

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.jfda-online.com

Original Article

The time-dependent effects of St John's wort on cytochrome P450, uridine diphosphate-glucuronosyltransferase, glutathione S-transferase, and NAD(P)H-quinone oxidoreductase in mice

Jin-Fu Yang^{a,b}, Yue-Rong Liu^a, Chiung-Chiao Huang^a,
Yune-Fang Ueng^{a,b,c,d,*}

^a National Research Institute of Chinese Medicine, Taipei, Taiwan, ROC

^b Department and Institute of Pharmacology, School of Medicine, National Yang-Ming University, Taipei, Taiwan, ROC

^c Institute of Biological Pharmacy, School of Pharmacy, National Yang-Ming University, Taipei, Taiwan, ROC

^d Institute of Medical Sciences, School of Medicine, Taipei Medical University, Taipei, Taiwan, ROC

ARTICLE INFO

Article history:

Received 12 September 2016

Received in revised form

21 December 2016

Accepted 18 January 2017

Available online 14 February 2017

Keywords:

cytochrome P450 2C

cytochrome P450 3A

Hypericum perforatum

liver

kidney

ABSTRACT

Hypericum perforatum [St. John's wort (SJW)] is known to cause a drug interaction with the substrates of cytochrome P450 (P450, CYP) isoforms, mainly CYP3A. This study aims to determine the dose response and time course of the effects of SJW extract on P450s, UDP-glucuronosyltransferase (UGT), glutathione S-transferase (GST), and NAD(P)H-quinone oxidoreductase (NQO) in mice. The oral administration of SJW extract to male mice at 0.6 g/kg/d for 21 days increased hepatic oxidation activity toward a Cyp3a substrate nifedipine. By extending the SJW treatment to 28 days, hepatic nifedipine oxidation (NFO) and warfarin 7-hydroxylation (WOH) (Cyp2c) activities were increased by 95% and 34%, respectively. Immunoblot analysis of liver microsomal proteins revealed that the Cyp2c protein level was elevated by the 28-day treatment. However, the liver microsomal activities of the oxidation of the respective substrates of Cyp1a, Cyp2a, Cyp2b, Cyp2d, and Cyp2e1 remained unchanged. In the kidney, SJW increased the NFO, but not the WOH activity. The extended 28-day treatment did not alter mouse hepatic and renal UGT, GST, and NQO activities. These findings demonstrate that SJW stimulates hepatic and renal Cyp3a activity and hepatic Cyp2c activity and expression. The induction of hepatic Cyp2c requires repeated treatment for a period longer than the initial induction of Cyp3a.

Copyright © 2017, Food and Drug Administration, Taiwan. Published by Elsevier Taiwan LLC. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

* Corresponding author. National Research Institute of Chinese Medicine, 155-1 Li-Nong Street, Section 2, Taipei 112, Taiwan, ROC.

E-mail address: ueng@nricm.edu.tw (Y.-F. Ueng).

<http://dx.doi.org/10.1016/j.jfda.2017.01.004>

1021-9498/Copyright © 2017, Food and Drug Administration, Taiwan. Published by Elsevier Taiwan LLC. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

The drug-metabolizing cytochrome P450 (P450, CYP)-dependent monooxygenase system primarily localizes at the membranes of endoplasmic reticulum and participates in the metabolism of about 96% of drugs under development or on the market [1]. Microsomal P450 isoforms catalyze the oxidation of structurally diverse drugs with broad substrate specificity. The oxidations catalyzed by P450s require NADPH-P450 reductase (CPR) to carry out the two-electron transfer to the P450 substrate and the oxygen-inserted P450-substrate complexes [2]. A hemoprotein cytochrome b_5 can initiate a second electron transfer. Cytochrome b_5 stimulates the activities of certain P450 isoforms, including CYP3A4; the stimulation of CYP3A4 activity is possibly through a protein–protein interaction without the direct influence of the electron transfer [3]. Alteration of the electron transfer partners may be involved in the changes of P450 activity. In human livers, CYP1, CYP2, and CYP3 are the main P450 families responsible for drug oxidations [1]. CYP3A4 is the most abundant human hepatic P450 and oxidizes about 27% of the drugs under development and used in patients. The CYP2C subfamily occupies about 20% of the total P450 content [4] and oxidizes about 24% of the drugs [1]. The functional changes of P450s, especially the CYP3A and CYP2C subfamilies, can be crucial for herb–drug interaction.

The extract of St. John's wort (SJW; *Hypericum perforatum*) is an over-the-counter botanical supplement widely used for the treatment of mild to moderate depression and sleep disorders in Europe and the United States [5]. The use of SJW in patients has been reported to decrease the therapeutic efficacy of a variety of drugs, including tolbutamide, warfarin, and indinavir, which are mainly metabolized by some P450 isoforms, including CYP2C9 and CYP3A4 [5]. In the *in vitro* system of the primary culture of human hepatocytes, it has been demonstrated that SJW extract induced CYP3A4 through the activation of the human pregnane X receptor (PXR), and the SJW ingredient hyperforin is identified as the primary PXR activator and induced CYP3A4 [6]. However, inconsistency exists in reports regarding the changes of the other P450 isoforms, such as CYP2C9. In a study involving 12 participants, treatment with SJW extract (25 mg hyperforin/g extract) at a daily dose of 0.9 g for 14 days caused a 52% decrease in the mean AUC (area under the curve of a plot of plasma drug concentration vs. time after administration) value of the CYP3A4 substrate midazolam [7]. This SJW treatment did not change the pharmacokinetic parameters of caffeine and tolbutamide, which are primarily metabolized by CYP1A2 and CYP2C9, respectively. However, in another pharmacokinetic study on 21 participants taking 0.9 g/d SJW extract (hyperforin content not shown) for 15 days prior to a CYP2C9/19 substrate gliclazide, the intrinsic clearance of gliclazide was elevated [8], suggesting that the SJW extract stimulated the CYP2C-mediated drug metabolism in humans. In the primary culture of human hepatocytes, a study of the effect of 48-hour exposure showed that hyperforin at 0.2 μ M and 1 μ M concurrently elevated the activities and/or expression of CYP3A4 and CYP2C9, whereas hyperforin had no influence on the mRNA and protein levels of CYP1A2 or CYP2D6 [9]. Reports regarding human samples suggested that CYP2C9, but not CYP1A2 and

CYP2D6, might be induced by SJW. Although the polymorphic expression of CYP2C9 can be associated with different activities [8], the inconsistency in the metabolic changes of CYP2C9 substrates revealed the importance of the information regarding the dose- and time-dependent functional changes of P450 isoforms by SJW extract *in vivo* or *ex vivo*.

Experimental animals provide an alternative biological system to assess the changes of drug-metabolizing enzymes *in vivo* and *ex vivo*, and a significant variation caused by genetic polymorphism can be excluded. In a study of drug-perfused livers in Wistar rats, unlike the potential induction of CYP2C by SJW in humans, the AUC value of a CYP2C substrate tolbutamide in perfusate was significantly increased by 80% in SJW (unknown hyperforin content; 100 mg/kg/d intraperitoneally for 10 days)-treated rats, suggesting a decrease in rat CYP2C activity [10]. In contrast, this treatment decreased the AUC values of dextromethorphan and midazolam, which are the substrates of CYP2D and CYP3A, respectively. In a study of 7-day, 14-day, and 21-day treatments, only the 21-day oral treatment of Swiss Webster mice with 0.14 g/kg and 0.28 g/kg SJW (2.3% hyperforin) induced the expression and activities of CYP3A and CYP2E1, whereas CYP1A activity remained unchanged [11]. However, although the oral treatment of Wistar rats with 0.4 g/kg SJW extract (unknown hyperforin content) for 10 consecutive days transiently increased the expression level of hepatic CYP1A2, the increase was eliminated after a 30-day consecutive treatment [12]. The time-dependent functional change of CYP1A2 remains unclear. In a study on rat livers perfused with saline containing a CYP1A2 substrate, phenacetin, the concentration of phenacetin in the perfusate was significantly higher after SJW treatment (100 mg/kg/d, 10 days), suggesting that CYP1A2 activity was decreased by SJW [13]. The changes in CYP1A2 and CYP2C activities/expression by SJW extract show inconsistent results in different treatment regimens and biological systems. The information on hyperforin content in the SJW extracts has not been fully provided, which reveals the difficulty of making a conclusion regarding the subchronic/chronic effects of SJW on P450s from the present reports of animal studies.

The oxidation metabolites, as generated from the oxidation by a P450, can be further subjected to conjugative metabolism. Among the varied conjugative metabolisms, glucuronidation catalyzed by the microsomal UDP-glucuronosyltransferase (UGT) family is quantitatively the most important metabolic process [14]. UGT participates in the glucuronidation of drugs or the metabolites of drugs, such as acetaminophen and warfarin. SJW extract stimulated the pGL₃-UGT1A1 reporter activity in CV-1 cells expressing human or mouse PXR [15]. However, hepatic UGT activity was not affected by 21-day treatment with SJW extract in Swiss Webster mice, whereas CYP3A was induced [10]. The importance of the cytosolic glutathione S-transferase (GST) family has been recognized in the detoxification of environmental toxins, such as benzo(a)pyrene and aflatoxin B₁ through the glutathione conjugation of their active metabolites [16]. The hepatic protein level of one type of GST, GST-P, can be increased in rats treated with 0.4 g/kg SJW extract daily for 10 consecutive days, and the induced level was decreased to a basal level after a 30-day treatment [12]. The functional change of GST remains unclear. The oxidative stress inducible

enzyme NAD(P)H-quinone oxidoreductase (NQO) catalyzes the two-electron reduction of a variety of therapeutic quinones, such as the chemotherapeutic agent mitomycin C [17]. Information regarding the functional alterations of GST and NQO activities after prolonged SJW treatment is sparse.

In addition to hepatic enzymes, extrahepatic enzymes can be important in the tissue-specific biological activity of physiologically occurring compounds and toxins [18,19]. The induction of drug-metabolizing enzymes can show tissue specificity. For example, the expression levels of hepatic and renal CYP3A were induced by 1,25-dihydroxyvitamin D₃ in sheep [20]. However, the hepatic level, but not the renal CYP3A mRNA level, was elevated after treatment of mice with 0.5 g/kg black cohosh for 28 days [21]. Thus, this SJW study aims to (1) examine the changes of the electron-transfer partners of P450, while CYP3A activity is stimulated; (2) reveal the dose- and time-dependent changes of P450 activities; and (3) elucidate the changes in hepatic and renal CYP2C/3A, UGT, GST, and NQO in mice.

2. Materials and methods

2.1. Chemicals and solvents

The capsules of SJW extract (Johanniskraut, Dr. Böhm) were purchased from APOMEDICA Pharmaceutical Product GmbH (Graz, Austria). Chlorzoxazone, coumarin, dextromethorphan, dextrophan, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, 6-hydroxychlorzoxazone, hyperforin, nicotinamide adenine dinucleotide phosphate (NADP⁺), 7-ethoxyresorufin, 7-hydroxycoumarin, 7-methoxyresorufin, 7-pentoxiresorufin, nifedipine, and warfarin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). 7-Hydroxywarfarin was purchased from Ultrafine Chemicals Ltd. (Manchester, UK). The oxidation product (2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester) of nifedipine was generously provided by Dr F. Peter Guengerich (Nashville, TN, USA) [22]. Acetonitrile and methanol were purchased from Merck KGaA (Darmstadt, Germany).

2.2. Determination of hyperforin content in SJW extract

SJW extract (100 mg) in a capsule was extracted three times with 1 mL methanol in the dark. After centrifugation at 14,100g for 15 minutes at room temperature, the supernatants were pooled and dried under nitrogen gas. The methanolic extract was dissolved in methanol and subjected to high performance liquid chromatography (HPLC) analysis following a method described by Bauer et al [23]. Quantitative analysis was performed using an HPLC system (Agilent 1100; Agilent Technology, Santa Clara, CA, USA) equipped with a photodiode array detector (G1315B) and a C18 column (4.6 mm × 250 mm, 5 μm; Nacalai Tesque, Kyoto, Japan). Hyperforin was separated and eluted using a mobile phase containing 80% acetonitrile in 0.01M monosodium phosphate buffer (pH 2.4) at a flow rate of 1 mL/min. The appearance of hyperforin was detected by measuring the absorbance at 273 nm.

2.3. Animal treatments

Male C57BL/6JNarl (B6) mice (5 weeks old, weighing 17–20 g) were purchased from the National Laboratory Animal Center (Taipei, Taiwan). All experimental protocols involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the National Research Institute of Chinese Medicine (IACUC#103-635-1; approved on December 26, 2013). Prior to experimentation, the mice were allowed a 1-week acclimation period at the animal quarters (25 ± 2°C, 12-hour day light cycle) with free access to laboratory rodent chow (no. 5P14; PMI Feeds Inc., Richmond, IN, USA) and water *ad libitum*. The human daily dose of SJW extract is 0.4–0.9 g. In humans, CYP3A4 activity was reported to have been increased after treatment with a daily dose of 0.9 g (0.3 g, t.i.d.) of SJW extract for 14 consecutive days [7]. A human equivalent dose of SJW extract in mice was estimated to be 0.18 g/kg/d based on a 60-kg person with a daily recommended dose of 0.9 g and a body surface area ratio of 12.3 for mouse dose to human equivalent dose (Table 3 in the Guidance for Industry, Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers, US Department of Health and Human Services, FDA, Center for Drug Evaluation and Research, 2005; <http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm078932.pdf>). Thus, mice were treated with SJW extract (extract in the capsule was suspended in water and mixed with vortex prior to each administration) at a minimal dose of 0.1 g/kg/d through gastrogavage. The control group received the same amount of water. Mice were euthanized by CO₂ asphyxiation 22 hours after the last treatment. Prior to asphyxiation, mice were anesthetized using ether (in a chemical safety hood); blood was collected by heart puncture, and the sera were separated by centrifugation at 9600g for 10 minutes at 4°C. The formalin-fixed mouse liver and kidney specimens were sent to the National Laboratory Animal Center (Taipei) or National Chung Hsing University (Taichung City, Taiwan) for paraffin embedding (3–5 μm sections) and were stained using hematoxylin and eosin for pathological examination. According to the lesion area, the degrees of the lesions were scored from 1 to 5. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities and blood urea nitrogen (BUN) and creatinine levels were determined using analysis kits designed for the Fujifilm Dri-Chem 3000 colorimetric analyzer (Fujifilm, Saitama, Japan).

2.4. Microsomal and cytosolic preparations, and enzyme assays

Microsomal and cytosolic fractions of liver and kidney tissues were prepared by differential centrifugation at 4°C [24]. To prevent the interference of hemoglobin, liver microsomal pellets were further resuspended using 1.15% potassium chloride and centrifuged at 100,000g for 1 hour at 4°C to prepare washed microsomes. The washed liver microsomes, kidney microsomes, and cytosols of liver and kidney tissues were used in enzyme assays. Microsomal contents of P450 and cytochrome b₅ were determined using spectrophotometric methods [25]. The reduction activity of CPR was determined by using cytochrome c as a substrate

following a method described by Phillips and Langdon [26]. 7-Ethoxyresorufin O-deethylation (EROD), 7-methoxyresorufin O-demethylation (MROD), and 7-pentoxyresorufin O-dealkylation (PROD) activities were determined by measuring the fluorescence of resorufin [27,28]. The 7-hydroxylation activities of microsomes on coumarin and warfarin 7-hydroxylation (WOH) were determined following the methods described by Souček [29] and Yamazaki and Shimada [30], respectively. Dextromethorphan O-demethylation (DMOD) activity was determined by HPLC with a fluorescence detector [31]. Nifedipine oxidation (NFO) and chlorzoxazone 6-hydroxylation activities were determined by HPLC with a UV detector [22,32]. Microsomal UGT activity was determined using *p*-nitrophenol as a substrate [33]. Cytosolic GST and NQO activities were determined using 1-chloro-2,4-dinitrobenzene and menadione as substrates, respectively [34,35]. Microsomal and cytosolic protein concentrations were determined using bovine serum albumin as a standard for establishing the linear relationship between protein concentration and absorbance, as described by Lowry et al [36].

2.5. Immunoblot analyses

Microsomal proteins (30 µg) were resolved by electrophoresis on a 10% or 7.5% (w/v) polyacrylamide gel (sodium dodecyl sulfate-polyacrylamide gel electrophoresis; 14 × 20 × 0.15 cm) and electrotransferred from the slab gel to a nitrocellulose membrane, as previously described [37]. Immunoblot analyses of mouse liver microsomal Cyp2c and CPR were performed using rabbit polyclonal antibodies against rat CYP2C11 (1:1000), CYP2E1 (1:1000), and CPR (1:2000) [diluted in phosphate-buffered saline (PBS) containing 1% nonfat milk], respectively. Rabbit polyclonal antibodies against rat CYP2C11 and CYP2E1 were purchased from NOSAN Co. (Yokohama, Japan). The antibody against CPR was kindly provided by Chul-Ho Yun (Korea) [38]. The prestained protein marker (9–245 kDa, PM2600) was purchased from SMOBiO (Hsinchu, Taiwan). After four washes (each for 15 minutes) of PBS containing 0.5% Tween 20, the immunoreactive proteins were detected using a goat antirabbit IgG conjugated with horseradish peroxidase and visualized using an enhanced chemiluminescence detection kit (PerkinElmer Life and Analytical Sciences, Inc., Shelton, CA, USA). Relative band intensity was analyzed with Multi Gauge software (Ver. 2.2; Fujifilm Co., Tokyo, Japan).

2.6. Data analyses

The differences in the experiments with more than two sets of data from mice treated with different doses of SJW extract were examined by one-way analysis of variance (ANOVA), followed by Dunnett's test for multiple comparisons (Prism 3.0, GraphPad Software, Inc., La Jolla, CA, USA). The statistical significance of the difference between the control and individual treatment group was evaluated with Student *t* test, where a *p* value of <0.05 was considered statistically significant.

3. Results

3.1. Dose response of Cyp3a induction by SJW extract in mice

The content of hyperforin in commercially available SJW extract varies from 0% to 6% [39]. Determination of the content of hyperforin in the SJW extract used in this study showed 1.3 mg hyperforin/g SJW extract (0.13%). Treatment of mice with 0.1–0.6 g/kg/d SJW for 14 days did not significantly increase mouse liver microsomal P450 content and NFO activity (data not shown). Thus, mice were treated with 0.3–0.9 g/kg of SJW extract for 21 days to illustrate the dose response of Cyp3a induction in B6 mice. Treatment with SJW extract at 0.3–0.6 g/kg had no effects on the spectrally determined content of P450 in liver microsomes (Figure 1). By increasing the dose to 0.9 g/kg, P450 content was elevated by 30% in the SJW-treated group ($p < 0.001$) (ANOVA, $F = 6.06$, $p = 0.0023$). The content of cytochrome *b*₅ and the cytochrome *c* reduction activity of CPR were not significantly changed by 0.3–0.9 g/kg SJW extract. Treatment with SJW extract at doses of 0.6 g/kg significantly increased liver microsomal NFO activity, without affecting the P450 content. SJW extract at 0.6 g/kg and 0.9 g/kg increased NFO activity by 91% ($p < 0.05$) and 95% ($p < 0.001$), respectively (ANOVA, $F = 6.63$, $p = 0.0015$). However, WOH activity was not affected by the 21-day treatment with SJW extract at a daily dose up to 0.9 g/kg. After the 21-day treatment, mouse serum ALT (control: 11.2 ± 1.7 U/L; SJW: 17.6 ± 3.4 U/L), AST (control: 81.6 ± 23.4 U/L; SJW: 69.2 ± 15.7 U/L), BUN (control: 26.0 ± 0.8 mg/dL; SJW: 25.1 ± 0.6 mg/dL), and creatinine (control: 1.0 ± 0.1 mg/dL; SJW: 0.7 ± 0.1 mg/dL) were not significantly changed by 0.6 g/kg SJW extract. Hematoxylin–eosin staining of liver sections revealed that the cytoplasm of hepatocytes was normally eosinophilic and finely granular (data not shown). SJW treatment did not cause any pathological changes in the kidney sections. Organ injury may not be a factor in the alterations of enzyme activities. Thus, mice were treated with 0.6 g/kg SJW extract in the following time-course study.

3.2. Time-dependent changes of P450 activities by SJW extract

After 14-day, 12-day, and 28-day consecutive treatments, the treatment of mice with 0.6 g/kg SJW did not change the P450 content and CPR activity (Figure 2A). The content of cytochrome *b*₅ was also unaffected by the 21-day and 28-day treatments with SJW extract. Liver microsomal Cyp1a marker activities related to 7-ethoxyresorufin and 7-methoxyresorufin were also unaffected by the SJW treatment. Microsomal activity of PROD (Cyp2b), coumarin 7-hydroxylation (Cyp2a), DMOD (Cyp2d), and chlorzoxazone 6-hydroxylation (Cyp2e1) remained unchanged. Treatment with SJW extract for 14 days did not cause a significant change in hepatic NFO activity. After the 21-day and 28-day treatments with 0.6 g/kg/d SJW extract, mouse hepatic NFO activity was increased by 88% ($p = 0.017$) and 95% ($p = 0.000$), respectively. Renal NFO activity was increased by 70% ($p = 0.009$) and 80% ($p = 0.016$) in mice treated with 0.6 g/kg/d SJW for 21 days and 28 days, respectively. After the 28-day consecutive

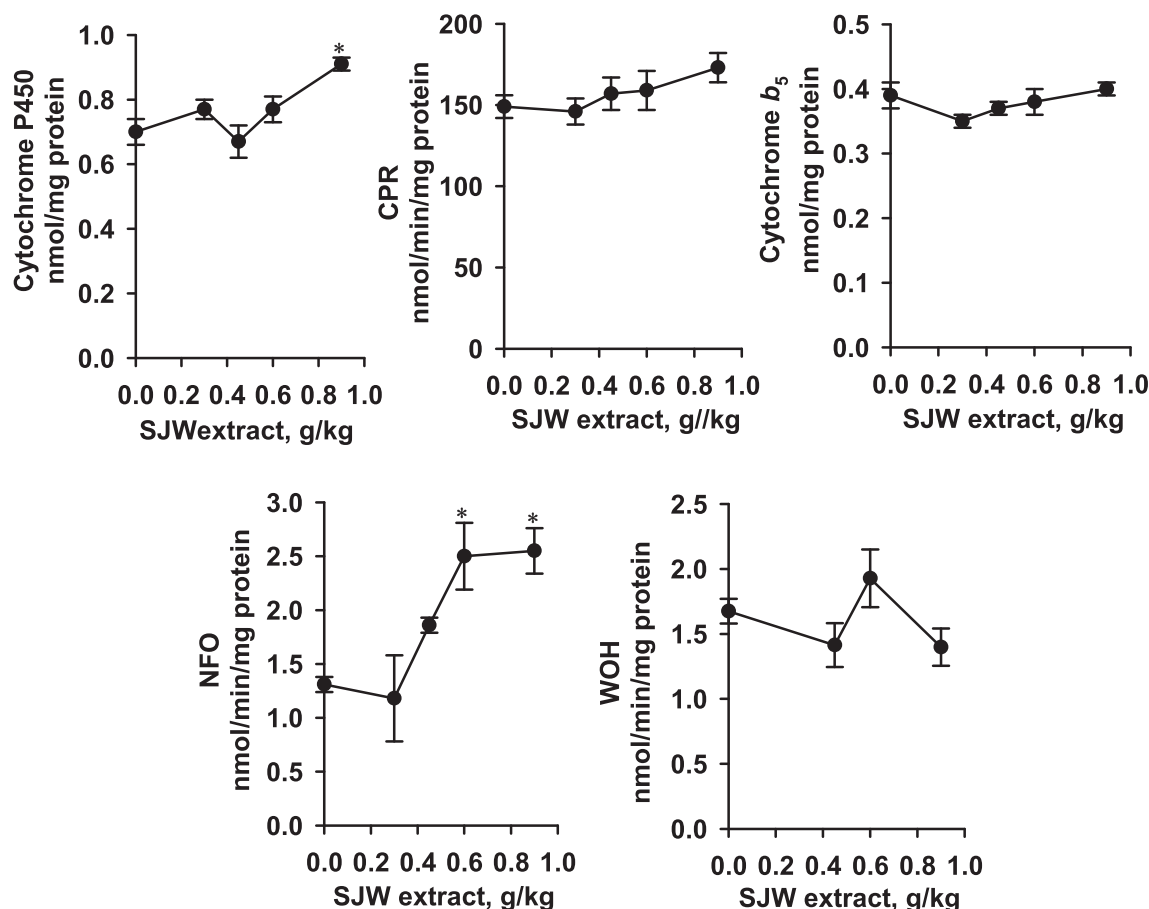


Figure 1 – The dose response of the effects of St John's wort (SJW) extract on liver microsomal content and activities of cytochrome P450-dependent monooxygenase in C57BL/6JNarl mice. Male mice were treated with increasing doses of SJW extract, as indicated, for 21 days. The results show the mean $\pm$ SEM of five mice. Asterisks (*) represent values that significantly differ from the control values, $p < 0.05$. CPR = NADPH-P450 reductase; NFO = nifedipine oxidation; SEM = standard error of the mean; WOH = warfarin 7-hydroxylation.

treatment, the SJW extract caused a 34% increase in WOH (Cyp2c) activity ($p = 0.033$). However, renal WOH activity remained unchanged (data not shown). After the 28-day treatment, mouse serum ALT (control: 7 ± 3.2 U/L; SJW: 5.4 ± 3.4 U/L), AST (control: 76.0 ± 27.1 U/L; SJW: 84.4 ± 19.4 U/L), BUN (control: 19.7 ± 3.2 mg/dL; SJW: 18.8 ± 2.7 mg/dL), and creatinine (control: 0.5 ± 0.1 mg/dL; SJW: 0.7 ± 0.1 mg/dL) were not significantly changed by the 0.6 g/kg SJW extract.

3.3. Lack of changes of hepatic and renal UGT, GST, and NQO activities by SJW extract

Treatment of mice with SJW extract at 0.6 g/kg for 21 days and 28 days did not change liver (Figure 3A) and kidney (Figure 3B) microsomal UGT activities related to *p*-nitrophenol. Liver and kidney cytosolic GST and NQO activities were not affected by the 21-day and 28-day treatments with SJW extract (Figures 3A and 3B).

3.4. Induction of protein level of Cyp2c by SJW extract

To examine the changes of P450 protein levels, which can be linked to the elevated WOH activity and unchanged

chlorzoxazone 6-hydroxylation activity, immunoblot analyses of Cyp2c and Cyp2e1 were performed. It has been suggested that polyclonal antirat CYP2C11 cross-reacted with mouse Cyp2c [40]. Immunoblot analysis of mouse liver microsomal proteins using polyclonal antirat CYP2C11 showed the three immunoreacted protein bands visualized by ECL staining. The upper band had an electrophoretic molecular weight of 49 kDa, which was similar to the reported mobility of mouse Cyp2c in the blot [41]. Cyp2c was elevated by 145% in mice treated with 0.6 g/kg SJW extract for 28 days ($p = 0.047$) (Figure 4). The other two protein bands with higher electrophoretic mobility had molecular weights of ≤ 45 kDa. Consistent with the lack of induction of the activities of Cyp2e1 and the electron-transfer partner CPR, the protein expression levels of Cyp2e1 and CPR remained unchanged.

4. Discussion

Our results of the dose–response study in B6 mice showed that the minimal dosing regimen to elicit an increase of hepatic Cyp3a activity was a consecutive 21-day treatment with 0.6 g/kg SJW extract. Compared to the preparations of SJW

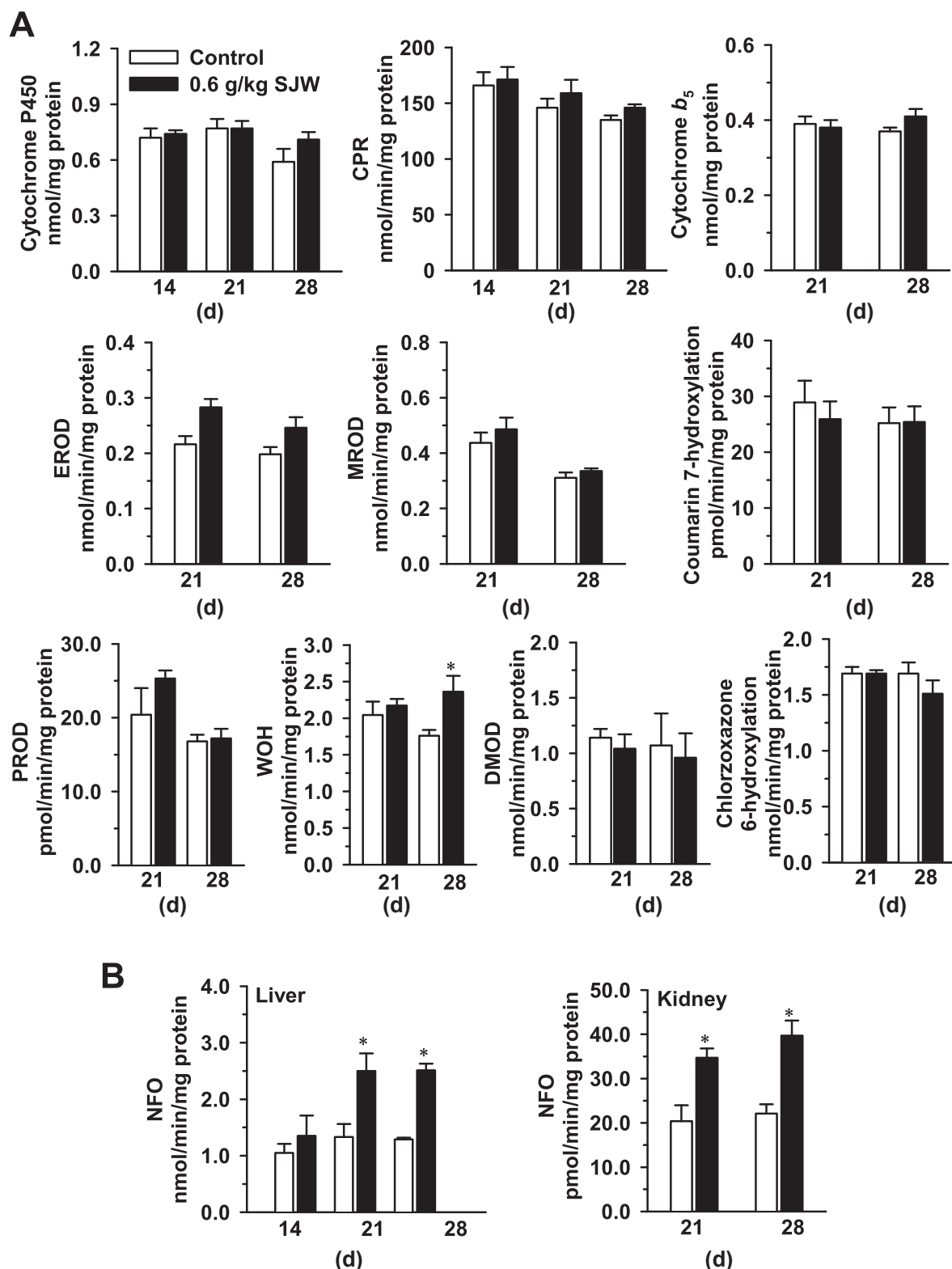


Figure 2 – The time course of effects of St John's wort (SJW) extract on the content/activity of the components of (A) the hepatic P450-dependent monooxygenase system and (B) the hepatic and renal NFO activities. Mice were treated with SJW extract at a daily dose of 0.6 g/kg for 14, 21, and 28 consecutive days. Data represent the mean $\pm$ SEM of four mice in the 14-day treatment groups and five mice in the 21-day and 28-day treatment groups. Asterisks (*) represent values that significantly differ from the control values, $p < 0.05$. CPR = NADPH-P450 reductase; DMOD = dextromethorphan O-demethylation; EROD = 7-ethoxyresorufin O-deethylation; MROD = 7-methoxyresorufin O-demethylation; NFO = nifedipine oxidation; PROD = 7-pentoxeresorufin O-dealkylation; SEM = standard error of the mean; WOH = warfarin 7-hydroxylation.

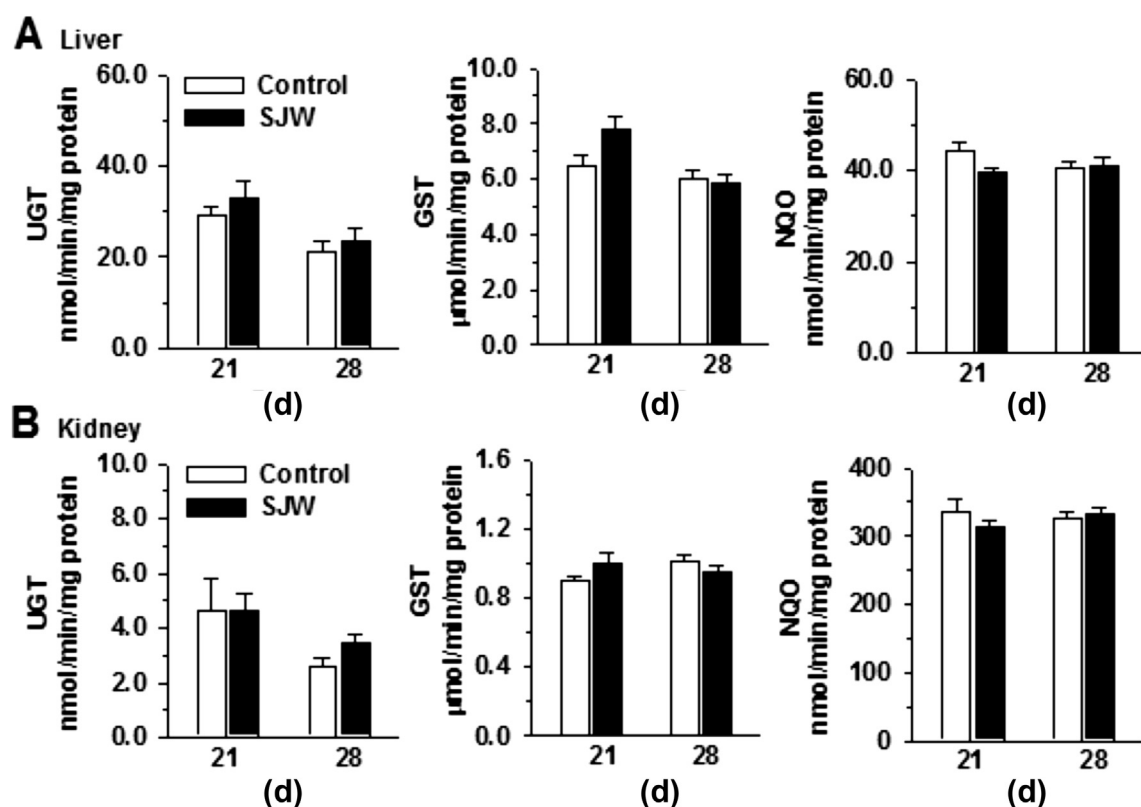


Figure 3 – Effects of St John's wort (SJW) on UGT, GST, and NQO activities in (A) mouse liver and (B) kidney. Mice were treated with 0.6 g/kg SJW extract for 21 days and 28 days. The data represent the mean $\pm$ SEM of five mice in each group. Asterisks (*) represent values that significantly differ from the control values, $p < 0.05$. GST = glutathione S-transferase; NQO = NAD(P) H-quinone oxidoreductase; SEM = standard error of the mean; UGT = UDP-glucuronosyltransferase.

extract used in the reported studies on human samples and experimental animals [7,8,11], the content of hyperforin in SJW extract used in this study was relatively low. The ligand-binding domains of human and mouse PXR share about 77% amino acid sequence identity [42]. Both human and mouse PXR can be activated by dexamethasone. However, human, but not mouse, PXR is activated by rifampicin. Mouse, but not human, PXR is activated by pregnenolone 16- α -carbonitrile. Human and mouse PXR have distinct ligand preference and may show differential activation capacity to one ligand [43]. In a receptor activation reporter assay on HepG2 cells, exposure to 1 μ M hyperforin resulted in 22-fold and 4-fold induction of the reporter gene expression activated by human and mouse PXR, respectively [43]. The hyperforin-mediated activation of mouse PXR was weaker than that of human PXR. As a result, the minimal daily dose for Cyp3a induction in this mouse study was 0.6 g/kg, which was higher than that (0.14 g/kg and 0.28 g/kg) in the previous mouse study reported by Bray et al [11] and was 3-fold greater than the estimated human equivalent dose in mice. Regarding the viewpoint of safety evaluation, a dose greater than the estimated human equivalent dose of a herbal extract shall be included in the assessment of the potential drug interaction using experimental animals.

While mouse hepatic NFO activity was increased by SJW extract after the 21-day treatment, renal NFO activity was also elevated. After the 28-day treatment, the increased hepatic and renal NFO activities remained. Unlike the universal

expression of CYP3A4 in human liver tissues, the level of CYP3A4 protein in 13 human kidney tissues were below the detection limit, and CYP3A5 protein could be determined in all kidney tissues in a study on 27 human samples [18]. Human CYP3A4 and CYP3A5 share the catalytic selectivity of some substrates including nifedipine [44]. Human renal CYP3A participates in the metabolism of xenobiotics, such as cocaine, and endogenous compounds, such as arachidonic acid and cortisol [18,19]. Renal CYP3A has been demonstrated to be crucial in the activation of the nephrotoxicity of cocaine [40] and the regulation of blood pressure [45]. By exposure to a CYP3A inhibitor ketoconazole, the cytotoxicity of cocaine was reduced in the primary culture of human proximal tubular epithelial cells. The potential impact of SJW-mediated renal CYP3A induction on physiological function and chemical toxicity is an important consideration for future studies.

Human CYP2C9 and mouse Cyp2c have overlapped specificity in substrates including warfarin [41]. Racemic warfarin is currently used in patients worldwide to prevent thrombosis and thromboembolism. The major metabolic pathway of most pharmacologically active S-warfarin is 7-hydroxylation, which is primarily catalyzed by CYP2C9 in human liver microsomes [46]. R-warfarin is primarily 6- and 10-hydroxylated by CYP1A2 and CYP3A4 in humans, respectively. Hydroxylated metabolites of warfarin can be further metabolized by UGT to form glucuronides. In healthy participants taking an SJW tablet containing 12.5 mg hyperforin (t.i.d.) for 14 days,

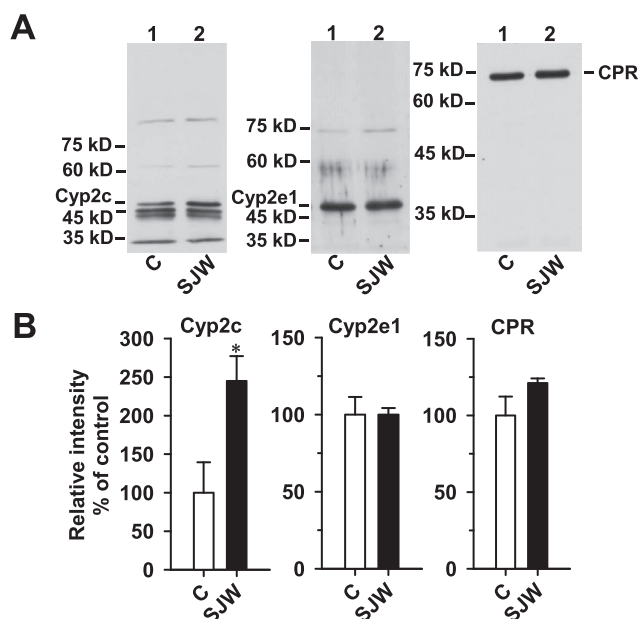


Figure 4 – Immunoblot analysis of liver microsomal Cyp2c, Cyp2e1 and NADPH-P450 reductase (CPR) in the control and St John's wort (SJW)-treated mice. Mice were treated with 0.6 g/kg SJW extract for 28 days. In the analyses of P450 and CPR, microsomal proteins were separated using 10% and 7.5% polyacrylamide gel, respectively. The representative blot and the results of band intensity scanning are shown on panels A and B, respectively. The results of band intensity scanning represent the mean $\pm$ standard error of the mean of the data from three mice. Asterisks (*) represent values that significantly differ from the control values, $p < 0.05$.

the clearances of S- and R-warfarin were increased [47]. Our findings first demonstrated that the treatment of mice with 0.6 g/kg SJW extract daily for 28 days increased the hepatic WOH activity and Cyp2c protein level, whereas the induction of Cyp3a initially occurred after the 21-day treatment. The induction of Cyp2c by repeated treatment of SJW extract required a period longer than the initial induction of Cyp3a. The results of the mouse study provide *ex vivo* evidence that the induction of CYP2C and CYP3A can contribute to the SJW-stimulated clearance of S- and R-warfarin in humans [47]. Hyperforin stimulated the CYP2C9 promoter transactivation and induced CYP2C9 mRNA, protein, and flurbiprofen oxidation activity in the primary culture of human hepatocytes [9,48]. Although the SJW extract contained >150 ingredients [6,13] and the effect of SJW extract on CYP2C expression and activity remained unclear regarding human hepatocytes, hyperforin can be crucial in the induction of Cyp2c by the SJW extract. The activation of PXR, constitutive androstane receptor (CAR), and glucocorticoid receptor have overlapped specificities in ligands and target genes, and the receptor activation can coordinately regulate the expression of CYP2C [49,50]. Hyperforin is metabolized by multiple P450 isoforms to form the hydroxylation metabolites, and CYP2C8/9/19 and CYP3A4 make the main contribution to the hydroxylation of hyperforin in human liver microsomes [51]. The potential

involvement of the cross talk in a receptor network [49], the modulatory effects of SJW metabolites, and the alterations of transcription factors in the distinct time course of SJW-mediated Cyp2c and Cyp3a induction requires further investigation. Meanwhile, unlike the induction of renal NFO activity, renal WOH activity was not affected by the extended SJW treatment. The induction of Cyp2c by SJW extract showed tissue differences.

In addition to the induction of CYP3A and CYP2C, some PXR activators are able to induce the expression and activities of CYP2B and UGT [49,50]. SJW extract stimulated the UGT1A1 promoter activity in CV-1 cells expressing mouse PXR, but not in cells expressing mouse CAR [15]. Consistent with the unchanged hepatic UGT activity by 21-day SJW treatment, as reported by Bray et al [11], the results of our mouse study revealed that hepatic and renal UGT activities remained unchanged even after the 28-day treatment with SJW extract. CYP2B, which is a target P450 subfamily in the induction through the activation of CAR [50], could not be induced by SJW. SJW showed preference in the activation of PXR. The metabolism capability and protein level of CYP1A2 have been reported to be either transiently induced or inhibited by SJW [12,13]. In another study of the primary culture of human hepatocytes, the mRNA and protein levels of CYP1A2 and CYP2D6 remained the same after hyperforin exposure for 48 hours [9]. The results of our mouse study support that hepatic CYP1A and CYP2D activities remained unchanged after prolonged SJW treatment. Although the impact of time-dependent changes and the relevance of transiently increased expression to functional change remained unclear, CYP1A2 was consistently unaffected by prolonged exposure to SJW. Hepatic CYP2E1 activity/protein level and GST-P protein level have been reported to be induced by SJW in mice and rats, respectively [11,12]. However, mouse hepatic CYP2E1 and GST activities remained unchanged in our findings. One of the factors for such inconsistent induction can be the difference in the constitution of different preparations of the SJW

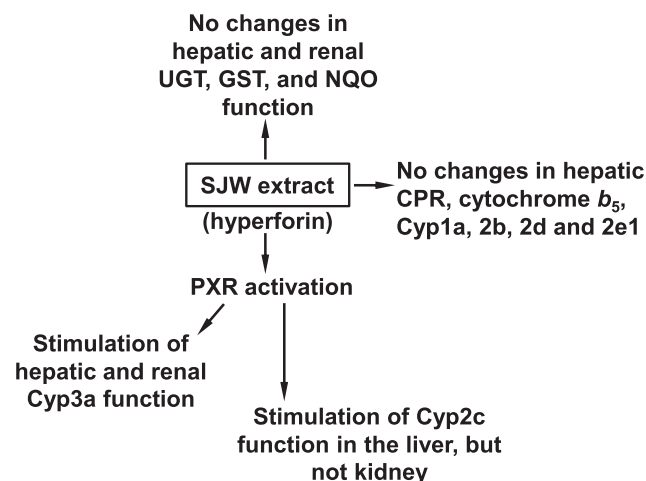


Figure 5 – Summary of the effects of SJW extract on mouse P450, UGT, GST, and NQO in mice. CPR = NADPH-P450 reductase; GST = glutathione S-transferase; NQO = NAD(P) H-quinone oxidoreductase; SJW = St John's wort; UGT = UDP-glucuronosyltransferase.

extract. Presently, no reports show the drug interaction of SJW with CYP2E1 substrates [5]. Our findings first showed that hepatic CYP2A activity and hepatic and renal NQO activities were not affected by SJW treatment. These findings suggest that the incidence of SJW-induced drug interaction through the functional changes of CYP2A, CYP2B, CYP2D, CYP2E1, UGT, GST, and NQO may be low.

In conclusion (Figure 5), our findings first demonstrate that Cyp2c can be induced by repeated treatment with SJW extract in B6 mice. The induction of Cyp2c required a treatment period longer than that for Cyp3a induction. The increases of Cyp2c and Cyp3a function cannot be attributed to the changes of CPR or cytochrome *b*₅. The difference in the time course of induction may be one of the causes for the inconsistency in CYP2C induction when different treatment regimens were used [7,8]. Our findings support that the increased CYP2C/3A activity, but not the UGT activity, may contribute to the interaction of SJW with warfarin [5,47]. Unlike the unchanged Cyp2c activity in mouse kidney, renal Cyp3a activity was stimulated by SJW extract. The influence of SJW on the renal CYP3A-linked toxicity or biological activities of xenobiotics, as well as physiologically occurring compounds, can prove important in future studies.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

This work was partly supported mainly by grants MM10414-0550 and MM10501-0265, and partly by grants MOST104-2320-B-077-001 from the Ministry of Science and Technology, Taiwan. Mr Yang and Ms Liu performed the experiments with the assistance of Miss Huang. Dr Ueng supervised this study and wrote this report.

REFERENCES

- [1] Rendic S, Guengerich FP. Survey of human oxidoreductases and cytochrome P450 enzymes involved in the metabolism of xenobiotic and natural chemicals. *Chem Res Toxicol* 2015;28:38–42.
- [2] Guengerich FP. Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. *Chem Res Toxicol* 2001;14:611–50.
- [3] Yamazaki H, Johnson WW, Ueng YF, Shimada T, Guengerich FP. Lack of electron transfer from cytochrome *b*₅ in stimulation of catalytic activities of cytochrome P450 3A4. Characterization of a reconstituted cytochrome P450 3A4/NADPH-cytochrome P450 reductase system and studies with apo-cytochrome *b*₅. *J Biol Chem* 1996;271:27438–44.
- [4] Shimada T, Yamazaki H, Mimura M, Inui Y, Guengerich FP. Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *J Pharmacol Exp Ther* 1994;270:414–23.
- [5] Russo E, Scicchitano F, Whalley BJ, Mazzitello C, Ciriaco M, Esposito S, Patanè M, Upton R, Pugliese M, Chimirri S, Mammì M, Palleria C, De Sarro G. *Hypericum perforatum*: pharmacokinetic, mechanism of action, tolerability, and clinical drug–drug interactions. *Phytother Res* 2014;28:643–55.
- [6] Moore LB, Goodwin B, Jones SA, Wisely GB, Serabjit-Singh CJ, Willson TM, Collins JL, Klier SA. St. John's wort induces hepatic drug metabolism through activation of the pregnane X receptor. *Proc Natl Acad Sci U S A* 2000;97:7500–2.
- [7] Wang Z, Gorski JC, Hamman MA, Huang SM, Lesko LJ, Hall SD. The effects of St John's wort (*Hypericum perforatum*) on human cytochrome P450 activity. *Clin Pharmacol Ther* 2001;70:317–26.
- [8] Xu H, Williams KM, Liauw WS, Murray M, Day RO, McLachlan AJ. Effects of St John's wort and CYP2C9 genotype on the pharmacokinetics and pharmacodynamics of glimepiride. *Brit J Pharmacol* 2008;153:1579–86.
- [9] Komoroski BJ, Zhang S, Cai H, Hutzler JM, Frye R, Tracy TS, Strom SC, Lehmann T, Ang CYW, Cui YY, Venkataramanan R. Induction and inhibition of cytochromes P450 by the St. John's wort constituent hyperforin in human hepatocyte cultures. *Drug Metab Dispos* 2004;32:512–8.
- [10] Dostalek M, Pistovcakova J, Jurica J, Tomandl J, Linhart I, Sulcova A, Hadasova E. Effect of St John's wort (*Hypericum perforatum*) on cytochrome P-450 activity in perfused rat liver. *Life Sci* 2005;78:239–44.
- [11] Bray BJ, Perry NB, Menkes DB, Rosengren RJ. St. John's wort extract induces CYP3A and CYP2E1 in the Swiss Webster Mouse. *Toxicol Sci* 2002;66:27–33.
- [12] Shibayama Y, Ikeda R, Motoya T, Yamada K. St John's wort (*Hypericum perforatum*) induces overexpression of multidrug resistance protein 2 (MRP2) in rats: a 30-day ingestion study. *Food Chem Toxicol* 2004;42:995–1002.
- [13] Dostalek M, Pistovcakova J, Jurica J, Linhart I, Sulcova A, Tomandl J. The effect of St John's wort (*Hypericum perforatum*) on cytochrome P450 1A2 activity in perfused rat liver. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub* 2011;155:253–8.
- [14] Jancova P, Anzenbacher P, Anzenbacherova E. Phase II drug metabolizing enzymes. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub* 2010;154:103–16.
- [15] Xie W, Teuh MF, Radominska-Pandya A, Saini SP, Negishi Y, Bottorff BS, Cabrera GY, Tukey RH, Evans RM. Control of steroid, heme, and carcinogen metabolism by nuclear pregnane X receptor and constitutive androstane receptor. *Proc Natl Acad Sci* 2003;100:4150–5.
- [16] Hengstler JG, Van der Burg B, Steinberg P, Oesch F. Interspecies differences in cancer susceptibility and toxicity. *Drug Metab Rev* 1999;31:917–70.
- [17] Siegel D, Yan C, Ross D. NAD(P)H: quinone oxidoreductase 1 (NQO1) in the sensitivity and resistance to antitumor quinones. *Biochem Pharmacol* 2012;83:1033–40.
- [18] Knights KM, Rowland A, Miners JQ. Renal drug metabolism in humans: the potential for drug–endobiotic interactions involving cytochrome P450 (CYP) and UDP-glucuronosyltransferase (UGT). *Br J Clin Pharmacol* 2013;76:587–602.
- [19] Valente MJ, Henrique R, Vilas-Boas V, Silva R, Bastos ML, Carvalho F, Guedes de Pinho P, Carvalho M. Cocaine-induced kidney toxicity: an in vitro study using primary cultured human proximal tubular epithelial cells. *Arch Toxicol* 2012;86:249–61.
- [20] Wilkens MR, Maté LM, Schnepel N, Klinger S, Muscher-Banse AS, Ballent M, Virkel G, Lifschitz AL. Influence of 25-hydroxyvitamin D₃ and 1,25-dihydroxyvitamin D₃ on expression of P-glycoprotein and cytochrome P450 3A in sheep. *J Steroid Biochem Mol Biol* 2015. <http://dx.doi.org/10.1016/j.jsbmb.2015.08.019>. pii: S0960–0760(15)30051-0.

- [21] Pang X, Cheng J, Krausz KW, Guo Da, Gonzalez FJ. Pregnane X receptor-mediated induction of Cyp3a by black cohosh. *Xenobiotica* 2011;41:112–23.
- [22] Guengerich FP, Martin MV, Beaune PH, Kremers P, Wolff T, Waxman DJ. Characterization of rat and human liver microsomal cytochrome P-450 forms involved in nifedipine oxidation, a prototype for genetic polymorphism in oxidative drug metabolism. *J Biol Chem* 1986;261:5051–60.
- [23] Bauer S, Störmer E, Graubau HJ, Roots I. Determination of hyperforin, hypericin, and pseudohypericin in human plasma using high-performance liquid chromatography analysis with fluorescence and ultraviolet detection. *J Chromatogr B* 2001;765:29–35.
- [24] Alvares AP, Mannering GJ. Two substrate kinetics of drug-metabolizing enzyme systems of hepatic microsomes. *Mol Pharmacol* 1970;6:206–12.
- [25] Omura T, Sato R. The carbon monoxide-binding pigment of liver microsomes. *J Biol Chem* 1964;239:2370–9.
- [26] Phillips AH, Langdon RG. Hepatic triphosphopyridine nucleotide-cytochrome c reductase: isolation, characterization, and kinetic studies. *J Biol Chem* 1962;237:2652–60.
- [27] Pohl RJ, Fouts JR. A rapid method for assaying the metabolism of 7-ethoxyresorufin by microsomal subcellular fractions. *Anal Biochem* 1980;107:150–5.
- [28] Lubet RA, Mayer RT, Cameron JW, Nims RW, Burke MD, Wolff T, Guengerich FP. Dealkylation of pentoxyresorufin: a rapid and sensitive assay for measuring induction of cytochrome(s) P-450 by phenobarbital and other xenobiotics in the rat. *Arch Biochem Biophys* 1985;238:43–8.
- [29] Souček P. Novel sensitive high-performance liquid chromatographic method for assay of coumarin 7-hydroxylation. *J Chromatogr B* 1999;734:23–9.
- [30] Yamazaki H, Shimada T. Human liver cytochrome P450 enzymes involved in the 7-hydroxylation of R-warfarin and S-warfarin enantiomers. *Biochem Pharmacol* 1997;54:1195–203.
- [31] Kerry NL, Somogyi AA, Mikus G, Bochner F. Primary and secondary oxidative metabolism of dextromethorphan: in vitro studies with female Sprague–Dawley and Dark Agouti rat liver microsomes. *Biochem Pharmacol* 1993;45:833–9.
- [32] Peter R, Böcker R, Beaune PH, Iwasaki M, Guengerich FP, Yang CS. Hydroxylation of chlorzoxazone as a specific probe for human liver microsomal P450 1A1. *Chem Res Toxicol* 1990;3:566–73.
- [33] Bock KW, Burchell B, Ditton GJ, Hanninen O, Mulder GJ, Owens IS, Siest G, Tephyl TR. UDP-glucuronosyltransferase activities: guidelines for consistent interim –terminology and assay condition. *Biochem Pharmacol* 1983;32:953–5.
- [34] Habig WH, Pabst MJ, Jascoby WB. Glutathione S-transferase: the first enzymatic step in mercapturic acid formation. *J Biol Chem* 1974;249:7130–9.
- [35] Liu XF, Yuan H, Haniu M, Iyanagi T, Shively JE, Chen S. Reaction of rat liver dt-diaphorase @ (NAD(P)H: quinone acceptor reductase) with 5'-[p-(fluorosulfonyl)benzoyl]-adenosine. *Mol Pharmacol* 1989;35:818–22.
- [36] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265–75.
- [37] Ueng YF, Chen CC, Huang YL, Lee IJ, Yun CH, Chen YH. Effects of aqueous extract of *Ruta graveolens* and its ingredients on cytochrome P450, uridine diphosphate (UDP)-glucuronosyltransferase, and reduced nicotinamide adenine dinucleotide (phosphate) (NAD(P)H)-quinone oxidoreductase in mice. *J Food Drug Anal* 2015;23:516–28.
- [38] Yim SK, Jung HC, Pan JG, Kang HS, Ahn T, Yun CH. Functional expression of mammalian NADPH-cytochrome P450 oxidoreductase on the cell surface of *Escherichia coli*. *Protein Exp Purif* 2006;49:292–8.
- [39] Butterweck V. St John's wort: quality issues and active compounds. In: Cooper R, Kronenberg F, editors. *Botanical medicine: from bench to bedside*. New York: Mary Ann Liebert, Inc.; 2009. p. 69–91.
- [40] Sodhi K, Inoue K, Gotlinger KH, Canestraro M, Vanella L, Kim DH, Manthathi VL, Koduru SR, Falck JR, Schwartzman ML, Abraham NG. Epoxyeicosatrienoic acid agonist rescues the metabolic syndrome phenotype of HO-2-null mice. *J Pharmacol Exp Ther* 2009;331:906–16.
- [41] Guo Y, Weller P, Farrell E, Cheung P, Fitch B, Clark D, Sy Wu, Wang J, Liao G, Zhang Z, Allard J, Cheng J, Nguyen A, Jiang S, Shafer S, Usuka J, Masjedizadeh M, Peltz G. In silico pharmacogenetics of warfarin metabolism. *Nat Biotech* 2006;24:531–6.
- [42] Jones SA, Moore LB, Shenk JL, Wisely GB, Hamilton GA, McKee DD, Tomkinson NC, LeCluyse EL, Lambert MH, Wilson TM, Kliever SA, Moore JT. The pregnane X receptor: a promiscuous xenobiotic receptor that has diverged during evolution. *Mol Endocrinol* 2000;14:27–39.
- [43] Vignati LA, Bogni A, Grossi P, Monshouwer M. A human and mouse pregnane X receptor reporter gene assay in combination with cytotoxicity measurements as a tool to evaluate species-specific CYP3A induction. *Toxicology* 2004;199:23–33.
- [44] Gillam EM, Guo Z, Ueng YF, Yamazaki H, Cock I, Reilly PE, Hooper WD, Guengerich FP. Expression of cytochrome P450 3A5 in *Escherichia coli*: effects of 5' modification, purification, spectral characterization, reconstitution conditions, and catalytic activities. *Arch Biochem Biophys* 1995;317:374–84.
- [45] Givens RC, Lin YS, Dowling AL, Thummel KE, Lamba JK, Schuetz EG, Stewart PW, Watkins PB. CYP3A5 genotype predicts renal CYP3A activity and blood pressure in healthy adults. *J Appl Physiol* 2003;95:1297–300.
- [46] Kaminisky LS, Zhang ZY. Human P450 metabolism of warfarin. *Pharmacol Ther* 1997;73:67–74.
- [47] Jiang X, Williams KM, Liauw WS, Ammit AJ, Roufogalis BD, Duke CC, Day RO, McLachlan AJ. Effect of St John's wort and ginseng on the pharmacokinetics and pharmacodynamics of warfarin in healthy subjects. *Br J Clin Pharmacol* 2004;57:592–9.
- [48] Chen Y, Ferguson SS, Negishi M, Goldstein JA. Induction of human CYP2C9 by rifampicin, hyperforin, and phenobarbital is mediated by the pregnane X receptor. *J Pharmacol Exp Ther* 2004;308:495–501.
- [49] Pascucci JM, Gerbal-Chaloin S, Maurel P, Vilarem MJ. The expression of CYP2B6, CYP2C9 and CYP3A4 genes: a tangle of networks of nuclear and steroid receptors. *Biochim Biophys Acta* 2003;1619:243–53.
- [50] Wang H, Negifshi M. Transcriptional regulation of cytochrome P450 2B genes by nuclear receptors. *Curr Drug Metab* 2003;4:515–25.
- [51] Hokkanen J, Tolonen A, Mattila S, Turpeinen M. Metabolism of hyperforin, the active constituent of St. John's wort, in human liver microsomes. *Eur J Pharm Sci* 2011;42:273–84.