Cytochrome P450 2D6 (CYP2D6) Inhibitory Constituents of Catharanthus roseus

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The MeOH-soluble fraction of the water extract of Catharanthus roseus from Indonesia, having shown potent inhibitory activity on the metabolism mediated by CYP2D6, was subjected to activity-guided isolation to yield two triterpenes, ursolic acid (1) and oleanolic acid (2), and three alkaloids, vindoline (3), ajmalicine (4), and serpentine (5). The isolated compounds were tested for their inhibitory activity on the metabolism mediated by CYP3A4 or CYP2D6 using [N-methyl-14C]erythromycin or [O-methyl-14C]dextromethorphan as a substrate, respectively. Ajmalicine (4) and serpentine (5) showed very potent inhibitory activity against CYP2D6 with IC50 values of 0.0023 and 3.51 μM, respectively. All isolated compounds showed weak or no inhibition against CYP3A4. On time-, concentration-, and NADPH-dependent assay, serpentine (5) appear to be the mechanism-based inhibitor for CYP2D6 enzyme in which the inhibition was irreversible and driven by catalytic process. Kt and k inact values for serpentine (5) were 0.148 μM and 0.090 min⁻¹, respectively. On the other hand, ajmalicine (4) showed no time-dependent inhibition or reversible inhibition, and thus appear to be not mechanism-based inhibitor.

Key words Catharanthus roseus; mechanism-based inhibition; serpentine; ajmalicine; cytochrome P450 2D6 (CYP2D6); Indonesian medicinal plant

Herbal medicines are used concomitantly with conventional prescription drugs, which is a situation that carries the risk of unanticipated adverse drug–herbal pharmacokinetic interactions. It is recognized that cytochrome P450 (CYP) enzyme inactivation is one of the main reasons for pharmacokinetic interactions.1,2 The inactivation of CYP can lead to serious clinical drug interactions, especially when concomitant drugs are metabolized by the same enzyme. The drug interactions cause symptoms of drug overdose, including an exaggerated pharmacological response and/or drug toxicity.3 One of the mechanism of CYP inactivation involves covalent binding of a reactive intermediate to the enzyme proteins and/or heme, which leads to irreversible inhibition of catalytic function, or a quasi-irreversible coordination of a reactive intermediate to the CYP.4 In Indonesia, herbal medicines “Jamu” have been used from ancient times until the present, and are consumed by people of different levels. Most of these herbal medicines are unregulated, and many patients do not inform their physician of the herbal medicines they consume. Therefore, interactions between herbal medicines and drugs prescribed clinically are becoming a concern. Thus, we investigated in vitro the cytochrome P450 3A4 (CYP3A4) and 2D6 (CYP2D6) inhibitory activity of Indonesian medicinal plants. We found that MeOH extracts of Zingiber aromaticum (IC50 102 μg/ml) and Piper cubeba (IC50 53 μg/ml) showed potent inhibitory activity against CYP3A4, while the MeOH extract of Catharanthus roseus revealed very potent inhibition against CYP2D6 with an IC50 value of 11 μg/ml.5 In addition, we reported the CYP3A4 and CYP2D6 inhibitory constituents of Z. aromaticum6) and P. cubeba.7)

Catharanthus roseus, also known as Vinca rosea, is a semiwoody evergreen perennial tree that originated in Madagascar. It has been widely cultivated for hundreds of years and can now be found growing wild in most warm regions of the world. The plant has historically been used to treat a wide assortment of diseases. It was used as a folk remedy for diabetes in Europe for centuries, while in Hawaii, the plant was boiled to make a poultice to stop bleeding. In China, it was used as an astringent, diuretic, and cough remedy, and in Central and South America it was used as a homemade cold remedy to ease lung congestion, inflammation, and sore throat.8) In Indonesia, on the other hand, it was used for treatment of malaria, diabetes, constipation, cancer, and hypertension.9) More than 70 alkaloids were isolated from C. roseus, including vincristine and vinblastine which have anticancer properties.5) Because of continued interest in the CYP study, we carried out phytochemical investigation of C. roseus from Indonesia. In this paper, we report the constituents of this plant and their inhibitory activity on the metabolism mediated by CYP3A4 and CYP2D6. We also examined the possibility of mechanism-based inhibition by these constituents.

MATERIALS AND METHODS

Extraction and Isolation The aerial parts of C. roseus (4.0 kg, voucher no. TMPW 22268), obtained at the GORO traditional market in Jakarta, Indonesia in May 2002, were extracted with H2O (201×2) under reflux for 3 h, and the insoluble portion was separated by filtration. The filtrate was concentrated under reduced pressure to give an H2O extract (554 g). A part of the H2O extract (300 g) was further fractionated into MeOH-soluble (106 g) and MeOH-insoluble (180 g) fractions. Half of the MeOH-soluble fraction (50 g) was subjected to silica gel column chromatography with EtOAc/hexane (5—100%) to give seven fractions. The fractions were tested for their inhibitory activity on the metabolism mediated by CYP3A4 or CYP2D6. Purification of active fraction 5 by repeated silica gel column chromatography with EtOAc/hexane (5—100%), followed, by preparative TLC with a CHCl3–Et2O–MeOH solvent system, gave ursolic acid10,11) (1, 5.4 g) and oleanolic
acid\(^{11}\) (2, 150 mg), while column chromatography of active fraction 7 with MeOH/CHCl\(_3\) (5—100%), followed by preparative TLC with a CHCl\(_3\)-Et\(_2\)O-MeOH solvent system, gave vindoline\(^{12}\) (3, 2.4 mg), ajmalicine\(^{13}\) (4, 3.6 mg), and serpentine\(^{14}\) (5, 31.3 mg) (Chart 1).

**Chemicals** \([\text{N-methyl}-\text{H}]\text{Erythromycin}\ (55 \text{ mCi}/\text{mmol}, >99\% \text{ pure})\) and \([\text{O-methyl}-\text{H}]\text{dextromethorphan}\ (55 \text{ mCi}/\text{mmol}, >99\% \text{ pure})\) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, U.S.A.). Human liver microsomes (HLM) were obtained from Xenotech, LLC (Kansas, KS, U.S.A.) and stored at \(-80^\circ\text{C}\) prior to use. \(\beta\)-Nicotinamide adenine dinucleotide phosphate (NADP\(^+\), oxidized form), glucose-6-phosphate (G-6-P), and G-6-P dehydrogenase were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). All other chemicals and solvents were of the highest grade available.

**CYP Inhibitory Assay** Inhibitory activity on the metabolism mediated by CYP3A4 or CYP2D6 in vitro was determined using a radiometric measurement of \(^{14}\text{C}\)formaldehyde formed by the reaction with \([\text{N-methyl}-\text{H}]\text{Erythromycin}\ or \([\text{O-methyl}-\text{H}]\text{dextromethorphan}\ as a substrate, respectively.\(^{15,16}\) Briefly, in disposable culture tubes (13×100 mm; Iwaki, Tokyo) containing phosphate buffer (0.1 M, pH 7.4), \([\text{N-methyl}-\text{H}]\text{Erythromycin}\ (0.1 \mu\text{Ci}/\text{incubation}; 1000 \mu\text{Ci} \text{in 5\% of MeOH}) or \([\text{O-methyl}-\text{H}]\text{dextromethorphan\ (0.1 \mu\text{Ci}/\text{incubation}; 100 \mu\text{Ci} \text{in 5\% of MeOH})\), and 50 \mu\text{l of HLM (4 mg/ml)} were added to varying concentrations of samples in a total incubation volume of 500 \mu\text{l} (final concentration of sample was 1%). After a preincubation period of 5 min in a shaking water bath at 37 \degree\text{C}, the reaction was initiated by adding 50 \mu\text{l of NADPH-generating system (4.20 mg/ml of NADP\(^+\), oxidized form), glucose-6-phosphate (G-6-P), and G-6-P dehydrogenase were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). All other chemicals and solvents were of the highest grade available.

**RESULTS AND DISCUSSION**

**Isolation and Identification of Constituents** The MeOH-soluble fraction of \(C.\text{roseus}\) which showed very potent inhibition against CYP2D6 was applied to silica gel column chromatography to give seven fractions. All fractions were tested for their inhibitory activity against CYP3A4 and CYP2D6. From fraction 5 which showed strong inhibition against CYP3A4 (73% inhibition at 25 \mu\text{g/ml}), two triter-
penes, ursolic acid (1) and oleanolic acid (2), were isolated. On the other hand, from fraction 7 which revealed very potent inhibition against CYP2D6 (94% inhibition at 25 μg/ml), three alkaloids, vindoline (3), ajmalicine (4), and serpentine (5), were obtained. The structures of the isolated compounds were identified by comparing their [α]D, MS, and 1H- and 13C-NMR data with those in the literature.10—14)  

**CYP Inhibitory Activity of Isolated Compounds** To clarify the selectivity of inhibition, all isolated compounds were tested for their inhibitory activity on the metabolism mediated by CYP3A4 and CYP2D6 using [N-methyl-14C]erythromycin or [O-methyl-14C]dextromethorphan as a substrate, respectively. The alkaloids, ajmalicine (4) and serpentine (5), strongly inhibited metabolism mediated by CYP2D6 with IC50 values of 0.0023 and 3.51 μM, respectively (Table 1). Previously, Strobl et al. reported that ajmalicine (4) and serpentine (5) were competitive inhibitors against CYP2D6 with the Ki values of 0.0046 and 2.2 μM, respectively.17) The reported IC50 value of ajmalicine against CYP2D6 was 0.0036 μM,18) identical to our data. The other compounds showed weak or no inhibition against CYP2D6. Furthermore, on the metabolism mediated by CYP3A4, all isolated compounds possessed only weak or no inhibitory activity, indicating that the activities of ajmalicine (4) and serpentine (5) were selective against CYP2D6. Moreover, ursolic acid (1) and oleanolic acid (2) isolated from fraction 5 showed no inhibition against CYP3A4, indicating the presence of other potent CYP3A4 inhibitor(s) in this fraction.  

**Mechanism-Based Inhibition** To determine the possibility of mechanism-based inhibition on CYP2D6 by ajmalicine (4) and serpentine (5), time-, concentration-, and NADPH-dependent inhibition assay were performed. Serpentine (5) decreased the rate of metabolism mediated by CYP2D6 in both concentration- and time-dependent manners (Fig. 1), and also showed NADPH-dependent inhibition (Fig. 2). On the other hand, ajmalicine (4) showed concentration-dependent inhibition, but no significant preincubation time-dependent decrease in the CYP2D6 activity was observed by 4 (Fig. 1). Indeed, the level of inhibition of CYP2D6 decreased as the length of the preincubation period increased. This result suggests that ajmalicine (4) is metabolized by CYP2D6 during the preincubation period, thereby lowering the level of inhibitor prior to the assay.  

Pseudo-first-order kinetics was observed on the inactivation of the enzyme activity. The inactivation rate constant at an infinite concentration of inactivator (k_inact) and the concentration of inactivator required for a half-maximal rate of inactivation (K_i) were determined from double-reciprocal plots of k_app values and inactivator concentrations.19) Based on time-, concentration-, and NADPH-dependent inactivation kinetics, serpentine (5) appear to be a mechanism-based inhibitor for CYP2D6. The apparent K_i and k_inact values were calculated to be 0.148 μM and 0.090 min⁻¹, respectively, which should

<table>
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<th>Compound</th>
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<tr>
<td>1</td>
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</tr>
<tr>
<td>2</td>
<td>&gt;100</td>
<td>&gt;100</td>
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![Fig. 1. Time- and Concentration-Dependent Inhibition of CYP2D6 by Ajmalicine (4) (A) and Serpentine (5) (B) and Double-Reciprocal Plots between Inactivation Rate Constants (k_app) and Serpentine Concentrations (C)](image)

Each point represents the mean of duplicate determinations. Compound concentrations are shown on the right of the plots. Other experimental details are described under Materials and Methods.

![Fig. 2. Time- and NADPH-Dependent Inhibition of CYP2D6 by Serpentine (5)](image)

Preincubation with CYP2D6 was conducted at 37°C, +/- NADPH for 0, 5, 10, and 20 min. In order to determine the extent of inactivation, CYP2D6 activity was normalized by the activity observed at 8 min, which was arbitrarily set as 100%. Each point represents the mean of duplicate determinations.
indicate that serpentine (5) was a more potent CYP2D6 inactivator than paroxetine ($K_i$, 4.85 μM; $k_{inact}$ 0.17 min$^{-1}$) or SCH66712 ($K_i$, 4.8 μM; $k_{inact}$ 0.14 min$^{-1}$), other reported mechanism-based inhibitors of CYP2D6 activity. To the best of our knowledge, this is the first report of the mechanism-based inhibition of serpentine (5) against CYP2D6.

In conclusion, these results have demonstrated that serpentine isolated from C. roseus can cause mechanism-based inhibition of CYP2D6, and that ajmalicine, a potent CYP2D6 inhibitor, can reversibly inhibit CYP2D6.

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