Furanocoumarin Derivatives in Kampo Extract Medicines Inhibit Cytochrome P450 3A4 and P-Glycoprotein

Kazunori Iwanaga, Manami Hayashi, Yukimi Hamahata, Makoto Miyazaki, Makio Shibano, Masahiko Taniguchi, Kimiye Baba, and Masawo Kakemi

Division of Pharmaceutics (K.I., M.H., Y.H., M.M., M.K.) and Division of Pharmacognosy (M.S., M.T., K.B.), Osaka University of Pharmaceutical Sciences, Nasahara, Takatsuki-city, Osaka, Japan

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ABSTRACT:

Furanocoumarins in grapefruit are known to show inhibitory effects against P-glycoprotein (P-gp) and CYP3A4 in intestinal epithelial cells; however, furanocoumarin derivatives are widely 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 12 furanocoumarins extracted from plants in the Umbelliferae family against P-gp and CYP3A4 activity. Furthermore, we studied their inhibitory effect on P-gp when furanocoumarins are used as Kampo extract medicine rather than as an isolated single compound. From screening of the CYP3A4 inhibitory effect, notopterol and rivulobirin A, the only dimer types of furanocoumarin, were found to be potent inhibitors of CYP3A4. On the other hand, byakangelicol and rivulobirin A showed strong P-gp inhibition from the screening of P-gp inhibitor evaluated by quinidine permeation through the Caco-2 monolayer; however, the chemical structural relationship of furanocoumarins between P-gp and CYP3A4 inhibitory effects could not be obtained. We also investigated the effect of these furanocoumarins on the transport of digoxin through the Caco-2 monolayer. The inhibitory effect of rivulobirin A was more potent than that of byakangelicol. Application of either Senkyu-cha-cho-san or Sokike-kaketsu-to, which are composed of herbal remedies in the Umbelliferae group, significantly decreased the efflux ratio of digoxin. In conclusion, it was found that some furanocoumarins extracted from the plants in the Umbelliferae family strongly inhibited P-gp and CYP3A4. Kampo extract medicines containing herbal remedies may cause a drug-drug interaction with P-gp or a CYP3A4 substrate drug.

P-Glycoprotein (P-gp) is widely expressed in the human blood-brain barrier, liver, kidney, intestine, and other organs (Cordon-Cardo et al., 1989, 1990) and is related to drug excretion from the body. CYP3A4, the main metabolic enzyme in humans, is known to be expressed not only in the liver but also in the intestine (Watkins et al., 1987; Kolars et al., 1992). Recently, it has been clarified that intestinal absorption of some orally administered drugs is cooperatively restricted by P-gp and CYP3A4 in intestinal epithelial cells because the substrates for both proteins overlapped (Zhang and Benet, 2001). It was also reported that the oral administration of substrates for P-gp or CYP3A4 with their substrates or inhibitors, such as talinolol (Schwarz et al., 2000), digoxin (Westphal et al., 2000), midazolam (Kanazu et al., 2005), verapamil (LeTT smuggling, 2005), dihydro-6-hydroxy-7H-furo[3,2-g][1benzopyran-4-yl]-4-hexenyl[oxyl]-3,7-dimethyl-2-oxenyl[oxyl]-7H-furo[3,2-g][1]benzopyran-7-one; MBI, mechanism-based inhibition; OATP/oatp, organic anion-transporting polypeptide; GF120918, N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinoliny]ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide.

ABBRIEVATIONS: P-gp, P-glycoprotein; DDI, drug-drug interaction; LC, liquid chromatography; MS, mass spectrometry; OH, hydroxy; HPLC, high-performance liquid chromatography; FC726, 4-{[6-hydroxy-7-{[1-{1-hydroxy-1-methyl}ethyl]-4-methyl-6-(7-oxo-7H-furo[3,2-g][1]benzopyran-4-yl]-4-hexenyl[oxyl]-3,7-dimethyl-2-oxenyl[oxyl]-7H-furo[3,2-g][1]benzopyran-7-one; MBI, mechanism-based inhibition; OATP/oatp, organic anion-transporting polypeptide; GF120918, N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinoliny]ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide.
Herbal medicines that are based on Chinese traditional medicines and adopted to Japanese culture are called Kampo medicines (Yu et al., 2006; Kono et al., 2009). Kampo medicines were approved for use by the National Health Insurance System in Japan in 1976 and are widely used for the treatment of cancer (Takegawa et al., 2008; Matsuda et al., 2009), dementia (Watanabe et al., 2003), metabolic diseases such as obesity and diabetes (Omiya et al., 2005; Shimada et al., 2008), hypertension (Kimura et al., 2006), neuralgia (Sunagawa et al., 2001), and dysmenorrhea (Oya et al., 2008). Moreover, complementary and alternative medicines including Kampo medicines are gradually being used in the hospital. However, therapeutic effects of Kampo medicines have not been well understood and an accumulation of information is desired to promote evidence-based medicine. The herbal remedies in the Umbelliferae family are commonly used and possibly concomitantly administered with substrate drugs for P-gp or CYP3A4; therefore, investigation of the possibility of inhibitory effects against P-gp and CYP3A4 by furanocoumarins is very important for safe drug therapy using Kampo extract medicines. Furthermore, the inhibitory potencies of bergamottin and 6',7'-dihydroxybergamottin, both isolated from grapefruit juice and classified as furanocoumarins, differ (Paine et al., 2004). Thus, the relationship of the effects of furanocoumarins with CYP3A4 and their chemical structure are not fully understood. In this study, we extracted 12 furanocoumarin derivatives from plants in the Umbelliferae family and investigated their inhibitory effects on P-gp and CYP3A4 activity (Fig. 1). Furthermore, we also studied their inhibitory effect on P-gp when furanocoumarins are used as Kampo extract medicines, not as an isolated single compound.

Materials and Methods

Materials and Chemicals. Quinidine was purchased from Sigma-Aldrich (St. Louis, MO). Digoxin was from Nacalai Tesque (Kyoto, Japan). Dulbecco’s modified Eagle’s medium, Hanks’ balanced salt solution, nonessential amino acid solution, HEPES, and trypsin were from Invitrogen (Carlsbad, CA). Fetal bovine serum was from HyClone (Logan, UT). Dimethyl sulfoxide 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%. Senkyu-cha-cho-san and Sokkei-kak-ketsu-to, granule Kampo extract medicines containing herbal remedies belonging to the Umbelliferae family were from Tsumura and Co. (Tokyo, Japan). Other reagents were commercially available and of analytical grade.

Cell Culture. Caco-2 (passage 27) cells were obtained from the European Collection of Cell Culture (Salisbury, UK) and cultured in a humidified atmosphere of 5% CO2 and 95% air at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 20% (v/v) heat-inactivated fetal bovine serum, 0.1 mM nonessential amino acid solution, 100 units/ml penicillin, and 100 μg/ml streptomycin. When the cell culture reached 80% confluence, 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) with an insert membrane pore size of 0.4 μm. The medium was changed every 2 days for 8 days, and the transport experiments were performed 17 days after seeding. One day before the experiment, transepithelial electrical resistance was ≥700 Ω · cm2.

FIG. 1. Chemical structures of furanocoumarins extracted from herbal remedies in the Umbelliferae family.
Incubation Experiment for CYP3A4 Inhibitor Screening. CYP3A4 inhibitory effects by furanocoumarins were evaluated using Supersomes containing cDNA-expressed CYP3A4 coexpressed with cytochrome P450 reductase (oxidoreductase) and cytochrome b5 (BD Gentest, Woburn, MA). Midazolam was used as a substrate for CYP3A4. Midazolam and furanocoumarin were dissolved in dimethyl sulfoxide and added to the incubations at 2 and 1 μM, respectively. The final concentration of dimethyl sulfoxide in the incubation was adjusted to 1%. Ketoconazole (100 nM), a potent CYP3A4 inhibitor, was used for comparison of the inhibitory effects (Guo et al., 2000b).

Incubation was performed in 100 mM potassium phosphate-buffered saline (pH 7.4) with 1 mM EDTA. The enzymatic reaction was initiated by adding an NADPH-regenerating system (1.3 mM β-NADP+, 3.3 mM glucose 6-phosphate, 0.4 units/ml glucose-6-phosphate dehydrogenase, and 3.3 mM MgCl2) to the incubations. Insect cell-expressed CYP3A4 Supersomes (coexpressed with cytochrome P450 reductase and cytochrome b5) were used in all incubations at a concentration of 10 pmol of CYP3A4/ml. The soft sonic ionization positive mode was performed on an M-8000 mass spectrometer (Hitachi High-Technologies Co.). The organic phase was transferred into a clean tube and evaporated in vacuo. The residue was reconstituted with methanol and injected into an LC/MS system.

LC/MS assay for 1'-Hydroxymidazolam. The rate of 1'-OH-midazolam formation from midazolam was used as an indicator of CYP3A4 activity. The quantification of 1'-OH-midazolam was performed using a LC/MS assay. Liquid chromatography was performed on a Hitachi L-7100 pump equipped with a Hitachi L-7000 autoinjector (Hitachi High-Technologies Co., Tokyo, Japan) fitted with a Mightysil RP-18 column (150 mm × 4.6 mm, 5 μm; Kanto Chemical Co., Inc., Tokyo, Japan). The column temperature was maintained at 30°C. Analysis was performed under isocratic conditions. The mobile phase was 10 mM ammonium acetate (pH 5.0)-methanol (45:55, v/v) and was run at a flow rate of 0.6 ml/min. The atmospheric pressure chemical ionization positive mode was used. The precursor ions [M + H+] of 1'-OH-midazolam and diazepam, an internal standard, were detected at m/z 342.4 and 285.4, respectively.

Calculation of CYP3A4 Inhibitory Ratio. The inhibitory ratio (percentage) was calculated by eq. 1, and the inhibitory effects of various furanocoumarins were compared:

\[
\text{Inhibitory ratio} (\%) = \frac{V_{\text{control}}}{V_{\text{cont}}} \times 100
\]

where \(V_{\text{control}}\) is the 1'-OH-midazolam formation rate (picomoles per minute per mole of CYP3A4) after incubation of midazolam alone and \(V\) is that after coinubcation of midazolam with furanocoumarin or ketoconazole. In this study, the furanocoumarins showing less than 75% or more than 125% in their inhibitory ratio were regarded as inhibitors or inducers of CYP3A4, respectively.

Transport Experiment for P-gp Inhibitor Screening. Quinidine was used as a P-gp substrate for the P-gp inhibitor screening experiment. Because quinidine has been reported to be a weak substrate (Troutman and Thakker, 2003), it was selected for the screening experiment to avoid false-negative results. Quinidine was dissolved with Hanks’ balanced salt solution containing 10 mM glucose and 0.5% dimethyl sulfoxide [transport medium (TM)]. Quinidine and furanocoumarin concentrations in TM were adjusted to 10 and 5 μM, respectively. 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 of TM, including quinidine alone or quinidine with furanocoumarin, was added to the apical side. Basal fluid was periodically withdrawn for 75 min. The sample was kept at ~80°C until analysis. We studied the transport of quinidine only from the apical-to-basal direction of Caco-2 cells for the screening experiment for reduce furanocoumarin use because of their scarcity.

Apparent permeability (\(P_{\text{app}}\)) of compound was calculated according to eq. 2:

\[
P_{\text{app}} = \frac{dQ/dt}{A \times C_0} \times 100
\]

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

On the basis of the screening results, the inhibitory effect of furanocoumarin against P-gp was evaluated by the increase ratio calculated from eq. 3:

\[
\text{Increase ratio} (\%) = \frac{P_{\text{app}, \text{cont}}}{P_{\text{app}, \text{control}}} \times 100
\]

where \(P_{\text{app}, \text{control}}\) is the apparent permeability after the application of quinidine alone and \(P_{\text{app}}\) is that with furanocoumarin or cyclosporine A. Cyclosporine A (20 μM) was used as a positive control for P-gp inhibition (Collett et al., 2005). In this study, the furanocoumarins showing more than 125% in their increase ratio were regarded as P-gp inhibitors.

Determination of Quinidine. To alkalinate the sample, 100 mM sodium bicarbonate aqueous solution was added to the sample obtained from the transport experiment. After the addition of ethyl acetate, the sample was vigorously shaken and centrifuged at 900 g for 10 min. The organic phase was transferred to the tube and evaporated in vacuo. The residue was reconstituted by 100 μl of methanol and injected into the LC/MS system.

LC/MS assay for Quinidine. Quinidine was quantified using the same LC/MS system and column used for the 1'-OH-midazolam determination described above. Column temperature was maintained at 30°C. Analysis was performed under isocratic conditions. The mobile phase was 10 mM ammonium acetate (pH 5.0)-methanol (45:55, v/v) and was run at a flow rate of 0.6 ml/min. The atmospheric pressure chemical ionization positive mode was used. The precursor ions [M + H+] of quinidine and dextromethorphan, an internal standard, were detected at m/z 325.4 and 272.4, respectively.

Transport Experiment of Digoxin for Quantitative Evaluation of P-gp Inhibitory Effect. Digoxin was used as a P-gp substrate for the experiment to characterize furanocoumarins as P-gp inhibitors because digoxin was reported as a sensitive substrate of P-gp (Troutman and Thakker, 2003). Digoxin was dissolved with TM as well as quinidine. The concentrations of both digoxin and furanocoumarin in the dosing solution were adjusted to 10 μM. For the experiment of digoxin transport from the apical-to-basal direction, 0.5 ml of dosing solution was applied to the apical side. Basal fluid was withdrawn at a predetermined time. For the experiment of transport from the basal-to-apical direction, 1.5 ml of dosing solution was applied to the basal side. Apical fluid was withdrawn at a predetermined time. Samples were taken until 150 min after the application.

To assess the situation of oral administration of furanocoumarins in vivo, we also investigated the transport using two Kampo extract medicines (granules) containing herbal remedies belonging to the Umbelliferae family. One was Senkyu-cha-cho-san, the regimen of which is as follows: 7.5 g (three packages) of Senkyu-cha-cho-san contains 3.25 g of dried extract from a mixture of Kyokatsu (2.0 g), Byakushi (2.0 g), Koubushi (4.0 g), and Senkyu-cha-cho-san, the regimen of which is as follows: 7.5 g (three packages) of Sokei-kakketsu-to contains 5.0 g of dried extract from the mixture of Kyogetsu (2.0 g), Byakushi (2.0 g), Koubushi (4.0 g), Senkyu (2.0 g), Keisai (2.0 g), Hakka (2.0 g), Boufu (2.0 g), Kanzo (1.5 g), and Chayou (1.5 g). The other is Sokei-kakketsu-to (TI-53), the regimen of which is as follows: 7.5 g (three packages) of Sokei-kakketsu-to contains 5.0 g of dried extract from the mixture of Kyogetsu (2.0 g), Byakushi (1.0 g), Syakuyaku (2.5 g), Giou (2.0 g), Senkyu (2.0 g), Soujutsu (2.0 g), Touki (2.0 g), Tounin (2.0 g), Bukuryou (2.0 g), Goshitsu (1.5 g), Chinpi (1.5 g), Ireisen (1.5 g), Bui (1.5 g), Boufuf (1.5 g), Ryutan (1.5 g), Kanzo (1.0 g), and Shoukyou (0.5 g). One package (2.5 g) of Senkyu-cha-cho-san or Sokei-kakketsu-to was added to 200 ml of TM and vigorously stirred for 2 h. The aqueous suspension obtained was filtered through filter paper (125-mm circle no. 3; Advantec Toyo Kaisha, Ltd., Tokyo, Japan). For the experiment using diluted Kampo extract medicine solution, the filtrate was diluted with TM to obtain (one-fifth diluted or one-tenth diluted) Kampo extract medicine solution. Digoxin was dissolved with this Kampo extract medicine solution, and cyclosporine A was also dissolved for the concomitant application experiment. Digoxin and cyclosporine A concentrations in these Kampo extract medicine solutions were adjusted to 10 and 20 μM, respectively. The transport experiment was performed using the same method as described above with these Kampo extract medicine solutions as dosing solutions.

To identify the P-gp inhibitory effect of furanocoumarins from digoxin transport experiment, the efflux ratio was calculated according to eq. 4:

\[
\frac{P_{\text{app}, \text{cont}}}{P_{\text{app}, \text{control}}} \times 100
\]
TABLE 1
CYP3A4 inhibitory effects of furanocoumarins

<table>
<thead>
<tr>
<th>Position</th>
<th>Addition</th>
<th>V</th>
<th>Inhibitory Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pmol (min · pmol) CYP3A4</td>
<td>%</td>
</tr>
<tr>
<td>Control</td>
<td>2 μM Midazolam alone</td>
<td>8.67</td>
<td>100</td>
</tr>
<tr>
<td>Positive control</td>
<td>+100 nM Ketoconazole</td>
<td>4.45</td>
<td>51</td>
</tr>
<tr>
<td>+10 μM Furanocoumarin</td>
<td>+Psoralen</td>
<td>10.60</td>
<td>122</td>
</tr>
<tr>
<td>Side chain</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+Bergapten</td>
<td>7.76</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>+Isomperatorin</td>
<td>9.71</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>+Oxypeucedanin</td>
<td>8.96</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>+Oxypeucedanin hydrate</td>
<td>8.32</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>+Notopterol</td>
<td>4.09</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+Imperatorin</td>
<td>9.13</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>+Heraclenin</td>
<td>7.32</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>+Heraclenol</td>
<td>9.06</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>5 and 8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+Byakangelicol</td>
<td>8.19</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>+Byakangelicin</td>
<td>8.13</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>+Rivulobirin A</td>
<td>3.68</td>
<td>42</td>
</tr>
</tbody>
</table>

where \( P_{\text{app, AB}} \) is the apparent permeability of digoxin from the apical-to-basal direction and \( P_{\text{app, BA}} \) is that from the basol-to-apical direction.

**Determination of Digoxin.** To extract digoxin from samples, saturated ammonium chloride aqueous solution and dichloromethane were added to the samples after the addition of prednisolone dissolved in methanol as an internal standard. The mixture was shaken for 15 min and centrifuged for 10 min at 900g. The organic phase was transferred into a clean tube and evaporated in vacuo, and the residue was reconstituted with the mobile phase of the HPLC assay.

**HPLC Assay for Digoxin.** Digoxin was assayed by reverse-phase HPLC on a Mightysil RP-18 GP column (150 × 4.6 mm, 5 μm; Kanto Chemical Co., Inc.). The HPLC system consisted of an LC-10ATvp pump, SPD-10AV spectrophotometric detector, and C-R8A integrator (Shimadzu Co., Kyoto, Japan). The mobile phase was acetonitrile-water (26:74) and was run at a flow rate of 1.5 ml/min. The UV detector was set at 221 nm. Column temperature was maintained at 50°C.

**Data Analysis.** All values are expressed as the mean ± S.E. except for the results of the screening experiment. Statistical analysis was performed using the Mann-Whitney U test. The level of significance was taken as \( p < 0.05 \).

**Results**

**Screening of Furanocoumarins Showing CYP3A4 Inhibitory Activity.** Screening experiments for CYP3A4 inhibitory activity of 12 furanocoumarins were performed using midazolam as a CYP3A4 substrate, and activity was evaluated by the production rate of 1'-OH-midazolam from midazolam. As listed in Table 1, the 1'-OH-midazolam production rate was 8.67 pmol/min·pmol of CYP3A4 when midazolam was incubated alone (control). Ketoconazole (100 nM), a well known potent CYP3A4 inhibitor, markedly decreased CYP3A4 activity to 51% of the control. Psoralen, a parent compound in a family of furanocoumarins, showed no inhibitory effect against CYP3A4. Among the group of furanocoumarins with a side chain at the 5-position of the furanocoumarin ring system (e.g., bergapten, isomperatorin, oxypeucedanin, oxypeucedanin hydrate, and notopterol), only notopterol decreased CYP3A4 activity to 54% of the control. All furanocoumarins with a side chain at the 8-position (e.g., imperatorin, heraclenin, and heracelenol) showed no CYP3A4 inhibitory effects. Furanocoumarins with a side chain at both the 5- and 8-positions did not show any inhibitory effect. In contrast, rivulobirin A, the only dimer type of furanocoumarin in this study, markedly decreased the production rate of 1'-OH-midazolam, and the inhibitory effect reached 42% of the control.

**Screening of Furanocoumarins Showing P-gp Inhibitory Activity.** Screening experiments for P-gp inhibitory activity of 12 furanocoumarins were performed by using quinidine as a P-gp substrate. Apparent permeability of quinidine from the apical-to-basal direction

<table>
<thead>
<tr>
<th>Position</th>
<th>Addition</th>
<th>( P_{\text{app}} ) ( \times 10^{-5} ) cm/s</th>
<th>Increase Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5 μM Quinidine alone</td>
<td>2.67</td>
<td>100</td>
</tr>
<tr>
<td>Positive control</td>
<td>20 μM Cyclosporine A</td>
<td>3.58</td>
<td>134</td>
</tr>
<tr>
<td>+10 μM Furanocoumarin</td>
<td>+Psoralen</td>
<td>2.32</td>
<td>87</td>
</tr>
<tr>
<td>Side chain</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+Bergapten</td>
<td>2.26</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>+Isomperatorin</td>
<td>2.82</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>+Oxypeucedanin</td>
<td>2.36</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>+Oxypeucedanin hydrate</td>
<td>2.63</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>+Notopterol</td>
<td>2.58</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+Imperatorin</td>
<td>3.07</td>
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<td>+Heraclenin</td>
<td>2.52</td>
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<td>+Byakangelicol</td>
<td>3.71</td>
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<td>2.90</td>
<td>108</td>
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<tr>
<td></td>
<td>+Rivulobirin A</td>
<td>3.85</td>
<td>144</td>
</tr>
</tbody>
</table>

TABLE 2
P-gp inhibitory effects of furanocoumarins

\( P_{\text{app}} \) values show the apparent permeability of quinidine through the Caco-2 monolayer from the apical-to-basal direction. The initial concentrations of quinidine and furanocoumarins were adjusted to 5 and 10 μM, respectively. The initial concentration of cyclosporine A (positive control) was adjusted to 20 μM. The increase rate was calculated from eq. 3. Each value represents the mean of two experiments.
through the Caco-2 monolayer ($P_{\text{app}}$) is shown in Table 2. In the presence of cyclosporine A (20 μM), a potent P-gp inhibitor, $P_{\text{app}}$ value increased compared with that for quinidine alone (1.34-fold increase versus control). Psoralen, a furanocoumarin without any side chains, showed no P-gp inhibitory activity. All furanocoumarins with a side chain at the 5-position showed no P-gp inhibitory effect. Furanocoumarins with a side chain at the 8-position also were not P-gp inhibitors. In contrast, in the group of furanocoumarins with side chains at both the 5- and 8-positions, only byakangelicol increased $P_{\text{app}}$ of quinidine by 1.39-fold compared with the control. Rivulobirin A markedly increased $P_{\text{app}}$ (1.44-fold increase versus control). This inhibitory effect was comparable to that of 20 μM cyclosporine A.

**Relationship between CYP3A4 and P-gp Inhibitory Effect of Furanocoumarins.** From the results of screening experiments, the relation between P-gp and CYP3A4 inhibitory effects by furanocoumarins was plotted to compare their potency. As shown in Fig. 2, most furanocoumarins used in this study showed neither P-gp nor CYP3A4 inhibition. Byakangelicol and notopterol showed potent inhibition against P-gp and CYP3A4, respectively. We were surprised to find that only rivulobirin A strongly inhibited both P-gp and CYP3A4. The potency of the inhibitory effects of P-gp and CYP3A4 by 10 μM rivulobirin A was greater than that for 20 μM cyclosporine A and 100 nM ketoconazole, respectively. From the screening experiments described above, the findings were as follows: 1) byakangelicol shows a P-gp inhibitory effect; 2) notopterol shows a CYP3A4 inhibitory effect; and 3) rivulobirin A, a dimer-type furanocoumarin, showed both P-gp and CYP3A4 inhibitory effects.

**Effects of Byakangelicol, Notopterol, and Rivulobirin A on the Transport of Digoxin by P-gp.** For further investigation of the P-gp inhibitory effects of the furanocoumarins, byakangelicol, notopterol, and rivulobirin A, a detailed study was performed using digoxin, which is more sensitive than quinidine as a substrate. Calculated values of $P_{\text{app, AB}}$ and $P_{\text{app, BA}}$ are shown in Fig. 3. $P_{\text{app, AB}}$ of digoxin (0.66 × 10⁻⁶ cm/s) alone was much lower than that of quinidine (2.67 × 10⁻⁶ cm/s). $P_{\text{app, BA}}$ (19.20 × 10⁻⁶ cm/s) was much greater than $P_{\text{app, AB}}$. Both $P_{\text{app, AB}}$ and $P_{\text{app, BA}}$ values after concomitant application with furanocoumarins are shown in Fig. 3. $P_{\text{app, AB}}$ of digoxin greatly increased in the presence of cyclosporine A (5.21 × 10⁻⁶ cm/s). The addition of rivulobirin A to the apical side significantly increased (2.88 × 10⁻⁶ cm/s) $P_{\text{app, AB}}$ of digoxin compared...
with the control. The addition of byakangelicol increased $P_{app, AB}$ (1.77 $\times$ 10$^{-6}$ cm/s); however, no change in $P_{app, BA}$ was observed when notopeterol was concomitantly applied to the apical side. Cyclosporine A, which was added to the basal side, inhibited digoxin from being pumped to the apical side and significantly decreased $P_{app, BA}$ values (5.50 $\times$ 10$^{-6}$ cm/s). Rivulobirin A and byakangelicol decreased $P_{app, BA}$ values to 10.70 $\times$ 10$^{-6}$ and 14.10 $\times$ 10$^{-6}$ cm/s, respectively. From these values, the efflux ratio was calculated for the evaluation of P-gp activity and is shown in Table 3. The efflux ratio of digoxin in the control condition was 29.2, showing that it is very sensitive to the P-gp substrate. Cyclosporine A decreased the efflux ratio to 1.06. Rivulobirin A and byakangelicol also significantly decreased the efflux ratio of digoxin and their inhibitory activity was on the order of 10 $\mu$M byakangelicol < 10 $\mu$M rivulobirin A < 20 $\mu$M cyclosporine A. Notopeterol slightly decreased the efflux ratio, but it was not significant compared with the control.

**Inhibitory Effect of Kampo Extract Medicines on P-gp.** Some Kampo extract medicines, such as Senkyu-cha-cho-san and Sokeikakketsu-to, contain herbs belonging to the Umbelliferae family. We also investigated the effect of these Kampo extract medicines on the permeability of digoxin through the Caco-2 monolayer. $P_{app, AB}$ and $P_{app, BA}$ values and the efflux ratio of digoxin after concomitant application with Senkyu-cha-cho-san are shown in Table 4. When Senkyu-cha-cho-san was concomitantly applied with digoxin to the apical side as a standard regimen, $P_{app, BA}$ increased (2.29 $\times$ 10$^{-6}$ cm/s) compared with the control. On the other hand, $P_{app, BA}$ of digoxin was greatly decreased by the addition of Senkyu-cha-cho-san to the basal side. From this result, the calculated efflux ratio of digoxin in the presence of Senkyu-cha-cho-san (standard regimen) significantly decreased compared with the control.

Next, we performed the same inhibition experiment using diluted Senkyu-cha-cho-san to check the dose dependence of the effect. $P_{app, AB}$ and $P_{app, BA}$ values and the efflux ratio after concomitant application with diluted Senkyu-cha-cho-san are shown in Table 4. When digoxin was applied with diluted Senkyu-cha-cho-san (one-fifth diluted or one-tenth diluted), $P_{app, AB}$ decreased compared with the standard regimen as the concentration of Senkyu-cha-cho-san in the apical side decreased. In contrast, $P_{app, BA}$ increased with the decrease of Senkyu-cha-cho-san concentration on the basal side. Consequently, the efflux ratios of digoxin after concomitant application of one-fifth- and one-tenth-diluted Senkyu-cha-cho-san increased from 1.60 of the normal regimen to 5.08 and 17.80, respectively; however, even one-tenth-diluted Senkyu-cha-cho-san still showed significant inhibition against P-gp. Likewise, we investigated the effect of Sokei-kakketsu-to on the transport of digoxin through the Caco-2 monolayer. When Sokei-kakketsu-to was used as a normal regimen, $P_{app, AB}$ of digoxin greatly increased (3.15 $\times$ 10$^{-6}$ cm/s), whereas $P_{app, BA}$ decreased to 8.58 $\times$ 10$^{-6}$ cm/s, as shown in Table 5. When diluted Sokei-kakketsu-to (one-fifth diluted or one-tenth diluted) was concomitantly applied with digoxin, P-gp activity was also inhibited and efflux ratios increased from 2.72 of the normal regimen to 7.40 and 15.61, respectively. Even one-tenth-diluted Sokei-kakketsu-to showed significant P-gp inhibition compared with the control.

**Discussion**

Recently, it was clarified that the ingredients in grapefruit juice that inhibited CYP3A4 and P-gp in the intestine were furanocoumarins (Guo et al., 2000a); however, furanocoumarins are included not only in grapefruit juice but also in the plants that are frequently used in Kampo extract medicine. Therefore, it is important for safe drug therapy using Kampo extract medicine to pay attention to the DDIs between Western drugs and Kampo extract medicines. Senkyu-cha-cho-san is used for alleviation of early symptoms of the common cold and is prescribed for many patients. On the other hand, Sokei-kakketsu-to is used for arthritis or neuralgia and often used by aged individuals. It is considered that the people who take Sokei-kak-

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**TABLE 3**

Effects of furanocoumarins on the permeability of digoxin

<table>
<thead>
<tr>
<th>Position</th>
<th>Addition</th>
<th>$P_{app, AB}$ $\times$ 10$^{-6}$ cm/s</th>
<th>$P_{app, BA}$ $\times$ 10$^{-6}$ cm/s</th>
<th>Efflux Ratio</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10 $\mu$M Digoxin</td>
<td>0.66 ± 0.05</td>
<td>19.20 ± 1.26</td>
<td>29.20</td>
<td>0.01</td>
</tr>
<tr>
<td>Positive control + 10 $\mu$M Furanocoumarin</td>
<td>+20 $\mu$M Cyclosporine A</td>
<td>5.21 ± 0.47</td>
<td>5.50 ± 1.18</td>
<td>1.06**</td>
<td>0.01</td>
</tr>
<tr>
<td>Side chain</td>
<td>5</td>
<td>0.96 ± 0.08</td>
<td>17.80 ± 1.71</td>
<td>18.50</td>
<td>0.02</td>
</tr>
<tr>
<td>Dimer</td>
<td>5 and 8</td>
<td>1.77 ± 0.16</td>
<td>14.10 ± 1.32</td>
<td>7.97**</td>
<td>0.001</td>
</tr>
</tbody>
</table>

**TABLE 4**

Effects of Kampo extract medicine (Senkyu-cha-cho-san) on the permeability of digoxin

<table>
<thead>
<tr>
<th>Addition</th>
<th>$P_{app, AB}$ $\times$ 10$^{-6}$ cm/s</th>
<th>$P_{app, BA}$ $\times$ 10$^{-6}$ cm/s</th>
<th>Efflux Ratio</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10 $\mu$M Digoxin</td>
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<td>19.20 ± 1.26</td>
<td>29.20</td>
</tr>
<tr>
<td>Positive control</td>
<td>+20 $\mu$M Cyclosporine A</td>
<td>5.21 ± 0.47</td>
<td>5.50 ± 1.18</td>
<td>1.06**</td>
</tr>
<tr>
<td>Kampo extract medicine</td>
<td>Standard regimen Senkyu-cha-cho-san</td>
<td>2.29 ± 0.25</td>
<td>3.67 ± 0.79</td>
<td>1.60**</td>
</tr>
<tr>
<td></td>
<td>one-fifth diluted Senkyu-cha-cho-san</td>
<td>1.30 ± 0.22</td>
<td>6.61 ± 0.49</td>
<td>5.08**</td>
</tr>
<tr>
<td></td>
<td>one-tenth diluted Senkyu-cha-cho-san</td>
<td>0.69 ± 0.02</td>
<td>12.30 ± 0.62</td>
<td>17.80*</td>
</tr>
</tbody>
</table>
ketsu-to are given many other drugs concomitantly. Therefore, we chose these two Kampo extract medicines to investigate the possibility of DDIs related to extract medicines.

Rivulobirin A and notopterol (10 μM) decreased the hydroxylation rate of midazolam with as much as 100 nM ketoconazole (Table 1). This result suggests that some furanocoumarins showing inhibitory effects against CYP3A4 are included in plants of the Umbelliferae family, and rivulobirin A and notopterol, in particular, may be potent inhibitors. Therefore, concomitant administration of CYP3A4 substrate drugs with herbal remedies, including furanocoumarins, may cause DDIs of metabolism, depending on the situation.

Rivulobirin A and byakangelicol at 10 μM increased the permeability of quinidine through the Caco-2 monolayer (Table 2). The increase in $P_{app}$ by furanocoumarins was comparable to that by 20 μM cyclosporine A, used as a positive control in this study. It was reported that cyclosporine A competitively and noncompetitively inhibited P-gp (Ambudkar et al., 1999) and 20 μM cyclosporine A completely inhibited P-gp expressed on the Caco-2 monolayer (Collett et al., 2005). Therefore, both rivulobirin A and byakangelicol might strongly inhibit P-gp transport of the concomitantly administered drug. It was also reported that other herbal remedies or phytochemicals inhibited P-gp (Nair et al., 2007; Engdal and Nilsen, 2008). Therefore, it is necessary to investigate the interaction with P-gp between not only Western drugs but also Western drugs and phytochemicals. Consequently, it was clarified that byakangelicol inhibited only P-gp, notopterol inhibited only CYP3A4, and rivulobirin A inhibited both. Then, the chemical structural relationship of furanocoumarins between P-gp and CYP3A4 inhibitory effects was studied.

A close relationship could not be identified under the experimental conditions in this study (Fig. 2). Almost all of the data on furanocoumarins were plotted around the origin of the coordinates, showing that they are neither P-gp nor CYP3A4 inhibitors. Psoralen, a parent compound in a family of furanocoumarins, showed no inhibitory effect against CYP3A4. From this result, it is speculated that the presence of a side chain in the structure of a furanocoumarin is significant to show inhibitory activity. On the other hand, among the group of furanocoumarins with a side chain at the 5-position, only notopterol, with a long side chain at the 5-position, showed very strong CYP3A4 inhibitory activity. It was also reported that among some furanocoumarins with a side chain at the 5-position only notopterol showed a potent CYP3A4 inhibitory effect (Guo et al., 2000b). Rivulobirin A, the only dimer-type furanocoumarin in this study, showed potent inhibition of both P-gp and CYP3A4. It was reported that 4-(6-hydroxy-7-[(1-[(1-hydroxy-1-methyl)ethyl]-4-methyl-6-(7-oxo-7H-furo[3,2-g][1]benzopyran-4-yl)-4-hexenyloxy]-3,7-dimethyl-2-octenyl]oxy)-7H-furo[3,2-g][1]benzopyran-7-one (FC726), a dimer-type furanocoumarin in grapefruit juice, strongly inhibited CYP3A4 (Ohtsuki et al., 2000). Rivulobirin A, similar to the dimer type of furanocoumarin, may have a potent inhibitory effect because the concentration of furanocoumarin increased by degradation to monomers; however, it was also reported that some dimer-type furanocoumarins showed more potent CYP3A4 inhibition than trimer-type furanocoumarins (Guo et al., 2000b). It was also reported that the IC₅₀ value of rivulobirin A (0.16 μM) was 3-fold lower than that of notopterol (0.44 μM) using human liver microsomes (Guo et al., 2000b). Although the microsomes used in their experiment differed from those we used, rivulobirin A seemed to be a more potent inhibitor than notopterol. From a different point of view, there is a possibility that the lipophilicity of the molecule might be related to the CYP3A4 inhibitory effect because the side chains of rivulobirin A and notopterol were longer than those of others. Furthermore, it was reported that some furanocoumarins in grapefruit juice showed mechanism-based inhibition (MBI) (Lin et al., 2005; Paine et al., 2005). Therefore, both notopterol and rivulobirin A might also show MBI. We are now performing investigations to clarify the possibility of MBI. Because the participation of these factors in inhibitory potency is complex, the detailed mechanism is still unclear.

Although quinidine is a good substrate for P-gp in screening experiments, it is not a sensitive substrate for experiments to study P-gp transport in detail. Therefore, we chose digoxin as the substrate for the following detailed transport study. Digoxin was classified as a high responder to P-gp in Caco-2 cells, but quinidine was classified as a low responder (Troutman and Thakker, 2003). When digoxin was applied with cyclosporine A, the efflux ratio decreased to 1.06, suggesting that 20 μM cyclosporine A completely inhibited P-gp activity. Rivulobirin A (10 μM) decreased the efflux ratio to 3.72 and potent inhibition of P-gp by rivulobirin A showed the same result as that obtained from the screening experiment (Table 3). An increase in the uptake amount of substrate drug by inhibition of efflux transporter such as P-gp leads to an increase in intestinal absorption. Therefore, the concentration of substrate drug in blood may unexpectedly increase, causing a severe side effect. However, byakangelicol (10 μM), which showed a strong inhibitory effect comparable to that for cyclosporine A (20 μM) and rivulobirin A by screening experiments, decreased the efflux ratio of digoxin to 7.97 (Table 3). This effect seemed to be much weaker than that speculated from the results of screening experiments. For the apical-to-basal transport of quinidine, the inhibitory effect of byakangelicol was similar to that of rivulobirin A; however, for digoxin transport, the inhibitory effect of byakangelicol was approximately 60% of that of rivulobirin A. This discrepancy might reflect differences in the transport mechanism of substrates. In addition to efflux transporters, such as P-gp and multidrug-resistant protein, many influx transporters are expressed on the intestinal epi-

### TABLE 5

**Effects of Kampo extract medicine (Sokei-kakketsu-to) on the permeability of digoxin**

<table>
<thead>
<tr>
<th>Addition</th>
<th>$P_{app, AB}$</th>
<th>$P_{app, RA}$</th>
<th>Efflux Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.66 ± 0.05</td>
<td>19.20 ± 1.26</td>
<td>29.20</td>
</tr>
<tr>
<td>Positive control</td>
<td>5.21 ± 0.47</td>
<td>5.50 ± 1.18</td>
<td>1.06**</td>
</tr>
<tr>
<td>Kampo extract medicine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard regimen</td>
<td>3.15 ± 0.37</td>
<td>8.58 ± 2.00</td>
<td>2.72**</td>
</tr>
<tr>
<td>One-fifth diluted</td>
<td>1.54 ± 0.38</td>
<td>11.40 ± 1.32</td>
<td>7.40**</td>
</tr>
<tr>
<td>One-tenth diluted</td>
<td>0.82 ± 0.11</td>
<td>12.80 ± 0.56</td>
<td>15.61*</td>
</tr>
</tbody>
</table>

* Significant at $p < 0.05$ vs. control.
** Significant at $p < 0.01$ vs. control.
therium (Ito et al., 2005). For example, organic anion transporting polypeptide B (OATP-B), known as an influx transporter on hepatocytes, was also expressed on the human intestinal epithelium (Kobayashi et al., 2003). If the substrate drug for P-gp was also that for the influx transporter, the membrane permeability of such a compound would be very complicated. Although quinidine transport by the organic cation transporter has not been completely rejected, it is thought to be a specific substrate for P-gp at present. Therefore, it can be assumed that quinidine permeates the Caco-2 monolayer by passive diffusion and transport via P-gp. It was reported that digoxin transport from the apical-to-basal direction through rat ileal epithelium increased with N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isooquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide (GF120918), a selective P-gp inhibitor, but concomitant application of bromosulfophthalein, a known oatp/OATP inhibitor, decreased quinidine transport (Yao and Chiou, 2006). This result suggests that oatp may contribute to digoxin transport in rat intestine as well as in the liver and brain. Furthermore, it was reported previously that OATP-B is expressed on the Caco-2 monolayer and contributes to drug transport (Saito et al., 2006). Inhibition of OATP-B by flavonoids and furanocoumarin derivatives was also reported (Dresser et al., 2005; Kamath et al., 2005; Satoh et al., 2005; Fuchikami et al., 2006). Therefore, as a reason for the unexpectedly reduced inhibitory effect of byakangelicol on P-gp shown in digoxin transport in Caco-2, it is possible that byakangelicol might inhibit not only P-gp but also OATP-B. The inhibitory effects of other P-gp by furanocoumarins is now under investigation.

Herbal remedies are usually taken as Kampo extract medicines, not as a single compound; therefore, we investigated the inhibitory effect of furanocoumarins as Kampo extract medicines applied as Kampo extract medicine on P-gp activity. Application of either Senkyu-cha-cho-san or Sokei-kakketsu-to significantly decreased the efflux ratio of digoxin. Surprisingly, efflux ratios after application of Kampo extract medicine were more decreased than with 10 μM concentrations of each single compound, i.e., byakangelicol or rivulobinin A (Tables 4 and 5). Furthermore, the inhibitory effects by Kampo extract medicines were dependent on their concentration in the dosing solution and even one-tenth-diluted solution compared with the normal regimen significantly inhibited P-gp activity. Kyokatsu and Byakus are common herbal remedies contained in Kampo extract medicines composed of many furanocoumarins, such as rivulobinin A, byakangelicol, norterol and so on; therefore, the total amounts of furanocoumarins in Kampo extract medicines may be greater than the dosing amounts of furanocoumarins applied as a single compound, resulting in an increased inhibitory effect on P-gp. The inhibitory effects of Senkyu-cha-cho-san were more potent than those of Sokei-kakketsu-to. According to the product information from the manufacturer, the content of Kyokatsu and Byakusi in Senkyu-cha-cho-san was 10% each, but in Sokei-kakketsu-to was 5.45 and 3.63%, respectively. The amounts of not only Kyokatsu and Byakusi but also of Senkyu and Boufu, which also contain furanocoumarins, are therefore higher in Senkyu-cha-cho-san than Sokei-kakketsu-to. The difference in the furanocoumarin amount in Kampo extract medicine might reflect differences in the potency of the inhibitory effect; however, it is still not known whether many furanocoumarins show an additive effect or a multiplicative effect on P-gp. Kampo extract medicine can usually be obtained in granule form, and the product instructions advise taking it a multiplier effect on P-gp. Kampo extract medicines can usually be obtained in granule form, and the product instructions advise taking it with a glass of water; however, the concentration of Kampo extract medicine might be lower in the gastrointestinal tract because of dilution by digestion fluid; therefore, we confirmed the inhibitory effect using Kampo extract medicine diluted to one-fifth or one-tenth of the normal regimen. Even the application of Kampo extract medicine diluted to one-tenth mildly inhibited P-gp activity, suggesting that intestinal absorption of P-gp substrate drugs might be changed by concomitant administration with Kampo extract medicines. This point is now under investigation using in vivo experiments.

In conclusion, it was found that some furanocoumarin derivatives extracted from herbs belonging to the Umbelliferae family strongly inhibited P-gp and CYP3A4 in the intestine. Kampo extract medicines containing Kyokatsu or Byakus, herbal remedies belonging to the Umbelliferae family, may cause drug-drug interactions when used concomitantly with P-gp or CYP3A4 substrate drugs.

References


**Address correspondence to:** Dr. Kazunori Iwanaga, Division of Pharmaceutics, Osaka University of Pharmaceutical Sciences, 4-20-1 Nasahara, Takatsuki-city, Osaka 569-1094, Japan. E-mail: iwanaga@gly.oups.ac.jp

