Effects of Kampo Medicines on P-Glycoprotein

Toshiyuki SATOH, Yuka WATANABE, Nobutomo IKARASHI, Kiyomi ITO, and Kiyoshi SUGIYAMA*  
Department of Clinical Pharmacokinetics, Hoshi University; 2–4–41 Ebara, Shinagawa-ku, Tokyo 142–8501, Japan.  
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The Kampo medicines are more and more often used in recent years, usually together with the western drugs. The need for the investigation of drug interactions between Kampo medicines and western drugs are, therefore, widely recognized. Among the various possible causes for the drug–drug interactions, those related to pharmacokinetics such as drug metabolism and transport are regarded as most frequent and clinically important. In the present study, the effects of Kampo medicines on the P-glycoprotein (P-gp), one of the major drug transporters, were investigated in in vitro studies using human P-gp membranes. The P-gp activity in the presence and absence of commonly used 50 Kampo medicines was evaluated by the ATPase assay detecting the inorganic phosphate produced by the ATP hydrolysis. The ATPase activity was inhibited by most of the Kampo medicines studied, indicating the possibility of their inhibiting the P-gp. The degree of inhibition in the presence of verapamil, a P-gp substrate, showed a significant correlation with that in the absence of verapamil. Furthermore, the inhibitory effect of the Kampo medicines on the ATPase activity correlated with their licorice root (kanzo) content, suggesting the contribution of licorice root in the P-gp inhibition. Because licorice root is one of the most common ingredients in the Kampo medicines and is also often used in the food as a sweetener, it might be necessary to pay attention on the interaction between the licorice root-containing drug/food and the number of drugs transported by P-gp.

Key words  P-glycoprotein; Kampo medicine; drug interaction; ATPase assay; licorice root

Multi-drug combination therapy is now common for a number of diseases and the drug–drug interactions have attracted more and more attention in the clinical practice. Among the various possible causes for the drug–drug interactions, those related to pharmacokinetics such as drug metabolism and transport are regarded as most frequent and clinically important. In contrast to the well-established information on the interactions involving drug metabolism, those involving drug transport are only recently recognized and are now under investigation by many researchers.1,2)

P-glycoprotein (P-gp), a member of the ABC transporter family, is located in the cell membrane and has a function of transporting drugs using the energy of ATP hydrolysis. P-gp is involved in the absorption, distribution and excretion of xenobiotics in various tissues.3) The inhibition of P-gp by another drug could cause the elevation of the plasma concentration of its substrates, leading to the unwanted side effects or toxicity.

Most of the interaction studies have focused on the interactions between western drugs or those between food and western drugs. Very few of them dealt with the Kampo medicines or herbs, the possibility of their causing interactions with western drugs being ignored. Since the Kampo medicines are more and more often used in recent years, usually together with the western drugs, the need for the investigation of drug interactions between Kampo medicines and western drugs are widely recognized. In the present study, the effects of Kampo medicines on P-gp were investigated in in vitro studies using human P-gp membranes.

MATERIALS AND METHODS

Materials  Human P-gp membranes were purchased from BD Gentest (Woburn, MA, U.S.A.). Dried extract of 50 Kampo medicines listed in Table 1 were provided by Tsumura & Co. (Tokyo, Japan). Adenosine-5’-triphosphate magnesium salt (MgATP), dl-dithiothreitol (DTT), ethylene glycol-bis-(beta-aminoethylether)-N,N,N’,N’-tetraacetic acid (EGTA), malachite green, 2-[N-morpholino]-ethane-sulfonic acid (Mes) hydrate, ouabain, polynvinyl alcohol, sodium molybdate dehydrate, sodium orthovanadate, trichloroacetic acid, (±)-verapamil hydrochloride, and vinblastine were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, U.S.A.). 2-Amino-2-hydroxyethylmethyl-1,3-propanediol (Tris), cyclosporin A, digoxin, dimethylsulfoxide (DMSO), hydrochloric acid (HCl), ketocanazole, potassium chloride (KCl), sodium azide, sodium dihydrogenphosphate dehydrate, and sulfuric acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

ATPase Assay  The ATPase activity of human P-gp membranes was determined by measuring inorganic phosphate liberation according to the procedure reported by Sarkadi et al.4) with some modifications.5) The human P-gp membranes (2 μg of protein) were suspended in 10 μl of the incubation medium containing 50 mM Tris–Mes (pH 6.8), 2 mM DTT, 50 mM KCl, 2 mM EGTA, 2 mM ouabain, and 5 mM sodium azide. This medium was mixed in a 96-well plate with 10 μl of a test compound solution and 10 μl of distilled water or 250 μM verapamil. The ATPase reaction was started by adding 20 μl of a 4 mM MgATP solution to the reaction mixture (30 μl) and the incubation was maintained at 37 °C for 30 min. The reaction was stopped by the addition of 20 μl of 5% trichloroacetic acid.

The liberated inorganic phosphate was measured by a method described by Carter and Karl.6) Briefly, 42 μl of solution A (2 M HCl : 0.1 M sodium molybdate = 4 : 3), 18 μl of solution B (0.042% (w/v) malachite green solution in 1% (w/v) polynvinyl alcohol), and 120 μl of solution C (7.8% (v/v) sulfuric acid) were added and the mixture was allowed to stand at room temperature for 1 h, after which the absorbance at a wavelength of 630 nm was measured using a microplate reader (Model 550, Bio-Rad, Hercules, CA, U.S.A.). The
ATPase activity was estimated by the difference obtained in the phosphate levels between 0-min (reaction stopped immediately) and 30-min incubation periods.

**Inhibitory Effects of Orthovanadate on the ATPase Activity**
Orthovanadate, a non-competitive inhibitor of P-gp ATPase, was prepared at final concentrations of 0, 5, 10, 30, 100, and 300 \( \mu \text{M} \) and the inhibitory effects on ATPase activity in the presence of 50 \( \mu \text{M} \) verapamil was examined.

**ATPase Activity in the Presence of P-gp Substrates**
The ATPase activity was measured in the presence of P-gp substrates (cyclosporin A, digoxin, ketoconazole, verapamil, and vinblastine) at final concentrations of 1.56, 3.13, 6.25, 12.5, 25, and 50 \( \mu \text{M} \). These substrates were dissolved in DMSO and the final concentration of DMSO in the assay medium was less than 1%. Control experiments indicated that 1% DMSO had no appreciable effect on the ATPase activity. The ATPase activity in the presence of above substrates (final 50 \( \mu \text{M} \)) was also measured with orthovanadate added in the assay mixture at a final concentration of 300 \( \mu \text{M} \).

The ATPase activity in the presence of each substrate was plotted against the substrate concentration and the kinetic parameters (\( K_m \) and \( V_{\text{max}} \)) were obtained by a nonlinear least-squares regression program MULTI7 according to Eq. 1 (ketoconazole, verapamil and vinblastine) or 2 (cyclosporin A and digoxin):

\[
v = \frac{V_{\text{max}} \times [S]}{K_m + [S]} \quad (1)
\]

\[
v = \frac{V_{\text{max}} \times [S]}{K_m + [S] \times \left(1 + \frac{[S]}{K_s}\right)} \quad (2)
\]

where \( v \), \( [S] \), \( V_{\text{max}} \), \( K_m \) and \( K_s \) represents the ATPase activity, substrate concentration, maximum ATPase activity, Michaelis constant and substrate inhibition constant, respectively.

**Effects of Kampo Medicines on the ATPase Activity**
The ATPase activity was measured in the presence of Kampo medicines (final 0.5 mg/ml) and compared with the positive control (50 \( \mu \text{M} \) verapamil) and negative control (distilled water).

The inhibitory effect of Kampo medicines (final 0.5, 1.0 mg/ml) on the ATPase activity was also measured in the presence of 50 \( \mu \text{M} \) verapamil as a P-gp substrate.

**Statistical Analyses**
The results were shown as means±S.E. Student \( t \)-test was used to evaluate the statistical significance.

**RESULTS**

**Inhibitory Effects of Orthovanadate on the ATPase Activity**
When the effects of orthovanadate on ATPase activity in the presence of 50 \( \mu \text{M} \) verapamil were examined, orthovanadate inhibited ATPase activity in a concentration-dependent manner (Fig. 1).

From this result, orthovanadate-sensitive ATPase activity was calculated by subtracting the amount of phosphoric acid formed in the presence of 300 \( \mu \text{M} \) orthovanadate from each of the measured values in subsequent experiments.

**ATPase Activity in the Presence of P-gp Substrates**
As shown in Fig. 2, the ATPase activity of human P-gp membranes was elevated by all of the P-gp substrates studied (cyclosporin A, digoxin, ketoconazole, verapamil, and vinblastine) in a concentration-dependent manner. The ATPase stimulating effects of these substrates at the concentration of 50 \( \mu \text{M} \) were almost completely reversed by the addition of orthovanadate (300 \( \mu \text{M} \)) in the assay mixture (Fig. 2).
The ATPase activity of human P-gp membrane was measured as described in Materials and Methods. Data are expressed as means±S.E. (n=3).

Fig. 3. Effect of 50 Kampo Medicines on the ATPase Activity of Human P-gp Membrane
Fifty micromolars verapamil was used as a positive control. The ATPase activity was measured as described in Materials and Methods. 50 Kampo medicines were added in the assay mixture at the final concentration of 0.5 mg/ml. The ATPase activity was measured in the presence of 0.5 or 1.0 mg/ml of Kampo medicines. The ATPase activity stimulated by verapamil was significantly reduced by 43 of the 50 Kampo medicines at both concentrations (Figs. 4A, B).

Correlation between the ATPase Inhibitory Effect and Licorice Root Content of Kampo Medicines As one of the herbal ingredients, licorice root is contained in 33 of the 50 Kampo medicines studied. The contents of licorice root in the individual Kampo medicines, calculated from the weight ratio of each herbal ingredient, are listed in Table 1. The inhibitory effects of Kampo medicines on the ATPase activity both in the presence and absence of verapamil showed a positive correlation with their licorice root content (Fig. 5).

DISCUSSION
The effects of Kampo medicines on P-gp were investigated in this study by the ATPase assay using human P-gp membranes. The concentration-dependent increases in the ATPase activity were observed by all of the 5 drugs known as P-gp substrates (cyclosporin A, digoxin, ketoconazole, verapamil, and vinblastine) (Fig. 2), indicating that P-gp substrates stimulate the ATPase activity in this assay system. Furthermore, these activities were inhibited by orthovanadate, a non-competitive inhibitor of P-gp ATPase (Figs. 1, 2), which confirms that the ATPase activity detected in this assay derives from the P-gp.

In the assay mixture used in the present study, the Na⁺/K⁺ ATPase, Ca²⁺-dependent ATPase, and mitochondrial ATPase were inhibited by ouabain, EGTA, and sodium azide, respectively. Therefore, the ATPase activity observed in the absence of drugs (Fig. 3; control) might be derived from the endogenous P-gp substrates such as membrane lipids.

The difference in the stimulating effects of 5 drugs on the ATPase activity possibly reflects the different transport ve-

Table 2. Estimated Michaelis–Menten Parameters for the ATPase Activity Stimulated by P-gp Substrates

<table>
<thead>
<tr>
<th>Drug</th>
<th>( K_m ) (( \mu M ))</th>
<th>( V_{max} ) (nmol/mg protein/min)</th>
<th>( K_s ) (( \mu M ))</th>
<th>( V_{max}/K_m ) (mM/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclosporin A</td>
<td>0.319±0.198</td>
<td>15.9±1.2</td>
<td>76.6±23.2</td>
<td>49.7±31.3</td>
</tr>
<tr>
<td>Digoxin</td>
<td>8.79±3.63</td>
<td>31.8±9.4</td>
<td>9.54±3.96</td>
<td>3.62±1.84</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>30.1±4.5</td>
<td>69.4±5.3</td>
<td>—</td>
<td>2.31±0.39</td>
</tr>
<tr>
<td>Verapamil</td>
<td>14.2±2.5</td>
<td>59.8±3.7</td>
<td>—</td>
<td>4.21±0.79</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>3.41±0.97</td>
<td>33.2±2.5</td>
<td>—</td>
<td>9.74±2.86</td>
</tr>
</tbody>
</table>

Data shown in Fig. 2 were analyzed according to the Eqs. 1 or 2 to determine the apparent \( K_m \), \( V_{max} \), and \( K_s \) values.

The kinetic parameters for the ATPase activity stimulated by each substrate are listed in Table 2.

Effects of Kampo Medicines on the ATPase Activity
The 50 Kampo medicines (0.5 mg/ml) were added in the assay mixture to evaluate their effects on the ATPase activity of human P-gp membranes. As a result, the ATPase activity was significantly reduced by 24 of the 50 Kampo medicines while none of them significantly increased the ATPase activity compared with the water control (Fig. 3).

Inhibition of Verapamil-Stimulated ATPase Activity by Kampo Medicines To evaluate the possibility of Kampo medicines inhibiting verapamil transport by P-gp, verapamil concentration in the assay mixture was fixed at 50 \( \mu M \) and the ATPase activity was measured in the presence of 0.5 or 1.0 mg/ml of Kampo medicines. The ATPase activity stimulated by verapamil was significantly reduced by 43 of the 50 Kampo medicines at both concentrations (Figs. 4A, B).
The values of $V_{\text{max}}/K_m$ obtained in the present study (Table 2) showed 2- to 7-fold difference from the reported values $^{12}$ though the relative order was the same for 4 substrates. Verapamil was used as a P-gp substrate in the further experiments because this drug showed the largest increase in the ATPase activity at a concentration of 50 μM.

Many of the 50 Kampo medicines reduced the ATPase activity (Fig. 3), suggesting the possibility of their inhibiting P-gp. The degree of inhibition correlated with their licorice root content, suggesting the contribution of licorice root in the P-gp inhibition. On the other hand, licorice root is one of the most common ingredients in the Kampo medicines and is also often used in the food as a sweetener. Although the clinical significance of the present in vitro study is yet to be cleared by searching for the active ingredients and investigating their pharmacokinetics, it might be necessary to pay attention on the possible interaction between the licorice root-containing drug/food and the number of drugs transported by P-gp.

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REFERENCES