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Influence of sesamin on CYP2C-mediated diclofenac metabolism: in vitro and in vivo analysis

Kaori Yasuda[a], Sera Ueno[a], Erika Ueda, Miyu Nishikawa, Kie Takeda, Masaki Kamakura, Shinichi Ikushiro & Toshiyuki Sakaki

Department of Biotechnology, Faculty of Engineering, Toyama Prefectural University, 5180 Kurokawa, Imizu, Toyama 939-0398, Japan

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Correspondence
Toshiyuki Sakaki, Department of Biotechnology, Faculty of Engineering, Toyama Prefectural University, 5180 Kurokawa, Imizu, Toyama 939-0398, Japan.
Tel: +81-766-56-7500 Ext.601;
Fax: +81-766-56-2498;
E-mail: tsakaki@pu-toyama.ac.jp

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[a]Both authors contributed equally to the study.

Abstract

Our previous studies revealed that sesamin caused a mechanism-based inhibition (MBI) of CYP2C9 in human liver microsomes. Additionally, we observed a similar MBI of CYP2C by sesamin in the rat liver microsomes. Sesamin-induced difference spectra of rat or human liver microsomes in the presence of NADPH showed a peak at 459 nm, suggesting the formation of a metabolic–intermediate (MI) complex of cytochrome P450 and the methylenedioxyphenyl group of sesamin. However, the peak disappeared in both liver microsomes within 30 min after the termination of the metabolism. These results suggest that the MI complex of cytochrome P450 and sesamin is unstable, and the effects of sesamin on human CYP2C9- or rat CYP2C-mediated drug metabolism may be small. To confirm this, in vivo studies using rats were performed. The pharmacokinetics of diclofenac, which is mainly metabolized by CYP2C11 in male rats, were investigated after a 3-days administration of sesamin (0, 10, and 100 mg/kg bw). No significant differences were observed among the three groups in the pharmacokinetic parameters, C_{max}, T_{max}, and AUC. Furthermore, administration of sesamin to rats for 7 days had no significant effects on diclofenac hydroxylation activity in rat liver microsomes. These results demonstrate that no significant interaction occurs between diclofenac and sesamin in rats. Moreover, the results of these in vitro and in vivo studies suggest that no significant interaction may occur between sesamin and diclofenac when sesamin is administered to humans as a supplement, since the standard sesamin dose in humans is much lower than that administered to rats in this study.

Abbreviations

$AUC_{0\text{-}24\,h}$, area under the concentration–time curves from 0 to 24 h; bw, body weight; DMSO, dimethylsulfoxide; MBI, mechanism-based inhibition; MDP, methylenedioxyphenyl; MI, metabolic–intermediate; P450 or CYP, cytochrome P450.

Introduction

Sesamin is a major lignan in sesame, and is known to have various biological effects such as antioxidant effects (Ikeda et al. 2003; Nakai et al. 2003), anticarcinogenic effects (Hirose et al. 1992; Miyahara et al. 2000), and protective effects against alcohol-induced liver injury (Akimoto et al. 1993). Thus, sesamin appears to be a reliable food component with predictable effects on individuals when taken as a supplement or remedy.

It is important to clarify drug–sesamin interactions with respect to safety because some food factors are known to affect xenobiotic metabolism. In particular, mechanism-based inhibition (MBI) should be assessed, because inhibitory effects often persist even after the disappearance of an inhibitory substance. Recently, we clarified the sequential metabolism of sesamin by drug-metabolizing enzymes in the human liver, and found that CYP2C9 (Yasuda et al. 2010) (Fig. 1A) and UGT2B7 (Yasuda et al. 2011) are crucial for its metabolism. In

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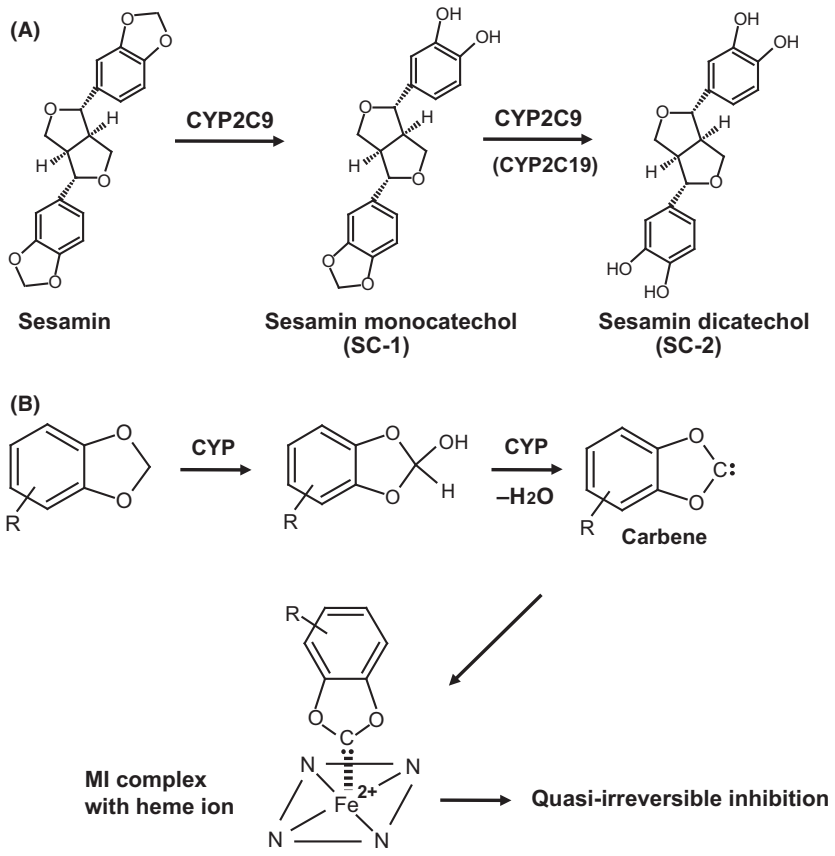

Figure 1. The metabolism of sesamin by CYP2C9 in human liver (A) and the metabolic–intermediate complex formation with a coordinate covalent bond between a carbine compound from sesamin and heme iron of CYP2C9 (B).

addition, it has also been suggested that sesamin could facilitate MBI of CYP2C9, since time- and concentration-dependent inactivation of CYP2C9-specific diclofenac hydroxylation activity by sesamin were observed in human liver microsomes or recombinant human CYP2C9 (Yasuda et al. 2010). Several reports have demonstrated that some compounds containing a methylenedioxyphenyl (MDP) group could cause MBI of cytochrome P450 (P450) (Nakajima et al. 1999; Bertelsen et al. 2003; Chatterjee and Franklin 2003; Heydari et al. 2004; Ring et al. 2005; Usia et al. 2005; Salminen et al. 2011). This finding suggests that sesamin could be a mechanism-based inhibitor of P450, because it has two MDP groups. The carbenes generated from P450-mediated metabolism of the MDP group could form a metabolic–intermediate (MI) complex with a heme iron of P450, thus resulting in the MBI (Murray 2000; Kamel and Harriman 2013; Taxak et al. 2013) (Fig. 1B). The absorbance spectrum of the MI complex shows a broad peak at 450–460 nm (Murray 2000). This type of inhibition is characterized as "quasi-irreversible" as opposed to the other two types, reversible or irreversible (Polasek and Miners 2007). It is noted that P450 could recover its activity when the quasi-irreversible inhibitor dissociates from the heme iron. 3,4-Methylenedioxymethamphetamine (MDMA, ecstasy) has an MDP group and is one of the most well-known quasi-irreversible inhibitors. O'Mathuna et al. (2008) reported that MDMA dramatically increases the serum concentration of dextromethorphan which is mainly metabolized by CYP2D6 in humans, and the half-life time of CYP2D6 recovery is approximately 46 h. They clarified that the dextromethorphan C_{max} and AUC values increases approximately 10-fold after MDMA administration. These findings suggest that not only irreversible inhibition but also quasi-irreversible inhibition should be carefully considered in the evaluation of drug–drug or drug–food interaction.

We have estimated the MBI parameters of CYP2C9 by sesamin in human liver microsomes (Yasuda et al. 2010; Yasuda and Sakaki 2012). The $k_{inact}/K_{i\,app}$ value indicates that sesamin is a weak inhibitor compared with other inhibitors such as bergamottin in grapefruit juice, which is a potent inhibitor of CYP3A4, or other lignans that have an MDP group (Usia et al. 2005; Yoo et al. 2008;

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Taesotikul et al. 2011). However, the effects of sesamin-dependent MBI on human CYP2C9 should be considered when sesamin is taken as a supplement, because it is a major human P450 isoform involved in metabolizing therapeutic drugs such as diclofenac, phenytoin, and S-warfarin.

Recently, Tomimori et al. (2013) reported the pharmacokinetics of a sesamin supplement including equal amounts of sesamin and episesamin in humans when it is administered daily for 28 days. They demonstrated no accumulation of sesamin or episesamin after 28 days for a dose of 50 mg/day, which is five-fold higher than the recommended daily intake (10 mg/day). This suggests that daily intake of the sesamin supplement has no significant toxic effects.

In this report, we describe the stability of the MI complex of sesamin and P450 in human or rat liver microsomes by spectral analysis. In addition, the effect of sesamin on the plasma concentration of diclofenac was examined in rats. To the best of our knowledge, this is the first analysis of the sesamin–drug interaction.

Materials and Methods

Materials

Sesamin for in vitro study was purchased from Sigma-Aldrich (St. Louis, MO), and sesamin for in vivo study was kindly provided by Suntory Wellness Ltd. (Osaka, Japan). The purity of the former was above 95.0% and that of the latter was 97.3%. NADPH was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). A 50 donor human liver microsomes pool and male Sprague–Dawley rat liver microsomes and recombinant rat CYP2C11 expressed in baculovirus-infected insect cells were purchased from BD Gentest (Woburn, MA, USA). Anti-human CYP2C9 antibody was purchased from Nihon Nosan Kogyo (Yokohama, Japan), and anti-Bip antibody was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). All other chemicals were purchased from standard commercial sources of the highest quality available.

Animals

Male Sprague–Dawley rats (5–8 weeks old) were obtained from Sankyo Laboratory Service Co. (Toyama, Japan). Rats were housed individually in a room with controlled temperature (22–24°C), humidity (55–65%), and lighting (turning on at 9:00 and off at 21:00), and fed on commercial diet (Oriental Yeast Co., Ltd). Rats were used in the experiments described as follows, after 7 days of acclimation to the housing conditions. Animal care and use conformed to US NIH published guidelines.

Preparation of the liver microsomes from rats administrated with sesamin

The liver microsomes of rats given sesamin were prepared according to the modified methods of previous report (Naritomi et al. 2001). Briefly, the liver was excised, rinsed with ice-cold saline solution, and homogenized with four volumes of ice-cold 1.15% KCl. The homogenate was centrifuged at 10,000*g* for 20 min, and the supernatant was then centrifuged at 100,000*g* for 60 min. The pellet was reconstituted with 20 mmol/L phosphate buffer (pH 7.4) containing 1.15% KCl, and the protein concentration was determined using Protein Assay Bicinchoninate Kit (Nacalai tesque, Inc., Kyoto, Japan).

Inhibition of diclofenac hydroxylation activity by sesamin in rat or human liver microsomes

The reaction mixture containing 0.5 mg protein/mL of a male Sprague–Dawley rat liver microsome or human liver microsomes, 1 mmol/L NADPH, and various concentrations of sesamin (0–50 μmol/L) in 100 mmol/L potassium phosphate buffer (pH 7.4) in a final volume of 100 μL was preincubated at 37°C for 0, 5, or 10 min. After the preincubation, diclofenac was added at the final concentration of 100 μmol/L, and the incubation was continued for 15 min under the same conditions. Reaction was stopped by addition of 100 μL of methanol, and then diclofenac and their metabolites were analyzed as described in the section "HPLC analysis of sesamin, diclofenac, and their metabolites." Kinetic parameters of inactivation process were calculated according to the method described by Waley (1980, 1985). The observed rate constant of inactivation (k_{obs}) was calculated from the initial slopes of the liner regression line of the "residual activity" versus "preincubation time" profile plotted on a semilogarithmic scale. The inactivation rate constant at infinite concentration of inhibitor (k_{inact}) and the appeared inhibitor constants (K_i) were calculated from the double reciprocal plots of k_{obs} versus sesamin concentration.

Spectral analysis of P450 MI complex during sesamin metabolism in rat or human liver microsomes

The reaction mixture containing 0.5 mg protein/mL of rat liver microsomes or 2.0 mg protein/mL of human liver microsomes or 50 pmol/mL recombinant rat CYP2C11, and 1 mmol/L NADPH in 100 mmol/L phosphate buffer (pH 7.4) in a final volume of 1.2 mL was put into the cuvette. DMSO solution containing sesamin

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was added to the sample cuvette to a final concentration of 20 μmol/L, while an equal volume of DMSO was added to the reference cuvette. Difference spectra in the range of 400 to 500 nm were recorded at 0, 1, 3, 5, 7, 10, 15, 20, 30, 40, 50, 60, 70, 90, and 120 min after incubation at 37°C. The final concentration of rat and human liver microsomes in the reaction mixture was adjusted to obtain a similar MI complex concentration. Sesamin and its metabolites, monocatechol (SC1) and dicatechol (SC2), in the reaction mixture containing rat or human liver microsomes were quantified under the same conditions as the spectral analysis. The reaction mixture (1 mL) was incubated at 37°C, and 0.05 mL aliquots of the reaction mixture was collected at 0, 1, 3, 5, 7, 10, 15, 20, 30, 40, 50, 60, 70, 90, and 120 min, and then 0.05 mL of methanol was added to them to stop the reaction. After a centrifugation at 20,000*g* for 15 min, the resultant supernatant was analyzed by HPLC as described in the section "HPLC analysis of sesamin, diclofenac, and their metabolites."

Recovery of the activity from sesamin-dependent MBI in rat and human liver microsomes

The reaction mixture containing 0.5 mg protein/mL of rat liver microsomes or 2.0 mg protein/mL of human liver microsomes, 1 mmol/L NADPH, and 20 μmol/L sesamin in 100 mmol/L phosphate buffer (pH 7.4) in a final volume of 100 μL was incubated for 60 min at 37°C. After incubation, the reaction mixture was centrifuged at 20,000*g* for 15 min, and the supernatant was removed. New reaction buffer (100 μL; 100 mmol/L phosphate buffer, pH 7.4) containing 1 mmol/L NADPH and 20 μmol/L sesamin was added to the resultant precipitation, and was further incubated for 5 min, and then 100 μL of methanol was added to the mixture to stop the reaction. After a centrifugation at 20,000*g* for 15 min, the resultant supernatant was analyzed by HPLC as described in the section "HPLC analysis of sesamin, diclofenac, and their metabolites." As a control, sesamin catecholization activity was measured under the same conditions (1 mmol/L NADPH, and 20 μmol/L sesamin in 100 mmol/L phosphate buffer [pH 7.4] for 5 min) without 60 min preincubation with sesamin. Protein concentration of each reaction mixture was measured using the Protein Assay Bicinchoninate Kit (Nacalai tesque, Inc., Kyoto, Japan).

Effects of sesamin on the pharmacokinetics of diclofenac in rats

A certain amount of sesamin and diclofenac sodium was suspended to 300 μL of corn oil and 0.9% saline solution, respectively. Sesamin (0, 10, and 100 mg/kg bw) was administered once a day to the rats using feeding needles for 3 days. At 3 h after the last sesamin administration, diclofenac sodium (10 mg/kg bw) was orally administered. After the last sesamin administration, rats were fasted until administration of diclofenac. Simultaneous administration of sesamin (0, 10, and 100 mg/kg bw) and diclofenac (10 mg/kg bw) was also performed.

Blood samples were collected from the jugular vein at 0, 2.5, 5, 7.5, 10, 15, 20, 30, 45, 60, 120, and 240 min and 24 h after administration of diclofenac sodium. All of the blood samples were immediately centrifuged at 800g for 10 min, and the resultant supernatant was stored at −80°C until analysis.

Determination of blood plasma diclofenac and sesamin concentration in rats

Diclofenac serum concentration was determined by HPLC under slightly modified conditions from those described previously (Leon-Reyes et al. 2009). Fifty microliters of 0.9% saline was added to 50 μL of blood plasma sample, and 1 mL of ethylacetate containing 10 mmol/L NaH_2PO_4 (pH 3.0) and 10 μmol/L testosterone (internal standard) was added to each sample, and then the samples were extracted by vigorously shaking with vortex mixer. After the centrifugation at 15,000*g* for 10 min, the organic phase was dried in a vacuum evaporator centrifuge (Thermo Fisher Scientific Inc., Waltham, MA, USA). The dry residue was solubilized with 100 μL of acetonitrile, and applied to HPLC under the condition described in the section "HPLC analysis of sesamin, diclofenac, and their metabolites."

Sesamin plasma concentration was determined by HPLC analysis with fluorescent detection (excitation 298 nm/emission 325 nm). Ethylacetate (2 mL) containing 10 mmol/L NaH_2PO_4 (pH 3.0) and 0.5 μmol/L bisphenol-A (internal standard) was added to 200 μL of blood plasma sample. Sesamin in the each sample was extracted with the same methods as diclofenac, and then analyzed by HPLC under the conditions described in the section "HPLC analysis of sesamin, diclofenac, and their metabolites."

Determination of hepatic sesamin concentration in rats

The livers from rats administered sesamin were homogenized in three volumes of 20 mmol/L phosphate buffer containing 1.15% KCl. The homogenate was centrifuged at 600*g* for 15 min, and the sesamin concentration of supernatant was determined with the same method

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described in the section "Determination of blood plasma diclofenac or sesamin concentration in rats."

Western blot analysis of rat CYP2C

To determine the contents of CYP2C in the liver microsomes prepared from the rats which took diclofenac and sesamin, western blot analysis of CYP2C and Bip was performed. Bip, which is known to be one of housekeeping proteins localized in microsomes, was used as a control protein. Proteins were separated by SDS-PAGE, and electrically transferred onto a nitrocellulose membrane. The membranes were incubated with anti-human CYP2C9 antibody (1:2000) or anti-BiP antibody (1:2000) at room temperature for 14 h. Next, the membranes were reacted with secondary anti-rabbit IgG alkaline phosphatase-linked antibodies (1:4000) and visualized using a BCIP-NBT solution kit for alkaline phosphatase (Nacalai tesque, Inc., Kyoto, Japan). The relative content of CYP2C to Bip was estimated from the intensities of both protein bands using Image J software (Schneider et al. 2012).

HPLC analysis of sesamin, diclofenac, and their metabolites

Sesamin and its metabolites were analyzed by HPLC under the following conditions. Column, YMC-pack ODS-AM (4.6 × 300 mm) (YMC Co., Tokyo, Japan); UV detection, 280 nm for in vitro study or fluorescent detection, excitation 298 nm/emission 325 nm for in vivo study; flow late, 1.0 mL/min; column temperature, 40°C; linear gradients of 10–95% methanol aqueous solution per 30 min containing 0.05% trifluoroacetic acid (TFA) followed by 100% methanol containing 0.05% TFA for 5 min. Diclofenac and its metabolite were analyzed by HPLC under the same conditions as those in sesamin with UV detection at 280 nm.

Results

Comparison of mechanism-based inhibition of P450s by sesamin between human and rat liver microsomes

As shown in Figure 2, diclofenac was converted to two metabolites designated as M1 and M2 at the retention time of 28.2 (M1) and 28.7 (M2) min in rat liver microsomes, whereas one metabolite in human liver microsomes at the retention time of 28.2 min. To determine the chemical structures of the metabolites, each metabolite in the effluent from HPLC was collected and subjected to LC/MS analysis. Relative intensities (%) of major ion fragments of authentic standard of diclofenac,

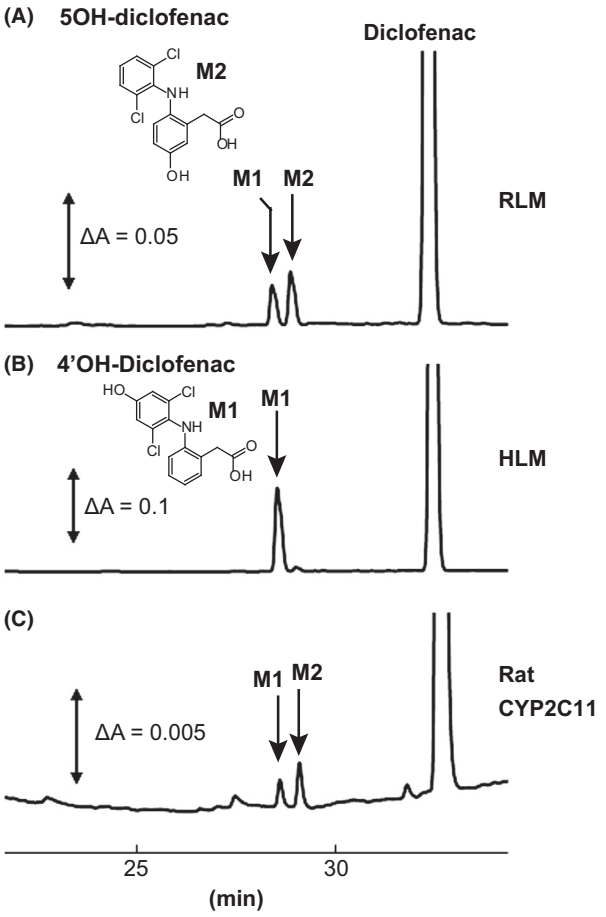

Figure 2. HPLC analysis of diclofenac and its metabolites produced in rat liver microsomes (A), human liver microsomes (B), and rat recombinant CYP2C11 (C). The metabolites M1 and M2 correspond to 4'- and 5-hydroxylated diclofenac, respectively.

and its metabolites were as follows. Diclofenac: m/z 250 (M-H-CO_2), 47%; m/z 294 (M-H), 100%. The metabolite M1: m/z 266 (M-H-CO_2) 15%; m/z 310 (M-H) 100%. The metabolite M2: m/z 266 (M-H-CO_2) 51%; m/z 310 (M-H) 100%. The metabolite in human liver microsomes showed nearly the same profile as M1. Previous studies demonstrated that diclofenac was converted to 4'OH-diclofenac in human liver microsomes by CYP2C9, and 4'-hydroxylated and 5-hydroxylated diclofenac in male rat liver microsomes by CYP2C11 based on the results that anti-rat CYP2C11 antibody inhibited both diclofenac 4'- and 5-hydroxylation activities in male rat liver microsomes (Masubuchi et al. 2001), These studies and our results suggest that M1 and M2 were 4'OH-diclofenac and 5-OH-diclofenac, respectively. Figure 2C shows HPLC profiles of diclofenac metabolites produced by rat recombinant CYP2C11, suggesting that both 4'-hydroxylated and 5-hydroxylated diclofenac were produced by

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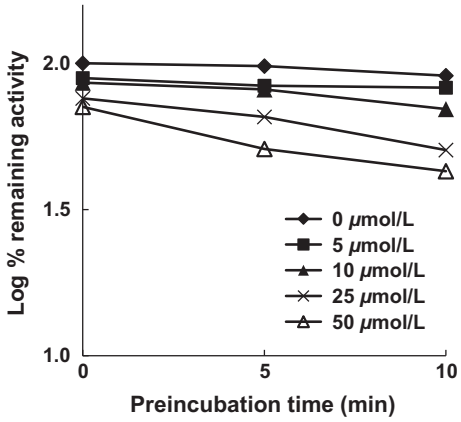

Figure 3. Time-dependent and sesamin concentration-dependent inactivation of CYP2C-mediated diclofenac hydroxylation activity in rat liver microsomes. The diclofenac hydroxylation activity was measured after preincubation with sesamin (0, 5, 10, 25, or 50 μmol/L) for 0, 5, or 10 min as described in the Materials and Methods section. The total of 4'- and 5-hydroxylation activity was plotted. Each point represents the mean of triplicate determinations.

mainly CYP2C11 in the rat liver microsomes. Both 4'- and 5-hydroxylation activities toward diclofenac in rat liver microsomes were time- and concentration-dependently inhibited by sesamin (Fig. 3). These results suggest that sesamin is an MBI inhibitor of CYP2C11 in rat liver microsomes, although contribution of other CYP2Cs could not be discarded. The $k_{inact}/K_{i\,app}$ value for rat liver microsomes was 0.0029 (Table 1).

Formation of MI complex of sesamin and CYP2C

Sesamin-induced difference spectra of rat or human liver microsomes in the presence of NADPH were measured. Figure 4 shows the difference spectra of rat or human liver microsomes after each period incubation in the presence of sesamin and NADPH at 37°C. After 7 min incubation, a remarkable peak was observed at 459 nm. Sesamin-induced difference spectrum of recombinant rat CYP2C11 in the presence of NADPH also showed a remarkable peak at 459 nm (Fig. 5). Figure 6A shows the time course of absorbance difference between 459 and 500 nm of rat liver microsomes (0.5 mg/mL), when it was incubated with sesamin (20 μmol/L) and NADPH (1 mmol/L) at 37°C. The absorbance difference increased up to 7 min incubation, but linearly decreased after 15 min. After 90 min incubation, no peak was detected at 459 nm (Fig. 6A). Figure 6C shows time courses of sesamin monocatechol (SC-1) and dicatechol (SC-2) formation. It is noted that almost all of sesamin was converted to SC-2 via SC-1 at 60 min. Thus, the peak at 459 nm completely disappeared within 30 min

Table 1. Comparison of mechanism-based inhibition parameters of sesamin between rat and human liver microsomes.

	Rat	Human	Rat/Human
k_{inact} (min^{-1})	0.079 ± 0.009	0.13 ± 0.01	0.61
$K_{i\ app}$ (μmol/L)	27.6 ± 3.4	22.4 ± 2.8	1.23
$k_{inact}/K_{i\,app}$	0.0029	0.0058	0.50

$K_{i\,app}$ and k_{inact} values represent mean ± SD from three separate experiments.

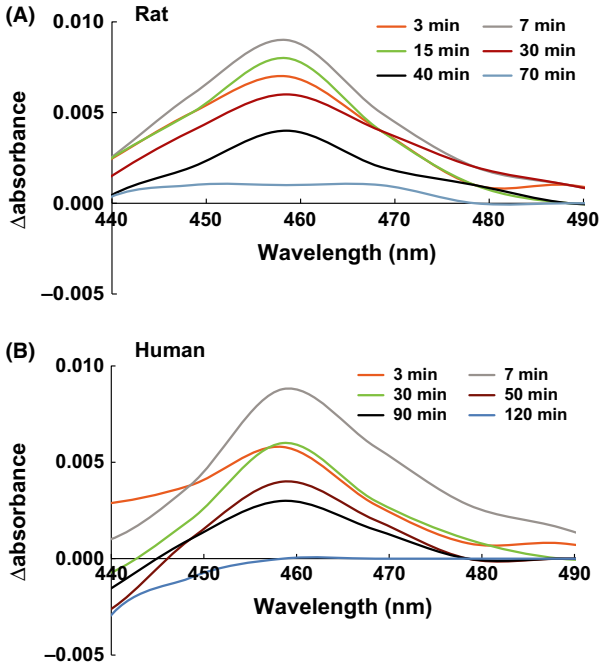

Figure 4. Difference spectra of rat (A) and human (B) liver microsomes (0.5 or 2 mg/mL) in the presence of NADPH (1 mmol/L) with and without sesamin (20 μmol/L) after incubation at 37°C for each period.

after the termination of the metabolism. As shown in Figure 6B, the peak at 459 nm rapidly decreased after 60 min, and almost disappeared after 90 min of incubation in the human liver microsomes. Sesamin was also converted into SC-2 via SC-1 in the human liver microsomes, although the reaction rate from SC-1 to SC-2 appeared to be significantly lower than that in the rat liver microsomes (Fig. 6D). As described in our previous report (Yasuda et al. 2012), SC1 also causes MBI, though SC1 is a weaker MBI inhibitor of CYP2C9 than sesamin, Thus, the MI complex concentration could be retained until all of the reactions are terminated. Almost no reaction occurs in rats and humans after 30 and 60 min, respectively (Fig. 6). The $\Delta A_{459\text{-}500}$ value is gradually decreased after the termination of the reaction. These results suggest the instability of the MI complex formed during sesamin metabolism in rat or human liver microsomes.

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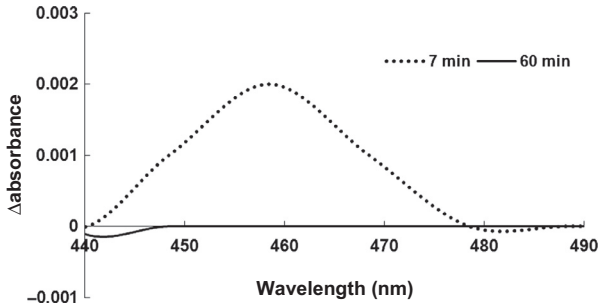

Figure 5. Difference spectra of recombinant rat CYP2C11 (50 pmol/mL) expressed in baculovirus-infected insect cells in the presence of NADPH (1 mmol/L) with and without sesamin (20 μmol/L) after incubation at 37°C for 7 min (broken line) or 60 min (solid line).

Recovery of the activity from sesamin-dependent MBI in rat and human liver microsomes

To examine the effects of the MBI, sesamin catecholization activity of rat or human liver microsomes was measured after sesamin metabolism for 60 min. No significant differences were observed between fresh and reused microsomes in the sesamin metabolism for 60 min, suggesting that CYP2C9 in human liver microsomes and CYP2C in rat liver microsomes were rapidly recovered from MBI. The half-life time of the recovery of CYP2C appears to be much shorter than that of CYP2D6 by MDMA (46 h) (O'Mathuna et al. 2008).

Pharmacokinetic interaction between sesamin and diclofenac

To examine the safety of sesamin, in vivo experiments using rats were performed. After oral administration of sesamin (0, 10, 100 mg/kg bw) for 3 days, 10 mg/kg bw dose of diclofenac was administered, and then the serum concentration of diclofenac were periodically measured (Fig. 7). The pharmacokinetic parameters of diclofenac, C_{max}, T_{max}, and $AUC_{0\text{-}24\,h}$ are summarized in Table 2. No significant differences were observed among three groups, though $AUC_{0\text{–}24\,h}$ value of rats taken 100 mg/kg bw dose of sesamin appears to be somewhat higher than those of the other groups.

On the other hand, sesamin (0, 10, 100 mg/kg bw) and 10 mg/kg bw dose of diclofenac were administered simul-

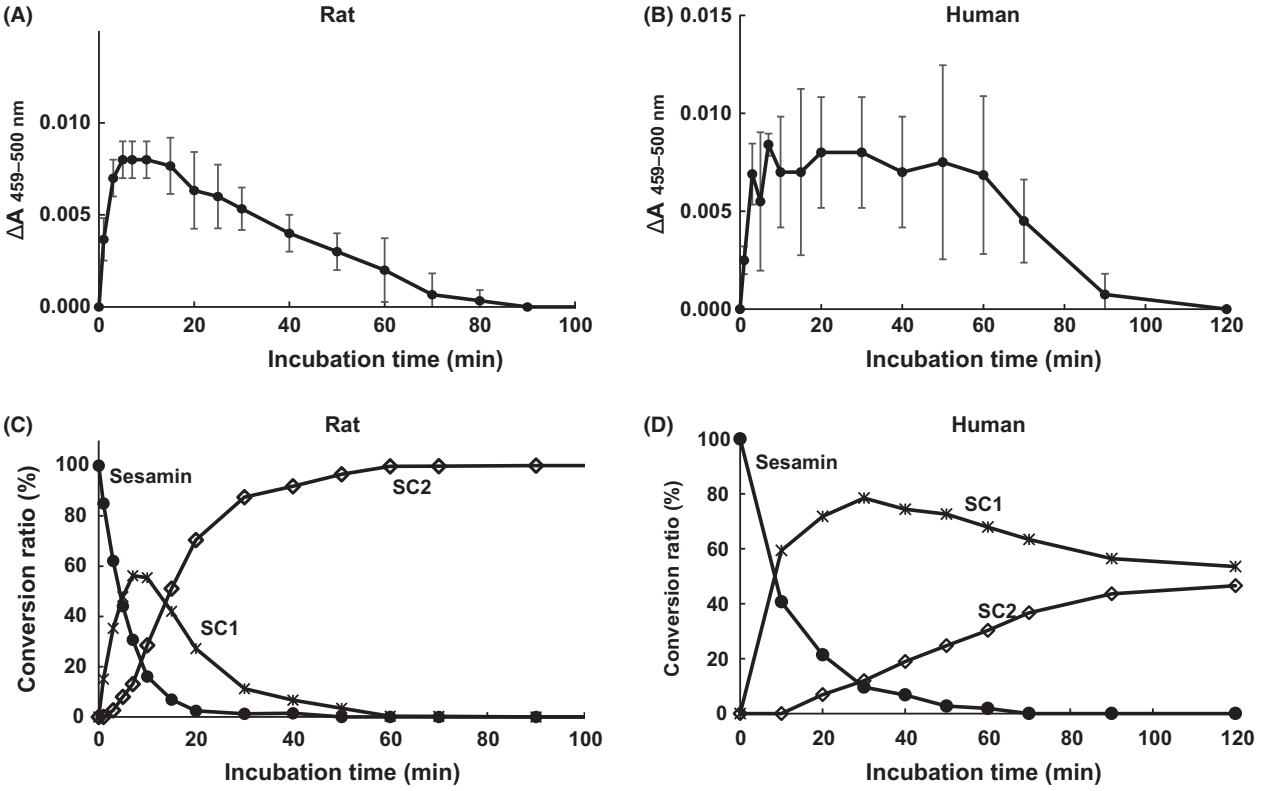

Figure 6. Time courses of absorbance differences between 459 and 500 nm of rat (A) or human (B) liver microsomes. Difference spectra in the range of 400 to 500 nm were recorded at 37°C at 0, 1, 3, 5, 7, 10, 15, 20, 30, 40, 50, 60, 70, 90, and 120 min. The relative ratio (%) of sesamin and its two metabolites SC-1 and SC-2 in rat (C) or human (D) liver microsomes were analyzed under the same conditions with the spectral analysis. Each point represents the mean ± SD of at least duplicate determinations.

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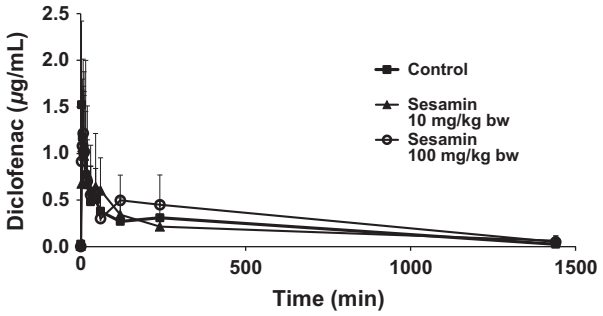

Figure 7. Time courses of serum concentration of diclofenac in rats after intragastric administration of 10 mg/kg bw diclofenac. Rats were preadministered with 0, 10, or 100 mg/kg bw of sesamin for 3 days. Each point represents the mean ± SD (n = 13 [control], n = 5 [10 mg/kg bw], and n = 10 [100 mg/kg bw]).

Table 2. Pharmacokinetic parameters of diclofenac with intragastric administration of 10 mg/kg bw diclofenac after taking 0, 10, and 100 mg/kg bw of sesamin for 3 days.

	T_{max} (min)	C_{max} (μg/mL)	$t_{1/2}$ (h)	$AUC_{0-24\,h}$ (μg min/mL)
Control	5.4 ± 3.2	1.8 ± 0.8	10.6 ± 2.9	293 ± 147
Sesamin, 10 mg/kg	7.0 ± 4.8	1.2 ± 0.5	14.8 ± 9.3	277 ± 93
Sesamin 100 mg/kg	7.2 ± 2.0	1.3 ± 0.6	11.1 ± 6.8	416 ± 221

Values are mean ± SD (n = 13 [control], n = 5 [10 mg/kg bw], and n = 10 [100 mg/kg bw]).

taneously, and then the serum concentration of diclofenac was measured periodically. As summarized in Table 3, no significant effects of sesamin were observed on the diclofenac pharmacokinetics. Furthermore, we examined the blood and hepatic sesamin concentration of rats given sesamin. The plasma concentration of sesamin in rats given 100 mg/kg bw of sesamin were periodically measured (Fig. 8). The C_{max} value of sesamin was 0.033 ± 0.020 μg/mL (94 ± 56 nmol/L), and T_{max} value was 1.2 ± 0.6 h. In contrast, the hepatic sesamin concentration at 1 h after sesamin administration was 0.39 μg/g tissue (1.1 μmol/L).

CYP2C expression and activity in the liver of rats taken sesamin

To examine the effects of sesamin administration on the expression of CYP2C, liver microsomes were prepared from the rats taken sesamin for 7 days, and the contents of CYP2C were determined by western blot analysis (Fig. S1). Diclofenac hydroxylation activity was also compared among the three groups. As shown in Figure 9, no significant differences were observed in both expression of CYP2C protein and diclofenac hydroxylation activity among the three groups (Fig. 9).

Table 3. Pharmacokinetic parameters of diclofenac with intragastric administration of 10 mg/kg bw diclofenac and 0, 10, and 100 mg/kg bw of sesamin at same time.

	T_{max} (min)	C_{max} (μg/mL)	$t_{1/2}$ (h)	$AUC_{0-24\,h}$ (μg min/mL)
Control	5.6 ± 2.9	2.1 ± 1.7	9.8 ± 5.1	329 ± 196
Sesamin, 10 mg/kg	5.4 ± 3.4	3.5 ± 2.3	11.8 ± 6.8	543 ± 205
Sesamin, 100 mg/kg	4.1 ± 1.9	2.6 ± 1.6	8.6 ± 3.9	385 ± 227

Values are mean ± SD (n = 8 [control], n = 7 [10 mg/kg bw], and n = 8 [100 mg/kg bw]).

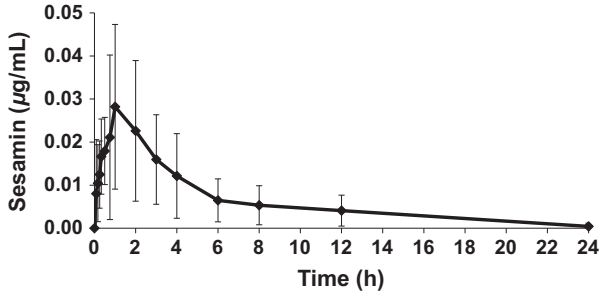

Figure 8. Time courses of plasma concentration of sesamin in rats after intragastric administration of 100 mg/kg bw sesamin. Each point represents the mean ± SD (n = 12).

Discussion

In our previous studies, we demonstrated that sesamin could cause MBI of human CYP2C9 using in vitro tests with human liver microsomes or recombinant human CYP2C9 (Yasuda et al. 2010). MBI of P450s is classified into two categories: (1) irreversible and (2) quasi-irreversible inhibition. Irreversible inhibition of P450s is usually caused by the alkylation of an apoprotein or a heme porphyrin ring of P450. In contrast, MBI by compounds containing an MDP group like sesamin is classified as quasi-irreversible inhibition, and forms an MI complex with a coordinate covalent bond between a carbene and a heme iron of P450 (Fig. 1). Quasi-irreversible inhibition could be serious when the MI complex has a long half-life time. Thus, the stability of the MI complex would be the most important factor for evaluating the seriousness of MBI, although the MBI kinetic parameters $K_{i\,app}$ and k_{inact} are also important. The stability of the MDP-P450 MI complex depends on

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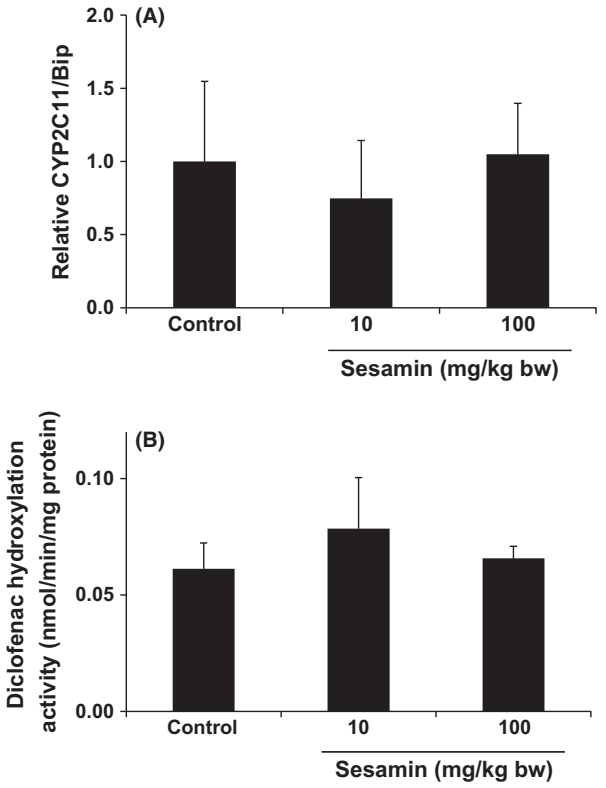

Figure 9. Relative CYP2C contents (A) and diclofenac hydroxylation activity (B) in liver microsomes prepared from rats administered with 0, 10, and 100 mg/kg bw of sesamin for 7 days. Bip localized on E.R. membrane was used as a control protein to estimate relative contents of CYP2C (Fig. S1). Each column represents the mean ± SD of triplicate determinations.

the chemical structures of MDP compounds and P450 species. For example, the stability of P450 MI complexes with 4-*n*-alkoxymethylenedioxybenzene compounds depends on their chain length (Murray et al. 1983, 1985). The MI complex of CYP2D6 and MDMA is known to cause serious MBI. The $AUC_{0\text{-}8\ h}$ of dextromethorphan, which is metabolized by CYP2D6, is increased approximately 10-fold when dextromethorphan is administered with MDMA in humans (O'Mathuna et al. 2008). Spectral difference scanning of yeast microsomes containing human recombinant CYP2D6 with MDMA in the presence of NADPH showed a time-dependent increase for 30 min (Heydari et al. 2004).

To evaluate the stability of the MI complex of sesamin and human CYP2C9 or rat CYP2C, difference spectrum analysis was performed during the incubation of rat or human liver microsomes and sesamin. As shown in Figure 6, the half-life time of the MI complex of sesamin in rat and human liver microsomes were estimated to be 10 to 20 min, respectively (Fig. 6). These results suggest that the MI complex of sesamin and human CYP2C9 or rat CYP2C was less stable than the MDMA-CYP2D6 MI complex (Fig. 10). The MBI kinetic parameters k_{inact} and $K_{i\,app}$ determined for CYP2D6 with MDMA in human liver microsomes were 0.12–0.26 min^{-1} and 8.8–45 μmol/L (Heydari et al. 2004), respectively, while k_{inact} and $K_{i\,app}$ determined for CYP2C9 with sesamin in human liver microsomes were 0.13 min^{-1} and 22.0 μmol/L, respectively (Yasuda et al. 2010). Thus, the $k_{inact}/K_{i\,app}$ value is similar for both MBI.

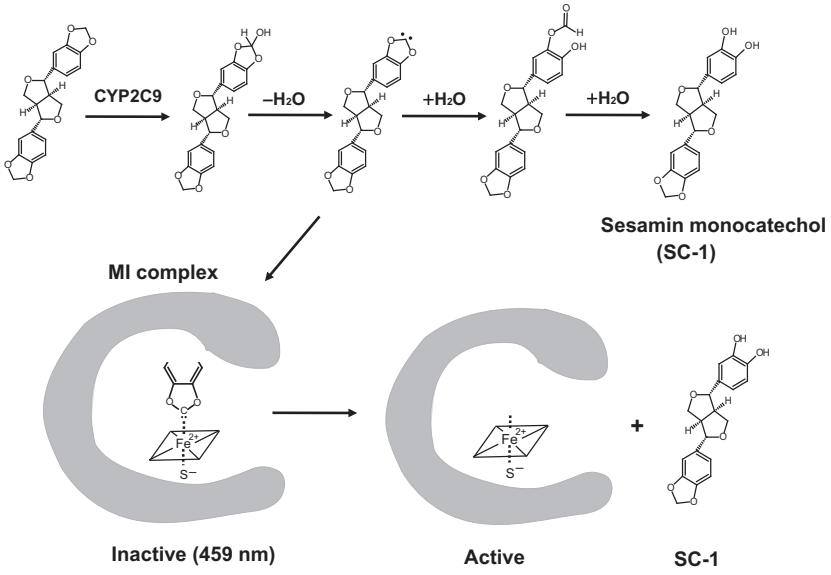

Figure 10. Proposed mechanism of MI complex formation between sesamin and rat CYP2C or human CYP2C9 and its dissociation with recovery of the enzyme activity. MI, metabolic–intermediate.

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In addition to the stability of the MI complex and the MBI kinetic parameters, the physiological concentration of sesamin is quite important for MBI evaluation in vivo. Tomimori et al. (2013) reported that the C_{max} of sesamin and episesamin in humans were 7.6 and 54 nmol/L, respectively, for a 50 mg/day sesamin supplement containing an equal amount of sesamin and episesamin. Assuming an absorption rate constant of 0.5 h^{-1} for sesamin and 1.5 h^{-1} for episesamin, which is roughly estimated from the data by Tomimori et al. (2013), and assuming a liver blood flow of 90 L/h and a fraction absorbed of 30% (Moazzami et al. 2007), the maximum inlet concentration of sesamin and episesamin to the liver following the 50 mg oral dose were estimated to be 130 and 410 nmol/L, respectively, which were estimated using the following equation (eqn. 1).

$$I_{in,\,max} = I_{max} + k_a \cdot D{\cdot}F_a/Q_H, \qquad (1)$$

I_{in}: the maximum concentration blood flowing into the liver;

I_{max}: the maximum concentration of the inhibitor in the circulation;

k_a: the absorption rate constant;

D: dose;

F_a: the fraction absorbed from the gastrointestinal tract into the portal vein;

Q_H: the hepatic blood flow rate.

Although the maximum inlet concentration of episesamin was higher than that of sesamin, the effect of episesamin could be smaller than that of sesamin, because our previous study demonstrated that the $k_{inact}/K_{i\,app}$ for CYP2C9 by episesamin is approximately one-fifth that of sesamin (Yasuda et al. 2012). It is noted that the values of 130 and 410 nmol/L for sesamin and episesamin are much lower than their $K_{i\,app}$ values.

In this study, we examined the effect of sesamin on diclofenac metabolism using rats at doses of 10 and 100 mg sesamin/kg bw. The hepatic sesamin concentration in the rats administered 100 mg/kg bw of sesamin was 1.1 μmol/L at 1 h after administration of sesamin, and it is noted that this value is much lower than the $K_{i\,app}$ value (27.6 μmol/L).

On the basis of low hepatic sesamin concentration and instability of the MI complex, we expected that sesamin hardly affects the drug metabolism in rats. As we expected, no significant differences were observed among the three groups (0, 10, and 100 mg sesamin/kg bw) in the pharmacokinetic parameters C_{max}, T_{max}, and AUC on diclofenac metabolism. In addition, no remarkable change was observed in the expression of CYP2C protein or CYP2C-mediated diclofenac hydroxylation activity in rat liver microsomes after administration of sesamin for 7 days (Fig. 9). As reported by Masubuchi et al. (2001), the major enzyme responsible for the diclofenac metabolism in male rats would be CYP2C11, but the other CYP2Cs such as CYP2C6, 2C7, 2C13, 2C22, and 2C23 might be also involved in the diclofenac metabolism.

In this study, we administered 50- or 500-fold higher doses of sesamin to the rats compared with daily intake of sesamin in humans (approximately 10 mg/day). Based on the low dose and instability of the MI complex, the usual intake of sesamin would give no serious effects on CYP2C9-dependent drug metabolism in humans, although further analysis would be needed to examine the possibility of MBI by metabolites with the MDP group such as sesamin monocatechol methylate. As mentioned earlier, commercially available sesamin supplements contains equal amounts of sesamin and episesamin. Our previous studies revealed that the k_{inact}/K_i value of episesamin for recombinant human CYP2C9 was five times smaller than that of sesamin. Recently, Tomimori et al. (2013) examined the repeated administration of sesamin supplement containing equal amounts of sesamin and episesamin at a dose of 50 mg/day for 28 days in humans, and did not detect the accumulation of sesamin and episesamin. Our results could be useful for safety evaluation regarding the use of sesamin supplements with diclofenac, although further analyses on the drugs other than diclofenac are required.

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Disclosures

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Western blot analysis of rat CYP2C in liver microsomes prepared from rats administered with 0, 10, and 100 mg/kg bw of sesamin for 7 days.

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