercial product of P. candollei. However, interaction could occur in case of administration of extremely high dose of miroestrol, and especially with intravenous administration. Further investigation concerning the long-term safety effect of using commercial product of P. candollei and miroestrol, and also, the effect on other efflux protein transporters should be performed.

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