Inhibitory Effects of Furanocoumarin Derivatives in Kampo Extract Medicines on P-Glycoprotein at the Blood–Brain Barrier

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Furanocoumarin derivatives, known as components of grapefruit juice, showing inhibitory effects against P-glycoprotein (P-gp) in the intestine are also contained in the plants of rutaceae and umbelliferae families, which are used as components of Kampo extract medicines. In this study, we investigated the inhibitory effects of byakangelicol and rivulobirin A, known as furanocoumarins showing P-gp inhibitory effect using Caco-2 monolayer, against P-gp at the blood–brain barrier (BBB) under both in vitro and in vivo conditions. First we studied the membrane permeability of furanocoumarins to clarify whether they can be absorbed from the intestine. Both furanocoumarins showed high permeability through the Caco-2 monolayer, suggesting that they can easily reach the systemic circulation after oral administration. Then, we evaluated the effect of these furanocoumarins on the uptake of calcein acetoxymethyl ester (calcein-AM), a P-gp substrate, into bovine brain microvascular endothelial cells (BBMEC). Both furanocoumarins significantly increased the uptake amount of calcein-AM into BBMEC by the inhibition of P-gp at the BBB in vitro. Next we also investigated the P-gp inhibitory effect of these furanocoumarins at the rat BBB in vivo using verapamil as a P-gp substrate. Both furanocoumarins increased the B/P ratio of verapamil compared to the control, even under in vivo conditions; however, the extent of the inhibitory effect was much lower than in vitro condition. In conclusion, byakangelicol and rivulobirin A may inhibit P-gp expressed at the BBB even under in vivo conditions. Further studies using Kampo extract medicines under in vivo condition are necessary for safe drug therapy.

Key words blood–brain barrier; P-glycoprotein; furanocoumarin; Kampo medicine; inhibition

P-Glycoprotein (P-gp) is expressed not only in tumor cells but also in normal human tissue, such as the blood–brain barrier (BBB), liver, kidney, intestine and other tissues, and is an ATP-dependent efflux transporter pumping many therapeutic medicines out of cells. Cytochrome P450 3A4 (CYP3A4), a main metabolic enzyme in humans, is also expressed in many tissues and is involved in the first-pass effect of various drugs. Thus, P-gp and CYP3A4 cooperatively limit intestinal absorption of some drugs, resulting in their low bioavailability, and making the development of new drugs for oral administration more difficult. In addition, organs expressing a transporter or metabolic enzyme involved in drug disposition, there is a possibility of drug–drug or drug–food interaction. Recently, the components of grapefruit juice have been intensively investigated and their inhibitory effects against intestinal CYP3A4 are well-known. However, the inhibitory activity of the components in grapefruit juice is not only against CYP3A4 but also against P-gp. Among the many components of grapefruit juice, furanocoumarin derivative is one of the compounds causing CYP3A4 and P-gp inhibition. Furanocoumarin derivatives are also widely contained in the plants of rutaceae and umbelliferae families. Byakuchi, Kyokatsu, Boufu and Hamaudo are herbal remedies used as components of Kampo extract medicines in the umbelliferae family. Herbal medicines based on Chinese traditional medicine and adopted to Japanese culture are called Kampo medicines. Today, Kampo medicines are widely used for the treatment of cancer, dementia, neuralgia and so on. Herbal remedies in the umbelliferae family are commonly used and possibly concomitantly administered with substrate drugs for P-gp; therefore, investigation of the possibility of inhibitory effects against P-gp by furanocoumarins is very important for safe drug therapy using Kampo extract medicines. By screening experiments using 12 furanocoumarins extracted from plants in the umbelliferae family, we clarified that byakangelicol and rivulobirin A, a dimer type of furanocoumarin, strongly inhibited P-gp. Moreover, we demonstrated that Kampo extract medicines including byakangelicol or rivulobirin A possibly inhibit P-gp using the Caco-2 monolayer. However, these results do not directly relate to the production of unexpected side effects under in vivo situation. If these furanocoumarins show the high membrane permeability, they are absorbed through the intestine and easily enter the systemic circulation. As a result, they may inhibit P-gp expressed in organs other than the intestine, such as the BBB, liver, kidney and so on. Among these, much attention should be paid to the inhibition of P-gp at the BBB. The brain is not an organ which intensively metabolizes drugs; therefore, the drug concentration in the brain might increase by even a slight inhibition of P-gp at the BBB. For example, it was previously reported that the concentration of verapamil, carvedilol, vinblastine in the brain increased with the inhibition of P-gp at the BBB.

In this study, we studied the membrane permeability of byakangelicol and rivulobirin A to clarify whether these furanocoumarins can be absorbed from intestine. Then, we investigated the inhibitory effect of these furanocoumarins against P-gp at the BBB using both in vitro and in vivo experimental methods.

MATERIALS AND METHODS

Materials and Chemicals Calcein acetoxymethyl ester...
(calcein-AM), carbamazepine and trioxalen were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Verapamil was from Nacalai Tesque (Kyoto, Japan). Dulbecco’s modified Eagle’s medium (DMEM), Hanks’ balanced salt solution (HBSS), non-essential amino acid solution (NEAA), N-2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES) and Trypsin RPMI 1640 were from Invitrogen (Carlsbad, CA, U.S.A.). Fetal bovine serum (FBS) was from Hyclone (Logan, UT, U.S.A.). Dimethyl sulfoxide (DMSO) and (±) α-tocopherol were from Wako Pure Chemicals (Osaka, Japan). All furanocoumarins used in this study were isolated from herbal remedies (Kyokatsu, Byakushi and Boufu) and purified in-house. The purity of all furanocoumarins was more than 99%. Other reagents were commercially available and of analytical grade.

Caco-2 Cell Culture Caco-2 (passage number 52—53) cells were obtained from the European Collection of Cell Culture Collection (Salisbury, U.K.) and cultured in a humidified atmosphere of 5% CO₂ and 95% air at 37°C in DMEM supplemented with 20% (v/v) heat-inactivated FBS, 0.1 mmol/l NEAA, 100 units/ml penicillin and 100 µg/ml streptomycin. When the cell culture reached 80% confluency, it was rinsed with phosphate-buffered saline and split using trypsin. For transport experiments, Caco-2 cells (5.5×10⁵ cells/cm²) were seeded on Costar 12-well Transwell plate inserts from Corning Inc. (Corning, NY, U.S.A.) with an insert membrane pore size of 0.4 µm. The medium was changed every 2 d for 8 d, and the transport experiments were performed 17 d post-seeding. One day before the experiment, transepithelial electrical resistance (TEER) was ≥700 Ω·cm².

Membrane Permeability of Furanocoumarins through Caco-2 Monolayer We used two furanocoumarins, byakangelicol and rivulobirin A (Fig. 1). Furanocoumarins were dissolved with HBSS containing 10 mmol/l glucose and 0.5% DMSO (transport medium (TM)). Furanocoumarin concentrations in TM were adjusted to 10 µmol/l. Apical and basal sides of the Caco-2 monolayer were filled with TM and prewarmed at 37°C for 10 min. After prewarming, TM on the apical side was discarded and 0.5 ml TM including furanocoumarin was added to the apical side. Basal fluid was periodically withdrawn for 120 min. The sample was kept at −80°C until analysis. Apparent permeability (P_app) of furanocoumarins from apical to basal side was calculated according to the following equation.

\[ P_{app} = \frac{dQ/dt}{A \times C_0} \]

where, \(dQ/dt\) is the slope of the linear portion of the permeated amount versus the time curve, A is the effective surface area of the Transwell insert and \(C_0\) is the initial concentration of furanocoumarin applied at \(t=0\).

LC/MS/MS Assay for Furanocoumarins Byakangelicol and rivulobirin A were quantified using a liquid chromatography/mass spectrometric (LC/MS/MS) assay. Analysis was carried out on a Waters ACQUITY UPLC™ system with an autosampler and column oven. An ACQUITY UPLC™ BEH C18 column (50 mm×2.1 mm, 1.7 µm (Waters Corp., Milford, MA, U.S.A.) was employed for separation with the column temperature maintained at 40°C. The gradient elution for UPLC analysis consisted of two solvent compositions: solvent A: 10 mM ammonium acetate in water and solvent B: acetonitrile. The gradient began with 10% eluent A and changed linearly to 90% A within 0.5 min, this component was maintained for 0.5 min, and then changed back to 10% A within 0.1 min. Throughout the UPLC process, the flow rate was set at 0.3 ml/min and the run time was 2 min. A Waters TQD™ tandem quadrupole mass spectrometer (Waters Corp.) equipped with an electrospray ionization (ESI) interface was used to determine furanocoumarins. The ESI source was set in positive ionization mode for both compounds. Quantification was performed using multiple reaction monitoring (MRM) of the transitions of m/z 317.46→231.00 for byakangelicol, m/z 573.20→203.00 for rivulobirin A and m/z 228.60→141.80 for trioxalen, an internal standard for furanocoumarins, with a scan time of 0.10 s per transition. The optimal MS parameters were as follows: collision energy 18 eV (byakangelicol), 37 eV (rivulobirin A) and 26 eV (trioxalen), cone voltage 34 V (byakangelicol), 32 V (rivulobirin A), 35 V (trioxalen), source temperature 120°C and desolvation temperature 350°C for all compounds. Nitrogen was used as the desolvation and cone gas with a flow rate of 500 and 6 l/h, respectively. Argon was used as the collision gas. All data collected in multi-channel analysis mode were acquired and processed using MassLynx™ ver 4.1 software (Waters Corp.).

Bovine Brain Microvascular Endothelial Cell (BBMEC) Culture Primary bovine brain microvascular endothelial cells were purchased from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). Cells were cultured and grown at 37°C in a humidified 5% CO₂ incubator on collagen-coated glass-bottomed dishes with further bovine fibronectin-coating (2.5 µg/cm²). The culture medium used was RPMI 1640 medium containing 100 µg/ml streptomycin, 100 unit/ml penicillin and 10% fetal bovine serum. After the cells had grown to confluence (4—6 d), the BBMEC monolayers were used for the uptake experiment of calcine-AM in the presence and absence of the furanocoumarins. Passages 6 through 9 were used for the experiments.

Uptake Experiment of Calcine-AM into BBMEC The inhibition of P-gp activity was achieved by using calcine-AM as the model substrate. Non-fluorescent calcine-AM is a lipophilic ester with high membrane permeability and is a substrate for P-gp. The ester bond is rapidly cleaved by intra-
cellular nonspecific esterases, generating highly fluorescent calcein with poor cell permeability; therefore, the P-gp inhibitory effect can be evaluated by an increase of intracellular fluorescent intensity. Calcein-AM and furanocoumarin were dissolved with HBSS containing 10 mmol/l glucose (TM). Calcein AM and furanocoumarin concentrations in TM were adjusted to 0.02 μmol/l and 1—10 μmol/l, respectively. Dimethyl sulfoxide (DMSO) was used as a dissolving agent for furanocoumarins and the concentration of DMSO in TM was adjusted to 0.1% for all experiments. Verapamil (1—10 μmol/l), a potent P-gp inhibitor, was used as a positive control. BBMEC monolayer was incubated with TM and prewarmed at 37 °C for 10 min. After prewarming, TM was discarded and 2 ml TM including calcein-AM that was not taken up. Then, 1% Triton X-100 was added to lyse the cells, the lysate was collected and centrifuged at 900 g for 10 min, and the fluorescent intensity of the supernatant was measured on a fluorometer (RF-540; Shimadzu, Kyoto, Japan) using 493 nm excitation and 515 nm emission filters. The protein concentration in each sample was measured by the method of Lowry.

**Uptake of Verapamil into the Rat Brain in Vivo** All animal experimentation protocols were approved by the institutional animal care and use committee at Osaka University of Pharmaceutical Sciences. Male Wistar rats weighing 270—320 g (SLC, Shizuoka, Japan) were used as experimental animals. Rats were freely fed and given drinking water, and housed under a 12 h light-dark cycle for more than one week prior to use. The rats were fasted for 18 h before the experiment, but drinking water was supplied ad libitum. Immediately before the experiment, the rats were anesthetized with an intraperitoneal injection of sodium pentobarbital solution (dose: 50 mg/kg BW) and were implanted surgically with a combination of Phicon (Fuji Systems Ltd., Tokyo, Japan) and PE50 (Clay Adams, Parsippany, NJ, U.S.A.) in a catheter, which was inserted into the jugular vein for furanocoumarin administration and for blood sampling. Byakangelic or rivulobirin A was injected as a bolus administration through the catheter at a dose of 0.2 mg/kg BW. At predetermined time, 0.2 ml blood was withdrawn and centrifuged at 900 g for 10 min. Plasma sample was deproteinized by the addition of acetonitrile. The furanocoumarins were extracted with the same methods of verapamil extraction from the brain samples mentioned above. The samples were injected into LC/MS/MS to determine the furanocoumarins concentration. The pharmacokinetic parameters of furanocoumarins were calculated by the fitting of furanocoumarin concentrations in plasma into the two-compartment open model.

**LC/MS/MS Assay for Verapamil** Verapamil was also quantified using LC/MS/MS assay. The equipment and column used for verapamil analysis were the same as used for furanocoumarins analysis. The isocratic elution for UPLC analysis consisted of 0.1% formic acid in water : methanol = 30 : 70. Throughout the UPLC process, the flow rate was set at 0.3 ml/min and the run time was 2 min. Quantification was performed using MRM of the transitions of m/z 455.30 → 164.90 and m/z 237.00 → 194.10 for verapamil and carbamazepine, respectively. The optimal MS parameters were as follows: collision energy 30 eV and 20 eV, cone voltage 50 V and 40 V for verapamil and carbamazepine, respectively. Source temperature was set at 120 °C and desolvation temperature at 360 °C. Nitrogen was used as the desolvation and cone gas with a flow rate of 600 ml/h and 50 l/h, respectively. Argon was used as the collision gas. All other parameters for the UPLC/MS/MS assay of verapamil were the same as those for furanocoumarins.

**Data Analysis** All values are expressed as the mean ± S.D. Statistical analysis was performed using the Dunnett’s test. For the comparison of P-gp inhibitory effects among byakangelic, rivulobirin A and verapamil, Two-way factorial analysis of variance (ANOVA) was performed. The level of significance was taken as p < 0.05.
RESULTS

Permeability of Furanocoumarins through Caco-2 Monolayer  
P-gp is expressed on many tissues such as the BBB, liver, kidney and so on; therefore, the intestinal membrane permeability of furanocoumarins closely related to P-gp inhibitory activity on those tissues since furanocoumarins are orally administered as components of Kampo extract medicines. The time courses of permeated furanocoumarins through the Caco-2 monolayer are shown in Fig. 2. The permeability of byakangelicol and rivulobirin A through the Caco-2 monolayer was 4.97±0.18×10⁻⁵ and 3.34±0.13×10⁻⁵ cm/s, respectively. Both furanocoumarins showed high permeability and the order was byakangelicol>rivulobirin A.

Increased Uptake of Calcein-AM into BBMEC  
Calcein-AM is a substrate of P-gp and is rapidly metabolized to calcein, a fluorescent probe, after being taken up by the cell. We evaluated the P-gp inhibitory effects of furanocoumarins by measuring the intracellular amount of calcein. The ratio of calcein concentration in BBMEC in the presence of furanocoumarin against that in the absence of furanocoumarin (control) is shown in Fig. 3. Verapamil was used as an inhibitor of P-gp in this experiment and the result is also shown for comparison. Intracellular calcein concentration increased with the increase of verapamil concentration. In the presence of 10μM verapamil, intracellular concentration of calcein reached 262% of the control. Both furanocoumarins increased the uptake of calcein-AM into BBMEC in a concentration-dependent manner. In the presence of 10μM byakangelicol and rivulobirin A, the intracellular concentration of calcein markedly increased to 391% and 535%, respectively. The potency of P-gp inhibitory effect by rivulobirin A was significantly higher than that by byakangelicol.

Uptake of Verapamil into the Rat Brain through the Blood–Brain Barrier  
Verapamil is not only an inhibitor but also a substrate of P-gp; therefore, we investigated the effects of furanocoumarins on the uptake of P-gp substrate into the brain using verapamil as a P-gp substrate. Verapamil concentrations in the plasma and brain 20 min after intravenously (i.v.) bolus injection are shown in Fig. 4. Verapamil concentrations in the brain after pretreatment with byakangelicol and rivulobirin were 1.33- and 1.38-fold higher than that of the control (no pretreatment). Among these data, only rivulobirin A significantly increased verapamil concentration in the brain compared to the control. On the other hand, verapamil concentration in plasma showed a tendency to decrease with furanocoumarin pretreatment, although no significant differences were observed compared to the control. From these data, the ratio of verapamil concentration in the brain to plasma (B/P ratio) was calculated and is also listed in Table 1. When verapamil was administered after pretreatment with furanocoumarins, B/P ratios were higher than that of the control; however, even rivulobirin A significantly but slightly increased the B/P ratio compared to the control.

Table 1. Effects of Furanocoumarins on Verapamil B/P Ratio in Rats in Vivo

<table>
<thead>
<tr>
<th></th>
<th>B/P ratio</th>
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<tbody>
<tr>
<td>Control</td>
<td>1.11±0.11</td>
</tr>
<tr>
<td>Byakangelicol</td>
<td>1.92±0.59</td>
</tr>
<tr>
<td>Rivulobirin A</td>
<td>2.12±0.21**</td>
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Each value is the mean±S.D. of 3—5 experiments. **Significant at \(p<0.01\) vs. control group.
Assistance-associated proteins (MRPs) are expressed at the more potent than verapamil. However, it is known that not the inhibitory effect against P-gp by these furanocoumarins was volume, CL\text{tot}: total body clearance. Each value is the mean ± S.D. of 3 experiments.

Each furanocoumarin was i.v. injected at a dose of 3.5 mmol/kg BW. λ\text{a}, elimination rate constant at initial phase; λ\text{e}, elimination constant at terminal phase; V: distribution volume, CL\text{tot}: total body clearance. Each value is the mean ± S.D. of 3 experiments.

** Significant at p<0.01 vs. byakangelicol group.

extent of inhibitory effect of both furanocoumarins was comparable.

**Pharmacokinetics of Both Furanocoumarins in Rats**
Pharmacokinetic parameters of both furanocoumarins after i.v. bolus administration to rats (0.35 μmol/kg BW) were calculated and listed in Table 2. Distribution volume of byakangelicol was significantly higher than that of rivulobirin A. On the other hand, the parameters of initial and terminal elimination rate constants and total body clearance were almost same between two furanocoumarins.

**DISCUSSION**
P-Glycoprotein is expressed on many tissues, such as the BBB, liver, kidney and so on; therefore, the intestinal membrane permeability of furanocoumarins closely related to P-gp inhibitory activity on those tissues since furanocoumarins are orally administered as components of Kampo extract medicines. Apparent permeability of furanocoumarins was in the order of 10^{-5} cm/s (Fig. 2), comparable to propranolol, known as a high permeability compound.\(^{24}\) In particular, the effect of rivulobirin A on the intestinal absorption of CYP3A substrate drug should be paid attention because rivulobirin A was also potent CYP3A inhibitor.\(^{14}\) The above result suggests that these furanocoumarins may inhibit P-gp at the BBB after being absorbed from the intestine when Kampo extract medicine was concomitantly administered with P-gp substrate drug. To clarify this point, we investigated the effect of furanocoumarins on P-gp expressed at the BBB even under in vivo conditions.

Both byakangelicol and rivulobirin A increased the uptake amount of calcein-AM into BBMEC in a concentration-dependent manner in the range of 1 to 10 μM (Fig. 3). Adams et al.\(^{25}\) also reported that furanocoumarins enhanced the uptake of calcein-AM into porcine brain microvascular endothelial cells and the inhibitory effect was dependent on their concentrations; therefore, it has been also confirmed that furanocoumarins inhibited P-gp at the BBB in spite of the species difference. When the extent of inhibition effect was compared, rivulobirin A much inhibited P-gp at the BBB than byakangelicol. This was consistent with the result previously shown for the P-gp on the Caco-2 monolayer.\(^{14}\) The calcein-AM amounts taken up into BBMEC in the presence of 10 μM byakangelicol and rivulobirin A were more than in the presence of 10 μM verapamil, suggesting that the inhibitory effect against P-gp by these furanocoumarins was more potent than verapamil. However, it is known that not only P-gp but also other transporters such as multidrug resistance-associated proteins (MRPs) are expressed at the BBB.\(^{26,27}\) It is also reported that calcein-AM may be a substrate for not only P-gp but also MRPs.\(^{28}\) Therefore, there is a possibility that the inhibition of multiple transporters by furanocoumarins may be involved in the results obtained in this study and we are planning to clarify the relationship between MRPs expressed on BBMEC and the inhibitory effect by furanocoumarins.

Recently, to predict the magnitude of P-gp-based drug interactions at the human BBB, many in vivo experiments, including non-invasive positron emission topography (PET) imaging, have been conducted\(^{29–31}\) because information obtained from only in vitro experiments is limited. We investigated the P-gp inhibitory effect of furanocoumarins against the rat BBB in an in vivo experiment. Verapamil concentration in the brain slightly increased by pretreatment with rivulobirin A, whereas no furanocoumarins affected verapamil concentration in plasma (Fig. 4). Calculated from these values, it was clarified that only rivulobirin A significantly increased the B/P ratio of verapamil compared to the control (Table 1), even under in vivo conditions; however, the extent of the inhibitory effect under in vivo condition was much lower than that under in vitro condition as shown in the experiment using BBMEC (Fig. 3). This discrepancy may have been caused by the difference in the experimental conditions. Namely, the disposition of furanocoumarins in the body may be affected to their inhibitory effect under in vivo condition; therefore, we examined the furanocoumarins disposition in rat's body after i.v. injection (Table 2). Both furanocoumarins showed similar pharmacokinetics in rat's body; however, only the distribution volume of byakangelicol was significantly higher than that of rivulobirin A. The protein binding of byakangelicol and rivulobirin A at 0.5 μM total concentration in plasma were 72.7±1.0% and 92.4±1.0%, respectively (data not shown). This may be the reason for the difference in the distribution volume between two furanocoumarins. On the contrary, the total body clearance of furanocoumarins were almost same regardless of their difference in protein binding. For this reason, we speculated that the unbound form of rivulobirin A might be more extensively metabolized than that of byakangelicol in rat's body. The consideration amount of furanocoumarin free fraction may also relate to the discrepancy of the extent of P-gp inhibitory effect between in vitro and in vivo conditions. Namely, the extent of P-gp inhibitory effect by rivulobirin A much higher than byakangelicol in vitro; however, the extent of both inhibitory effect were similar under in vivo condition. This might be caused by the fact that free fraction of rivulobirin A was approximately 1/3 of byakangelicol.

Moreover, up to now, we have had no information about the metabolites of these furanocoumarins showing inhibitory effects against P-gp. Bergamottin, a furanocoumarin in grapefruit juice, is metabolized by CYP2B6 and 6',7'-dihydroxybergamottin, one of the metabolites, shows more potent inhibitory activity against P-gp than bergamottin\(^{22}\); therefore, we cannot rule out the possibility of P-gp inhibition by active metabolites of the furanocoumarins only from the results of i.v. injection data without per os (p.o.) administration data.

In conclusion, byakangelicol and rivulobirin A may inhibit P-gp expressed at the BBB even under in vivo conditions. Since the amounts of these furanocoumarins in Kampo ex-
tract medicines vary from product to product; therefore, further studies using Kampo extract medicines under in vivo condition are necessary for safe drug therapy.

REFERENCES


