Furanocoumarin derivatives in Kampo extract medicines inhibit cytochrome P450 3A4 and/or 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, 4-20-1 Nasahara, Takatsuki-city, Osaka 569-1094, Japan
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Corresponding author: Kazunori Iwanaga, Ph. D.,
Division of Pharmaceutics, Osaka University of Pharmaceutical Sciences, 4-20-1 Nasahara, Takatsuki-city, Osaka 569-1094, Japan.
Phone & FAX: +81-72-690-1049
e-mail: iwanaga@gly.oups.ac.jp

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ABSTRACT
Furanocoumarins in grapefruit are known to show inhibitory effects against P-glycoprotein and/or 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, not 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 Sokei-kakketsu-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/or CYP3A4. Kampo extract medicines containing herbal remedies belonging to the umbelliferae family may cause drug–drug interaction with P-gp or CYP3A4 substrate drug.
INTRODUCTION

P-glycoprotein (P-gp) is widely expressed in the human blood–brain barrier, liver, kidney, intestine and other organs (Cordon-Cardo et al., 1989; Cordon-Cardo et al., 1990), and related to the drug excretion from the body. Cytochrome P-450 3A4 (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 et al., 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 (Lemma et al., 2006) and cyclosporine A (Kageyama et al., 2005), increased the bioavailability and maximum concentration of substrates in blood. These are typical examples of drug–drug interaction (DDI) in intestinal drug absorption between Western drugs; however, such DDI is not a special case for the combination of Western drugs. Some ingredients in food and dietary supplements, such as grapefruit, hops, tea (Rodriguez-Proteau et al., 2006), St. John’s Wort (Johne et al., 1999), ginkgo leaf (Wang et al., 2005) and red clover (Peng et al., 2006) affect the activities of P-glycoprotein and/or CYP3A4, resulting in the alteration of the pharmacokinetics of Western drugs.

Among these, grapefruit juice has been intensively investigated and inhibitory effects against CYP3A4 are well-known. It is shown that ingredients in grapefruit juice inhibit P-gp and CYP3A4 and affect the absorption and metabolism of midazolam (Veronese et al., 2003), vinblastine (Ohnishi et al., 2000), talinolol (S-Langguth and Langguth, 2001) and digoxin (Becquemont et al., 2001) both in vitro and in vivo. At the first, naringin, one of the flavonoids in grapefruit, was thought to be a cause of the inhibitory effect on CYP3A4 in the intestinal epithelium; however, it
has been reported that some furanocoumarin derivatives in grapefruit showed more potent inhibitory effect later (Guo et al., 2000). Furanocoumarin derivatives are widely contained in the plants of rutaceae and umbelliferae families. Byakushi, Kyokatsu, Boufu and Hamaudo are herbal remedies used as components of Kampo extract medicines in the umbelliferae family.

Recently, many herbal medicines were used all over the world. Among these, Chinese traditional medicines (CTM) are well-known. Herbal medicines which are based on CTM 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 widely used for the treatment of cancer (Takegawa et al., 2008; Matsuda et al., 2009), dementia (Watanabe et al., 2003), metabolic disease such as obesity and diabetes (Omiya et al., 2005; Shimada et al., 2008), hypertension (Kimura et al., 2006), neuralgia (Sunagawa et al., 2001) and dysmenorrhoeal (Oya et al., 2008). Moreover, complementary and alternative medicines including Kampo medicines are gradually provided in the hospital. However, the therapeutic effects using Kampo medicines have not been well-understood and the 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, the 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 potency of bergamottin and 6’ 7’-dihydroxybergamottin, both classified as furanocoumarins isolated from grapefruit juice, differed from each other (Paine et al., 2004). Thus, the relation of the effects of furanocoumarins to CYP3A4 and their chemical structure has not been 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. Furthermore, we also studied their inhibitory effect on P-gp when furanocoumarins are used as Kampo extract medicine, not as an isolated single compound.
Materials and Methods

Materials and Chemicals

Quinidine was purchased from Sigma-Aldrich (St. Louis, MO, USA). Digoxin was from Nacalai Tesque (Kyoto, Japan). Dulbecco’s modified Eagle’s medium (DMEM), Hanks’ balanced salt solution (HBSS), non-essential amino acid solution (NEAA), HEPES and Trypsin were from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) was from Hyclone (Logan, UT, USA). 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%. Senkyu-cha-cho-san and Sokei-kakketsu-to, granule Kampo extract medicines containing herbal remedies belonging to the umbelliferae family were from Tsumura & Co. (Tokyo, Japan). Other reagents were commercially available and of analytical grade.

Cell Culture

Caco-2 (Passage number 27) cells were obtained from the European Collection of Cell Culture Collection (Salisbury, UK) 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, USA) with an insert membrane pore size of 0.4 µm. The medium was changed every two days for 8 days, and the transport experiments were performed 17 days post-seeding. One day before the experiment, transepithelial electrical resistance (TEER) was ≥ 700Ω•cm².
Incubation experiment for CYP3A4 inhibitor screening

CYP3A4 inhibitory effects by furanocoumarins were evaluated using Supersomes™ containing cDNA-expressed CYP3A4 coexpressed with P450-reductase (oxidoreductase) and cytochrome b5 (BD Gentest, Woburn, MA). Midazolam was used as a substrate for CYP3A4. Midazolam and furanocoumarin were dissolved in dimethylsulfoxide and added to the incubations at 2 µmol/L and 1 µmol/L, respectively. Final concentration of dimethylsulfoxide 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., 2000). Incubation was performed in 100 mM potassium phosphate-buffered saline (pH 7.4) with 1 mM EDTA. The enzymatic reaction was initiated by adding NADPH regenerating system (1.3 mmol/L β-NADP+, 3.3 mmol/L glucose-6-phosphate, 0.4 units/mL glucose-6-phosphate dehydrogenase and 3.3 mmol/L MgCl₂) to the incubations. Insect cell-expressed CYP3A4 Supersomes™ (coexpressed with P450 reductase and cytochrome b5) were used in all incubations at a concentration of 10 pmol CYP3A4/mL. Two minutes after starting, ice-cold 100 mmol/L sodium bicarbonate solution was added to the incubation to terminate the reaction. One hundred milliliters of diazepam (5 µmol/L), an internal standard, and 1.5 mL ethyl acetate were added to 500 µL of the incubation. The mixture was vigorously shaken for 15 min and centrifuged at 900 G for 10 min. The organic phase was transferred into a clean tube and evaporated in vacuo. The residue was reconstituted with methanol and injected into LC/MS.

LC/MS assay for 1’-hydroxy midazolam (1’-OH midazolam)

The rate of 1’-OH midazolam formation from midazolam was used as an indicator of CYP3A4
activity. The quantification of 1’-OH midazolam was carried out using a liquid chromatography/mass spectrometric (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, 150 mm φ 4.6 mm, 5 µm column (Kanto Chemical Co., Inc., Tokyo, Japan). The column temperature was maintained at 30 °C. Analysis was carried out under isocratic conditions. The mobile phase was 10 mM ammonium acetate (pH5.0): methanol = 20 : 80 (v/v) and was run at a flow rate of 0.5 mL/min. Mass spectrometry was performed on a M-8000 mass spectrometer (Hitachi High-Technologies Co., Tokyo, Japan). The soft sonic ionization (SSI) positive mode was employed. The precursor ions [M-H+] of 1’-OH midazolam and diazepam, an internal standard, was detected at m/z 342.1 and 285.4, respectively.

Calculation of CYP3A4 inhibitory ratio

Inhibitory ratio (%) was calculated by the following equation, and the inhibitory effects of various furanocoumarins were compared.

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

(1)

where, \(V_{\text{control}}\) is the 1’-OH midazolam formation rate (p mol/min/p mol CYP3A4) after incubation of midazolam alone and \(V\) is that after co-incubation 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 to 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 as a weak substrate (Troutman and Thakker, 2003), it was selected for the screening experiment to avoid false-negative results. Quinidine was dissolved with HBSS containing 10 mmol/L glucose and 0.5% DMSO (Transport medium (TM)). Quinidine and furanocoumarin concentrations in TM were adjusted to 10 µmol/L and 5 µmol/L, respectively. Apical and basal sides of the Caco-2 monolayer were filled with TM and prewarmed at 37°C for 10 minutes. After prewarming, TM on the apical side was discarded and 0.5 mL 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_{app}) of compound was calculated according to the following equation.

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

(2)

where, dQ/dt is the slope of the linear portion of permeated amount versus time curve, A is the effective surface area of insert of Transwell and C_0 is 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 the following equation.

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

(3)

where, P_{app, control} is the apparent permeability after the application of quinidine alone and P_{app} is that
with furanocoumarin or cyclosporine A. Cyclosporin 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 alkalinize the sample, 100 mmol/L 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 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 carried out under isocratic conditions. The mobile phase was 10 mM ammonium acetate (pH5.0): methanol = 45 : 55 (v/v) and was run at a flow rate of 0.6 mL/min. The atmospheric pressure chemical ionization (APCI) positive mode was employed. The precursor ions [M-H+] of quinidine and dextromethorphan, an internal standard, was 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 µmol/L. For the experiment of digoxin transport from the apical to basal direction, 0.5 mL 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 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: 7.5g (3 packages) of Senkyu-cha-cho-san (TJ-124) contains 3.25g dried extract from a mixture of Kyokatsu (2.0g), Byakushi (2.0g), Koubushi (4.0g), Senkyu (3.0g), Keigai (2.0g), Hakka (2.0g), Boufu (2.0g), Kanzo (1.5g) and Chayou (1.5g). The other is Sokei-kakketsu-to (TJ-53), the regimen of which is as follows: 7.5g (3 packages) of Sokei-kakketsu-to contains 5.0g dried extract from the mixture of Kyokatsu (1.5g), Byakushi (1.0g), Syakuyaku (2.5g), Jiou (2.0g), Senkyu (2.0g), Soujutsu (2.0g), Touki (2.0g), Tounin (2.0g), Bukuryou (2.0g), Goshitsu (1.5g), Chinpi (1.5g), Ireisen (1.5g), Boui (1.5g), Boufu (1.5g), Ryutan (1.5g), Kanzo (1.0g) and Shoukyou (0.5g). One package (2.5g) of Senkyu-cha-cho-san or Sokei-kakketsu-to was added to 200 mL TM and vigorously stirred for 2 hours. The obtained aqueous suspension 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 (1/5- diluted) or one tenth-diluted (1/10- 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 using 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 the following equation,

$$\text{Efflux ratio} = \frac{P_{\text{app, BA}}}{P_{\text{app, AB}}}$$  \hspace{1cm} (4)

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 basal 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 900 G. 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 reversed phase HPLC on a Mightysil RP-18 GP column (150 × 4.6 mm, 5 µm; Kanto Chemical Co., Inc., Tokyo, Japan). HPLC 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 ± SE. 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 carried out 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 p mol/min/p mol 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. Psolaren, 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, isoimperatorin, 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 heraclenol) 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 carried out by using quinidine as a P-gp substrate. Apparent permeability of quinidine from the apical to basal direction through the Caco-2 monolayer (P_{app}) is shown in Table 2. In the presence of cyclosporine A (20 μM), a potent P-gp inhibitor, P_{app} value increased compared to quinidine alone (1.34-fold...
increase vs control). Psolaren, 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 were also 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_app of quinidine by 1.39-fold compared to the control. Rivulobirin A markedly increased P_app (1.44-fold increase vs control). This inhibitory effect was comparable to that of 20 μM cyclosporin 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. Surprisingly, 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 those of 20 μM cyclosporin 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, 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 P-gp inhibitory effects of those furanocoumarins, byakangelicol,
notopterol and rivulobirin A, a detailed study was carried out using digoxin, which is more sensitive than quinidine as a substrate. Calculated values of $P_{appAB}$ and $P_{appBA}$ were shown in Fig. 3. $P_{appAB}$ of digoxin ($0.66 \times 10^{-6}$ cm/sec) alone was much lower than that of quinidine ($2.67 \times 10^{-5}$ cm/sec). Apparent permeability of digoxin from basal to apical ($P_{appBA}$; $19.20 \times 10^{-6}$ cm/sec) was much greater than $P_{appAB}$. Both $P_{appAB}$ and $P_{appBA}$ values after concomitant application with furanocoumarins are shown in Fig. 3. $P_{appAB}$ of digoxin greatly increased in the presence of cyclosporin A ($5.21 \times 10^{-6}$ cm/sec). The addition of rivulobirin A to the apical side significantly increased ($2.88 \times 10^{-6}$ cm/sec) $P_{appAB}$ of digoxin when compared to the control. The addition of byakangelicol increased $P_{appA}$ ($1.77 \times 10^{-6}$ cm/sec); however, no change in $P_{appA \rightarrow B}$ was observed when notopterol was concomitantly applied to the apical side. Cyclosporin A, which was added to the basal side, inhibited digoxin from being pumped to the apical side and significantly decreased $P_{appBA}$ values ($5.50 \times 10^{-6}$ cm/sec). Rivulobirin A and byakangelicol decreased $P_{appBA}$ values to $10.70 \times 10^{-6}$ and $14.10 \times 10^{-6}$ cm/sec, 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. Cyclosporin 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 in the order of 10 µM byakangkelicol < 10 µM rivulobirin A < 20 µM cyclosporin A. Notopterol slightly decreased the efflux ratio, but it was not significant compared to the control.

Inhibitory effect of Kampo extract medicines on P-gp

Some Kampo extract medicines, such as Senkyu-cha-cho-san and Sokei-kakketsu-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. $P_{appAB}$, $P_{appBA}$ values and the efflux ratio of digoxin after concomitant application with Sekyu-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, $P_{appAB}$ increased ($2.29 \times 10^{-6}$ cm/sec) compared to the control. On the other hand, $P_{appBA}$ 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 to the control.

Next, we carried out the same inhibition experiment using diluted Senkyu-cha-cho-san to check the dose dependency of the effect. $P_{appAB}$, $P_{appBA}$ values and the efflux ratio after concomitant application with diluted Senkyu-cha-cho-san are also shown in Table 4. When digoxin was applied with diluted Senkyu-cha-cho-san (1/5-diluted or 1/10-diluted), $P_{appAB}$ decreased compared to the standard regime as the concentration of Senkyu-cha-cho-san in the apical side decreased. In contrast, $P_{appBA}$ increased with the decrease of Senkyu-cha-cho-san concentration on the basal side. Consequently, the efflux ratios of digoxin after concomitant application of 1/5-diluted and 1/10-diluted Senkyu-cha-cho-san increased from 1.60 of normal regimen to 5.08 and 17.80, respectively; however, even 1/10-diluted Senkyu-cha-cho-san still showed significant inhibition against P-gp. Similarly, 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, $P_{appAB}$ of digoxin greatly increased ($3.15 \times 10^{-6}$ cm/sec), whereas $P_{appBA}$ decreased to $8.58 \times 10^{-6}$ cm/sec, as shown in Table 5. When diluted Sokei-kakketsu-to (1/5-diluted or 1/10-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 1/10-diluted Sokei-kaketsu-to showed significant P-gp inhibition compared to the control.
Discussion

Recently, it was clarified that the ingredients in grapefruit juice which inhibited CYP3A4 and P-gp in the intestine were furanocoumarins (Guo et al., 2000); however, furanocoumarins were 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 DDI between Western drugs and Kampo extract medicines. Senkyu-cha-cho-san is used for alleviation of early symptom of the common cold and prescribed for many patients. On the other hand, Sokei-kakketsu-to is used for arthritis or neuralgia and often used by the aged. It is considered that the people who take Sokei-kakketsu-to are concomitantly given many other drugs. Therefore, we chose these two Kampo extract medicines to investigate the possibility of DDI 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, in particular, rivulobirin A and notopterol may be potent inhibitors. Therefore, concomitant administration of CYP3A4 substrate drugs with herbal remedies, including furanocoumarins, may cause DDI 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 of P_app by furanocoumarins was comparable to that by 20 μM cyclosporine A, used as a positive control in this study. It is reported that cyclosporine A comparatively or non-comparatively 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 may strongly inhibit P-gp transport of the concomitantly administered drug. It is also reported that other herbal remedies or phytochemicals inhibited P-gp (Nair et al., 2007; Engdal and Nilsen, 2008). Therefore, it is necessary to inclusively investigate the interaction with P-gp between not only Western drugs but also Western drug 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 the data on furanocoumarins were plotted around the origin of the coordinates, showing that they are neither P-gp nor CYP3A4 inhibitors. Interestingly, psolaren, a parent compound in a family of furanocoumarins, showed no inhibitory effect against CYP3A4. From this result, it is speculated that the presence of side-chain in furanocoumarin in its structure 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 is also reported that only notopterol showed a potent CYP3A4 inhibitory effect among some furanocoumarins with a side-chain at the 5 position (Guo et al., 2000). Rivulobirin A, the only dimer-type furanocoumarin in this study, showed potent inhibition of both P-gp and CYP3A4. It was reported that FC-726, a dimer-type furanocoumarin in grapefruit juice, strongly inhibited CYP3A4 (Ohnishi 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 is also reported that some dimer-type furanocoumarins showed more potent CYP3A4 inhibition than trimer-type furanocoumarins (Guo et al., 2000). It is
also reported that IC50 values of rivulobirin A (0.16 µM) was 3-fold lower than that of notopterol (0.44 µM) using human liver microsomes (Guo et al., 2000). Although the microsomes used for in their experiment was differed from that we used, rivulobirin A seemed to be 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-chain length of rivulobirin A and notopterol was longer than that of others. Furthermore, it is reported that some furanocoumarins in grapefruits juice showed mechanism-based inhibition (MBI) (Lin et al., 2005; Paine et al., 2005). Therefore, both notopterol and/or rivulobirin A might also show MBI. We are now under investigation to clarify the possibility of MBI. Because these factors showed complex participation in inhibitory potency, the detailed mechanism is still unclear.

Although quinidine is quite 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. It is reported that digoxin has been classified as a high responder to P-gp in Caco-2 cells, but quinidine 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 cyclosporin 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). Increase in the uptake amount of substrate drug by inhibition of efflux transporter such as P-gp leads to the increase of intestinal absorption. Therefore, the concentration of substrate drug in blood may unexpectedly increase, causing the severe side effect. However, byakangelicol (10 µM), which showed a strong inhibitory effect comparable to 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 about 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 (MRP-2), many influx transporters are expressed on the intestinal epithelium (Ito et al., 2005). For example, organic anion transporter 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 were also that for the influx transporter, the membrane permeability of such a compound would be very complicated. Although quinidine transport by 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 is reported that digoxin transport from the apical to basal direction through rat ileal epithelium increased with 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 the liver and brain. Furthermore, it was previously reported that OATP-B is expressed on the Caco-2 monolayer and contributes to drug transport (Sai et al., 2006). Inhibition of OATP-B by furavonoids and furanocoumarin derivatives was also reported (Fuchikami et al., 2006; Kamath et al., 2005; Dresser et al., 2005; Satoh et al., 2005). 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 inhibition of transporters other than 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 applied as Kampo extract medicine on P-gp activity. Application of either Senkyu-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 of each single compound, i.e., byakangelicol or rivulobirin A (Tables 4, 5). Furthermore, inhibitory effects by Kampo extract medicines were dependent on their concentration in dosing solution and even 1/10-diluted solution compared to the normal regimen significantly inhibited P-gp activity. Kyoukatsu and Byakushi are common herbal remedies contained in Kampo extract medicines composed of many furanocoumarins, such as Rivulobirin A, byakangelicol, notopterol 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 the increased inhibitory effect of P-gp. Inhibitory effects by Senkyu-cho-san were more potent than by Sokei-kakketsu-to. According to the product information from the manufacturer, the content of Kyoukatsu and Byakushi in Senkyu-cho-san was 10% each, but that in Sokei-kakketsu-to was 5.45 % and 3.63%, respectively. The amounts of not only Kyoukatsu and Byakushi but also Senkyu and Boufu, which also contain furanocoumarins, are therefore higher in Senkyu-cho-san than Sokei-kakketsu-to. The difference in furanocoumarin amount in Kampo extract medicine might reflect differences in the potency of the inhibitory effect; however, it is still unknown whether many furanocoumarins show an additive effect or a multiplier effect on P-gp. Kampo extract medicine can
be usually 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 1/5 or 1/10 of the normal regimen. Even the application of Kampo extract medicine diluted to 1/10 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/or CYP3A4 in the intestine. Kampo extract medicines containing Kyoukatsu or Byakushi, herbal remedies belonging to the umbelliferae family, may cause drug–drug interactions when concomitantly used with P-gp or CYP3A4 substrate drugs.
References


Lin HL, Kent UM, and Hollenberg PF (2005) The grapefruit juice is not limited to cytochrome P450 (P450) 3A4: evidence for bergamottin-dependent inactivation, heme destruction, and covalent binding to protein in P450s 2B6 and 3A5. *J Pharmacol Exp Ther* **313**: 154–164.


in rats. *Int J Pharm* **322**: 79–86.


Figure legends

Fig. 1 Chemical structures of furanocoumarins extracted from herbal remedies in the Umbelliferae family

Fig. 2 Relationship between the potency of P-gp and CYP3A4 inhibitory effects by 12 furanocoumarins

CYP3A4 inhibitory effect (Inhibitory rate (%)) was calculated from equation (1) based on the results of the incubation experiment for CYP3A4 inhibitor screening. P-gp inhibitory effect (Increase rate (%)) was calculated from equation (3) based on the results of the transport experiment for P-gp inhibitor screening. Each value represents the mean of 2 experiments.

Fig. 3 Effects of furanocoumarins on the permeability of digoxin through the Caco-2 monolayer in both apical to basal (A to B) and basal to apical (B to A) directions.

Initial concentration of each furanocoumarin and digoxin was adjusted to 10 µM. Initial concentration of cyclosporin A was adjusted to 20 µM. Each value represents the mean ± S.E. (n=3-6). **Significant at p < 0.01 vs Control.
Table 1. CYP3A4 inhibitory effects of furanocoumarins

V values show midazolam hydroxylation rate by CYP3A4 of Supersomes™ determined from the CYP3A4 inhibitor screening experiment. Initial concentrations of midazolam and each furanocoumarin were adjusted to 2 µM and 10 µM, respectively. Initial concentration of ketoconazole (positive control) was adjusted to 100 nM. Inhibitory ratio was calculated from equation (1).

<table>
<thead>
<tr>
<th></th>
<th>V (pmol/min/pmol CYP3A4)</th>
<th>Inhibitory ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2 µmol/L Midazolam alone</td>
<td>8.67</td>
</tr>
<tr>
<td>Positive control</td>
<td>+ 100 nmol/L Ketoconazole</td>
<td>4.45</td>
</tr>
<tr>
<td>+10 µmol/L furanocoumarin</td>
<td>+ Psoralen</td>
<td>10.60</td>
</tr>
<tr>
<td>side-chain 5 position</td>
<td>+ Bergapten</td>
<td>7.76</td>
</tr>
<tr>
<td></td>
<td>+ Isoimperatorin</td>
<td>9.78</td>
</tr>
<tr>
<td></td>
<td>+ Oxypeucedanin</td>
<td>8.96</td>
</tr>
<tr>
<td></td>
<td>+ Oxypeucedanin hydrate</td>
<td>8.32</td>
</tr>
<tr>
<td></td>
<td>+ Notopterol</td>
<td>4.09</td>
</tr>
<tr>
<td>8 position</td>
<td>+ Imperatorin</td>
<td>9.13</td>
</tr>
<tr>
<td></td>
<td>+ Heraclenin</td>
<td>7.32</td>
</tr>
<tr>
<td></td>
<td>+ Heraclenol</td>
<td>9.06</td>
</tr>
<tr>
<td>5 and 8 positions</td>
<td>+ Bykangelicol</td>
<td>8.19</td>
</tr>
<tr>
<td>dimer</td>
<td>+ Byakangelic</td>
<td>8.13</td>
</tr>
<tr>
<td></td>
<td>+ Rivulobirin A</td>
<td>3.68</td>
</tr>
</tbody>
</table>

Each value represents the mean of 2 experiments.
Table 2. P-gp inhibitory effects of furanocoumarins

$P_{\text{app}}$ values show the apparent permeability of quinidine through Caco-2 monolayer from apical to basal direction. Initial concentrations of quinidine and furanocoumarins were adjusted to 5 µM and 10 µM, respectively. Initial concentration of cyclosporine A (positive control) was adjusted to 20 µM. Increase rate was calculated from equation (3).

<table>
<thead>
<tr>
<th></th>
<th>$P_{\text{app}}$ ($\times 10^{-5}$ cm/s)</th>
<th>Increase ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5 µmol/L Quinidine alone</td>
<td>2.67</td>
</tr>
<tr>
<td>Positive control</td>
<td>20 µmol/L Cyclosporin A</td>
<td>3.58</td>
</tr>
<tr>
<td>+10 µmol/L furanocoumarin</td>
<td>+ Psoralen</td>
<td>2.32</td>
</tr>
<tr>
<td>side-chain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 position</td>
<td>+ Bergapten</td>
<td>2.26</td>
</tr>
<tr>
<td></td>
<td>+ Isoimperatorin</td>
<td>2.82</td>
</tr>
<tr>
<td></td>
<td>+ Oxypeucedanin</td>
<td>2.36</td>
</tr>
<tr>
<td></td>
<td>+ Oxypeucedanin hydrate</td>
<td>2.63</td>
</tr>
<tr>
<td></td>
<td>+ Notopterol</td>
<td>2.58</td>
</tr>
<tr>
<td>8 position</td>
<td>+ Imperatorin</td>
<td>3.07</td>
</tr>
<tr>
<td></td>
<td>+ Heraclenin</td>
<td>2.52</td>
</tr>
<tr>
<td></td>
<td>+ Heraclenol</td>
<td>2.31</td>
</tr>
<tr>
<td>5 and 8 positions</td>
<td>+ Bykangelicol</td>
<td>3.71</td>
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<td></td>
<td>+ Byakangelicin</td>
<td>2.90</td>
</tr>
<tr>
<td>dimer</td>
<td>+ Rivulobirin A</td>
<td>3.85</td>
</tr>
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</table>

Each value represents the mean of 2 experiments.
Table 3. Effects of furanocoumarins on the permeability of digoxin

$P_{\text{appAB}}$ and $P_{\text{appBA}}$ values show the apparent permeability of digoxin through Caco-2 monolayer from apical to basal and basal to apical direction, respectively. Initial concentrations of both digoxin and furanocoumarins were adjusted to 10 µM. Initial concentration of cyclosporine A (positive control) was adjusted to 20 µM. Efflux ratio was calculated from equation (4).

<table>
<thead>
<tr>
<th></th>
<th>Papp$_{\text{AB}}$ (×10$^{-6}$ cm/s)</th>
<th>Papp$_{\text{BA}}$</th>
<th>Efflux ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10 µmol/L Digoxin</td>
<td>0.66 ± 0.05</td>
<td>19.20 ± 1.26</td>
</tr>
<tr>
<td>Positive control + 20 µmol/L Cyclosporin A</td>
<td>5.21 ± 0.47</td>
<td>5.50 ± 1.18</td>
<td>1.06**</td>
</tr>
<tr>
<td>+10 µmol/L furanocoumarin side-chain 5 position + Notopterol</td>
<td>0.96 ± 0.08</td>
<td>17.80 ± 1.71</td>
<td>18.50</td>
</tr>
<tr>
<td>5 and 8 positions + Bykangelicol</td>
<td>1.77 ± 0.16</td>
<td>14.10 ± 1.32</td>
<td>7.97**</td>
</tr>
<tr>
<td>dimer</td>
<td>+ Rivulobirin A</td>
<td>2.88 ± 0.31</td>
<td>10.70 ± 2.12</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.E. (n=3).

**Significant at $p < 0.01$ vs Control.
Table 4. Effects of Kampo extract medicine (Senkyu-cha-cho-san) on the permeability of digoxin

$P_{appAB}$ and $P_{appBA}$ values show the apparent permeability of digoxin through Caco-2 monolayer from apical to basal and basal to apical direction, respectively. Initial concentration of digoxin was adjusted to 10 µM. Standard regimen of Kampo extract medicine (Senkyu-cha-cho-san) was prepared by adding 1 package (2.5 g) of Kampo extract medicine to 200 mL transport medium, vigorously stirring for 2 hours and filtered with filter paper. For the diluted regimen, the obtained standard regimen solution was diluted with TM to adjust 1/5 or 1/10 concentration of Kampo extract medicine in standard regimen.

<table>
<thead>
<tr>
<th></th>
<th>$P_{appAB}$</th>
<th>$P_{appBA}$</th>
<th>Efflux ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(×10(^{-6}) cm/s)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 µmol/L Digoxin</td>
<td>0.66 ± 0.05</td>
<td>19.20 ± 1.26</td>
<td>29.20</td>
</tr>
<tr>
<td><strong>Positive control</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 20 µmol/L Cyclosporin A</td>
<td>5.21 ± 0.47</td>
<td>5.50 ± 1.18</td>
<td>1.06**</td>
</tr>
<tr>
<td><strong>Kampo extract medicine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>standard regimen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Senkyu-cha-cho-san</td>
<td>2.29 ± 0.25</td>
<td>3.67 ± 0.79</td>
<td>1.60**</td>
</tr>
<tr>
<td>1/5-diluted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Senkyu-cha-cho-san</td>
<td>1.30 ± 0.22</td>
<td>6.61 ± 0.49</td>
<td>5.08**</td>
</tr>
<tr>
<td>1/10-diluted</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Senkyu-cha-cho-san</td>
<td>0.69 ± 0.02</td>
<td>12.30 ± 0.62</td>
<td>17.80*</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.E. (n=3-6).

**Significant at $p < 0.01$ vs Control., *Significant at $p < 0.05$ vs Control.
Table 5. Effects of Kampo extract medicine (Sokei-kakketsu-to) on the permeability of digoxin

$P_{\text{appAB}}$ and $P_{\text{appBA}}$ values show the apparent permeability of digoxin through Caco-2 monolayer from apical to basal and basal to apical direction, respectively. Initial concentration of digoxin was adjusted to 10 µM. Standard regimen of Kampo extract medicine (Sokei-kakketsu-to) was prepared by adding 1 package (2.5 g) of Kampo extract medicine to 200 mL transport medium, vigorously stirring for 2 hours and filtered with filter paper. For the diluted regimen, the obtained standard regimen solution was diluted with TM to adjust 1/5 or 1/10 concentration of Kampo extract medicine in standard regimen.

<table>
<thead>
<tr>
<th></th>
<th>$P_{\text{appAB}}$ ($\times 10^{-6}$ cm/s)</th>
<th>$P_{\text{appBA}}$ ($\times 10^{-6}$ cm/s)</th>
<th>Efflux ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10 µmol/L Digoxin</td>
<td>0.66 ± 0.05</td>
<td>19.20 ± 1.26</td>
</tr>
<tr>
<td>Positive control</td>
<td>+ 20 µmol/L Cyclosporin A</td>
<td>5.21 ± 0.47</td>
<td>5.50 ± 1.18</td>
</tr>
<tr>
<td>Kampo extract medicine</td>
<td>Sokei-kakketsu-to</td>
<td>3.15 ± 0.37</td>
<td>8.58 ± 2.00</td>
</tr>
<tr>
<td>standard regimen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/5 -diluted</td>
<td>Sokei-kakketsu-to</td>
<td>1.54 ± 0.38</td>
<td>11.40 ± 1.32</td>
</tr>
<tr>
<td>1/10 -diluted</td>
<td>Sokei-kakketsu-to</td>
<td>0.82 ± 0.11</td>
<td>12.80 ± 0.56</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.E. (n=3-6).

**Significant at $p < 0.01$ vs Control., *Significant at $p < 0.05$ vs Control.
Fig. 1

- Side chain at the 5 position
  - Bergapten
  - Isoimperatorin
  - Oxypeucedanin
  - Oxypeucedanin hydrate
  - Notopterol

- Side chain at the 8 position
  - Imperatorin
  - Heraclenin
  - Heraclenol
  - Dimer

- Side chain at the 5 and 8 position
  - Byakangelicol
  - Byakangelicin
  - Rivulobirin A
Fig. 2
Fig. 3