Time-Dependent Induction of Midazolam-1-hydroxylation Enzymes in Rats Treated with St. John’s Wort

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Received January 20, 2005; accepted May 16, 2005; published online May 30, 2005

Time-dependent effects of St. John’s wort (SJW) on midazolam 1-hydroxylation were investigated in Wistar rats. Wistar rats treated with SJW (1000 mg/kg/d) for 1, 3, and 7 d were administered midazolam orally at a dose of 10 mg/kg. Oral clearance of midazolam in the SJW treated rats increased time dependently, and was significant after 7 d of treatment with SJW. The midazolam-1-hydroxylation activity in liver microsomes obtained from the SJW treated rats was significantly higher than in the control group. Linear correlation was observed between oral clearance and midazolam-1-hydroxylation activity in the liver microsomes, suggesting that CYP3A induction in liver mainly decreased the midazolam concentration in plasma. Immunoblotting revealed that the protein amount of CYP3A was induced within 3 d of SJW treatment. Since the midazolam-1-hydroxylation activity continuously increased for at least 7 d, the induction of CYP3A by SJW continued to cause interactions with drugs metabolized by CYP3A. It is important for persons receiving SJW for an extended time to consider its interactions with prescription drugs.

Key words drug interaction; induction; St. John’s wort; midazolam; pharmacokinetics

St. John’s wort (SJW) (Hypericum perforatum) is an herbal remedy which has become increasingly popular as a remedy for mild depression. Traditionally, it has also been used in treatment of wounds. In recent years, there have been numerous case reports of interactions between SJW and prescription drugs which are metabolized by cytochrome P450 (CYP), such as indinavir, cyclosporin A, warfarin, digoxin, and theophylline.1 Several in vitro and in vivo studies have evaluated the effects of SJW extract on the expression and activity of hepatic CYP3A4 and MDR1, the drug efflux protein.2–6 These data suggest that SJW induces CYP3A4 activity, which could explain most of these drug interactions. Additionally, SJW may also induce intestinal MDR1 expression and activity, and influence pharmacokinetics of drugs such as indinavir and digoxin.2,4,5 The studies performed in vitro have demonstrated that SJW extract is a potent inducer of CYP2B6 and CYP3A4, and the responsible constituent of SJW is hyperforin.5,7,8 CYP2B6, CYP3A4 and MDR1 genes have transcriptional binding sites for the pregnane X receptor, an orphan receptor which regulates expression of CYP3A4.9–11 Binding of a specific ligand to this receptor increases transcription of these genes with the cognate recognition sites in their 5′-regulatory region.

Concerning other CYPs, the effects of SJW are controversial. The induction of CYP2D6 seems to be minimal.6 Decreased plasma concentrations of theophylline following SJW therapy, has been reported.12 Since theophylline is metabolized mainly by CYP1A2, it is plausible that induction of CYP1A2 by SJW may explain the reduced theophylline concentrations and loss of therapeutic efficacy. SJW increased the expression of CYP1A2 in a concentration- and time-dependent manner in LS180 cells.13 The pharmacokinetics of theophylline however did not change following SJW administration in another study.14 The increase of S-warfarin clearance by SJW has been also reported, suggesting that the induction of CYP2C9.15 Hydroxylation of omeprazole by CYP2C19 is also increased by SJW administration for 14 d.15

The time dependent induction16 and the direct inhibitive activity17 of SJW to CYPs make the issue more complicated.

In this study, we investigated the effect of SJW in rats on the pharmacokinetics of midazolam, a benzodiazepine adaptable for preoperative sedation, anxiolysis and anterograde amnesia. The time-dependent change of hydroxylation activities of midazolam, a typical substrate of CYP3A, in liver microsomes was investigated. We also examined the effect of SJW on the amount of CYP3A2, and that of CYP2C11, another major CYP in rats.

MATERIALS AND METHODS

Chemicals and Materials Dried extract of St. John’s Wort was generously supplied from Fancel Co. (Yokohama, Japan). Midazolam injection (Dormicum Injections) was purchased from Yamanouchi Pharmaceuticals (Tokyo, Japan). Clonazepam, an internal standard for HPLC analysis, was generously supplied from Sumitomo Pharmaceuticals (Osaka, Japan). Rabbit anti-rat CYP3A2 and Goat anti-rat CYP2C11 antibodies were purchased from Daichi Pure Chemical (Tokyo, Japan). All other chemicals and reagents of analytical grade were obtained from commercial sources and used without further purification.

Animal Experiments All animal experimental procedures were performed in accordance with the ethic guidelines for the Institutional Committee on Care and Use of Laboratory Animals. Male Wistar rats (7 weeks of age, weighing 300—350 g) were purchased from Charles River (Tokyo, Japan). The rats were kept under a 24 h cycle of 12 h light and dark at 24 °C, and allowed free access to the standard laboratory rodent chow and water.

Dried SJW extract was suspended in saline and was orally administered to the rats in SJW group at the dose of 1000 mg/kg every 24 h for 1, 3 or 7 d. The same volume of saline was administered to the rats in the control group. On the final day of SJW treatment, the rats were fasted overnight. After

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24 h of the final administration of SJW, 10 mg/kg of midazolam in saline was orally administered, and 300 μl of blood sample was collected from the femoral artery at 2, 5, 10, 15, 20, 30, 45, 60, 90, 120, 150 and 180 min after the administration of midazolam. The blood samples were immediately centrifuged at 1000 g for 10 min and the plasma samples were stored at −20 °C until analyzed by HPLC. At 180 min after administration of midazolam, the rats were decapitated and the whole liver was immediately removed, weighed and kept at −80 °C until further analysis.

**Determination of Midazolam and Its Metabolites by HPLC** The plasma concentrations of midazolam and its metabolites, 1-hydroxy-midazolam and 4-hydroxy-midazolam were measured by HPLC. Briefly, 100 μl of the plasma was mixed with 0.1 ml of internal standard solution (clonazepam 1 μg/ml) and 1.0 ml of 0.1 M borate buffer (pH 10.0). The mixture was extracted with 2.5 ml of n-hexane : dichloromethane (1 : 1, v/v). The mixture was centrifuged at 1600 g for 5 min after shaking for 10 min. The upper organic phase (2.0 ml) was transferred to a glass tube and evaporated to dryness at 40 °C. The dried residue was dissolved with 100 μl of 65% methanol, and 20 μl of the sample was applied to HPLC (Waters 2960, U.S.A.) detecting absorbance at 245 nm by Waters 996 photodiode-array detector. The column was a YMC-Pack Pro C18 ODS column with 250 mm length and 4.6 mm diameter (YMC, Japan) kept at 40 °C. Mobile phase was 65% methanol at the flow rate of 1 ml/min. The detectable concentrations of midazolam by HPLC are 50 ng/ml.

In order to determine the concentrations of 1-hydroxy-midazolam and 4-hydroxy-midazolam in liver microsomal suspension, diazepam as an internal standard was added after the addition of acetonitrile to stop the reaction. The mixture was centrifuged at 2000 g for 20 min, and the supernatant was applied to HPLC. The mobile phase for 1-hydroxy-midazolam and 4-hydroxy-midazolam were 20 mM acetate buffer (pH 4.5) : acetonitrile = 60 : 40 v/v and 50 : 50 v/v, respectively. Other HPLC conditions are the same as for the midazolam analysis. We used two different conditions for the separation of parent drug and each metabolite. The detectable concentration of 1-hydroxy-midazolam and 4-hydroxy-midazolam are 0.5 μM and 0.2 μM, respectively.

**Preparation of Microsomes** Liver microsomal fraction was prepared as described by Kamataki and Kitagawa. Briefly, the rat livers were homogenized in a 1.15% (w/v) KCl solution using a Teflon homogenizer and centrifuged at 9000 g for 20 min. The supernatant fraction was centrifuged at 105000 g for 1 h to yield a microsomal pellet and cytosol. The microsomal pellet was washed once and resuspended in a cold 0.1 M Tris–HCl buffer (pH 7.4). After determining the protein content by the method of Lowry et al., the microsomal fractions were frozen, and kept at −80 °C until use.

**Assay of Midazolam 1- or 4-Hydroxylase Activity** The midazolam 1- or 4-hydroxylase activity was determined as described by Yamaoi et al. 20 The reaction mixture (500 μl) for the assay using rat liver microsomes consisted of 0.1 M Na+, K+-phosphate buffer (pH 7.4), 0.1 mM EDTA, midazolam (1—100 μM) and microsomes (0.5 mg protein/ml). After preincubating at 37 °C for 5 min, the reaction was initiated by addition of 500 μl of NADPH-generating system (5.0 mM glucose-6-phosphate, 0.5 mM Na2HPO4, 1 unit/ml glucose-6-phosphate dehydrogenase and 5 mM MgCl2) as described in the previous study, and the reaction mixture was incubated at 37 °C for 7 min. Ice cold acetonitrile (0.5 ml) was added to terminate the reaction, and 1.0 μg diazepam was added as an internal standard. The concentration of 1-hydroxy-midazolam and 4-hydroxy-midazolam was determined by HPLC.

The formation of 1-hydroxy-midazolam by the rat liver microsomes was linear at 37 °C for 20 min for the substrate concentrations between 0 and 100 μM. The product formation by rat liver microsomes was also linear for 60 min at 50 μM substrate. Another product, 4-hydroxy-midazolam, was not detected in our experimental format.

**Pharmacokinetic Analysis** Pharmacokinetics of midazolam after oral administration was analyzed by the model-independent moment analysis method. Area under the plasma concentration curve (AUC) and area under the moment curve (AUMC) was calculated by the trapezoidal method between 0 and 180 min. Oral clearance (CLoral) and mean residence time (MRT) of midazolam were calculated by the following equations;

\[
\text{CLoral} = \frac{\text{Dose}}{\text{AUC}}
\]

\[
MRT = \frac{AUMC}{\text{AUC}}
\]

Apparent volume of distribution \((V_d)\) was calculated by following equation.

\[
V_d/F = MRT - \text{CLoral}
\]

Since hepatic clearance of midazolam is substantially blood-flow limited, induction of metabolic enzyme(s) would not increase total body clearance of midazolam when administered intravenously. On the other hand, CLoral is proportional to intrinsic clearance, which is considered to reflect the metabolic activity in liver.

**Analysis of Kinetics** Enzyme kinetic parameters \((K_m, V_{max}, V_{max}/K_m)\) were estimated by the nonlinear regression analysis using simple Michaelis–Menten equation.

**Immunoblotting Analysis** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blotting were performed according to the methods of Guengerich et al. 

12.5% polyacrylamide gel. Immunodetection was performed using anti-Rat CYP2C11 and CYP3A2 antibodies according to the manufacturer’s instructions using 4-chloro-1-naphthol as the substrate upon the peroxidase. The contents of each subtype of CYPs were determined by scanning the immunostained bands using the public domain NIH image program on a Macintosh computer.

**RESULTS**

The plasma concentration profiles and pharmacokinetic parameters of midazolam in the rats treated with SJW for 1, 3 and 7 d are shown in Fig. 1 and Table 1, respectively. Midazolam concentrations in the SJW treated rats were lower at day 3 and at day 7 than in the control rats. The oral clearance of midazolam increased time-dependently to 265% of the control group after 7 d treatment of SJW, while MRT did not change during 7 d treatment. CLoral and \(V_d/F\) of the SJW group were significantly lower than in the control group on day 1, while they were higher on day 3 and day 7.

The effects of SJW on the metabolic enzymes of midazolam were examined. The first step of metabolism of midazo-
In human liver is the hydroxylation to 1-hydroxy-midazolam or 4-hydroxy-midazolam. The 1-hydroxylase activity in the liver microsomes and the enzyme kinetic parameters were compared in Table 2. Maximum production velocity ($V_{\text{max}}$) of 1-hydroxy-midazolam was 1.4 times higher than in the control group after 7 d of treatment. In our experimental format, we could hardly detect 4-hydroxy-midazolam as a product of midazolam metabolism.

The profiles of enzyme induction in vivo and in vitro experiments are shown in Fig. 2. Both CLoral and $V_{\text{max}}/K_m$ linearly increased in a time-dependent manner for 7 d. CLoral and $V_{\text{max}}/K_m$ showed significant correlation as shown in Fig. 3.

To confirm the effects of SJW on the induction of CYP3A and CYP2C, the amounts of these proteins were compared with western blotting followed by detection using antibodies to CYP3A2 and CYP2C11. Figure 4 shows the amount of CYP3A induced by SJW treatment after 3 d. By contrast, the amount of CYP2C11 did not seem to be induced by SJW treatment.

**DISCUSSION**

Interactions between SJW and medicines have recently been identified by many pharmacokinetic studies. These studies have shown that SJW is capable of inducing drug metabolizing enzymes, CYP3A4, 1A2 and 2C9, as well as a transporter, MDR1, in humans. Most of the reported interactions are the decrease of drug concentrations in blood and/or therapeutic efficacy.

It has been thought that midazolam is metabolized extensively in the liver and intestine by CYP3A4 in human. Midazolam rapidly undergoes hydroxylation via hepatic microsomal enzymes to form 1-hydroxy-midazolam. It was reported that CYP2B6 and CYP2C9 do not metabolize midazolam in humans. The present study, we demonstrated that $AUC$ of
midazolam in Wistar rats was significantly decreased after 7 d treatment with SJW (Fig. 1). Since the hepatic clearance of midazolam is substantially blood-flow limited, increase of intrinsic clearance may not appear as the increase of total body clearance after intravenous administration. Therefore, midazolam was orally administered in this study since oral clearance, or total clearance divided by bioavailability, is proportional to intrinsic clearance. Induction of hepatic enzyme activity leads to a decrease of bioavailability, but does not decrease the elimination half life. Pharmacokinetic results showing no change in MRT and an increase in $V_d/F$ are consistent with the increase of hepatic first pass metabolism.

Oral clearance of midazolam increased time dependently and the induction of the activities of the drug metabolizing enzymes by SJW could generally explain this phenomenon. The increase of CYP3A protein by immunoblotting analysis (Fig. 4) supported the induction of CYP3A by SJW within 3 d in Wistar rats. Since the CYP3A2 antibody used in our study was polyclonal one, and might have cross-reactivity to CYP3A1, we were unable to identify the molecular species of CYP3A. Midazolam-1-hydroxylation activity after SJW treatment for 7 d was 1.4 times larger than in the control group. Durr et al. reported a 2.5 fold increase of hepatic expression of CYP3A2 in Wistar rats after the administration of the same amount of SJW for 14 d, suggesting that induction of CYP3A activity was initiated early after SJW treatment and should go on increasing for more than 2 weeks.

Because it was reported that CYP2C11 is one of the major enzymes in Wistar rats, we investigated CYP2C11 induction after the treatment of SJW by immunoblotting, and could find no clear induction of the enzyme protein within 7 d. Induction of CYP families other than CYP3A by SJW is controversial. Decrease of S-warfarin concentration and the therapeutic effects suggested the induction of CYP3A activity in Wistar rats after the administration of the same amount of SJW for 14 d, suggesting that induction of CYP3A activity was initiated early after SJW treatment and should go on increasing for more than 2 weeks.

Therefore, midazolam was orally administered in this study since oral clearance, or total clearance divided by bioavailability, is proportional to intrinsic clearance. Induction of hepatic enzyme activity leads to a decrease of bioavailability, but does not decrease the elimination half life. Pharmacokinetic results showing no change in MRT and an increase in $V_d/F$ are consistent with the increase of hepatic first pass metabolism.
exclude the possibility of false-negative error in detection of possible drug interactions with SJW by short-term experiments.

The phenomenon showing little change of CYPs activity in the early period of SJW treatment might be explained by the inhibition of CYPs activity by SJW. SJW extracts and its major constituents have been reported to inhibit the activities of CYP1A2, 2C9, 2C19, 2D6 and 3A4.26) If the experimental period is short, the effect of SJW in inhibiting the enzyme activity may cancel the slow induction of the CYPs. In our study, results showing significantly lower CLoral in the SJW group after 1 d of treatment (Table 1) might be due to the inhibition of CYP enzymes by SJW. The difference between day 1 and other days may be a chance observation and the deviation may be within the experimental error margins.

When comparing the in vivo oral clearance and in vitro enzyme activity, it is necessary to consider the first-pass metabolism at the intestine. The difference of increasing ratio of oral clearance (2.7 times) and that in microsomal enzyme activity (1.4 times) may be due to the induction of intestinal metabolic activity could be insignificant.

In conclusion, our data showed that SJW induced CYP3A activity in the early period of SJW treatment might be explained by the inhibition of CYPs activity by SJW. The difference between day 1 and other days may be a chance observation and the deviation may be within the experimental error margins.

Acknowledgements We greatly appreciate the contribution of Ms. T. Senbongi for her technical assistance in western blotting, and also thank to Dr. T. Yano and the Rotary Yoneyama Memorial Foundation, Inc. for their financial support to complete our study.

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