Comparison of Metabolism of Sesamin and Episesamin by Drug-Metabolizing Enzymes in Human Liver

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Received March 27, 2012; accepted June 29, 2012

ABSTRACT:

Sesamin and episesamin are two epimeric lignans that are found in refined sesame oil. Commercially available sesamin supplements contain both sesamin and episesamin at an approximate 1:1 ratio. Our previous study clarified the sequential metabolism of sesamin by cytochrome P450 (P450) and UDP-glucuronosyltransferase in human liver. In addition, we revealed that sesamin caused a mechanism-based inhibition (MBI) of CYP2C9, the P450 enzyme responsible for sesamin monocatecholization. In the present study, we compared the metabolism and the MBI of episesamin with those of sesamin. Episesamin was first metabolized to the two epimers of monocatechol, S- and R-monocatechols in human liver microsomes. The P450 enzymes responsible for S- and R-monocatechol formation were CYP2C9 and CYP1A2, respectively. The contribution of CYP2C9 was much larger than that of CYP1A2 in sesamin metabolism, whereas the contribution of CYP2C9 was almost equal to that of CYP1A2 in episesamin metabolism. Docking of episesamin to the active site of CYP1A2 explained the stereoselectivity in CYP1A2-dependent episesamin monocatecholization. Similar to sesamin, the episesamin S- and R-monocatechols were further metabolized to dicatechol, glucuronide, and methylate metabolites in human liver; however, the contribution of each reaction was significantly different between sesamin and episesamin. The liver microsomes from CYP2C9 ultra-rapid metabolizers showed a significant amount of episesamin dicatehol. In this study, we have revealed significantly different metabolism by P450, UDP-glucuronosyltransferase, and catechol-O-methyltransferase for sesamin and episesamin, resulting in different biological effects.

Introduction

Sesamin and episesamin are major lignans in refined sesame oil, and they are epimers of each other. Episesamin is rarely found in unrefined sesame oil but is generated from sesamin in equivalent amounts during acid-clay bleaching of the oil (Li et al., 2005); therefore, commercially available sesamin supplements contain both sesamin and episesamin at a ratio of approximately 1:1. Thus, effects of sesamin supplements depend on both sesamin and episesamin, which are known to have biological effects such as antioxidant effect (Ikeda et al., 2003; Nakai et al., 2003), anticarcinogenic effects (Hirose et al., 1992; Miyahara et al., 2000), and suppression of hypertension (Miyawaki et al., 2009).

The biological effects of sesamin and episesamin were compared previously (Kushiro et al., 2002; Ide et al., 2009). Kushiro et al. (2002) demonstrated that episesamin was much stronger at increasing the activity and gene expression of the enzymes involved in fatty acid oxidation in the liver of rats fed 0.2% of sesamin or episesamin for 15 days. Furthermore, Ide et al. (2009) compared the impact of sesame lignans containing sesamin and episesamin on hepatic gene expression of rats by using DNA microarray. They demonstrated that the changes in the gene expression were generally greater with episesamin than with sesamin and speculated that the difference between episesamin and sesamin is caused by the differences in their rates of metabolism. The concentration of episesamin in each tissue and serum in rats taking sesame lignans (a mixture of almost the same amount of sesamin and episesamin) was higher than that of sesamin, whereas no significant difference existed in lymphatic absorption between sesamin and episesamin (Umeda-Sawada et al., 1999). From a previous study (Shimizu et al., 1991), it had been proven that not only the metabolic rates but also the inhibitory effects for Δ5 desaturase in rats are different between sesamin and episesamin. They demonstrated that sesamin has a stronger inhibitory effect than episesamin for Δ5 desaturase using rat liver microsomes. According to these findings, it is possible that the biological effects of episesamin are significantly different from those of sesamin in humans as well as rats.

Several reports on the metabolism of sesamin have been published previously (Nakai et al., 2003; Peñalvo et al., 2005; Liu et al., 2006; Moazzami et al., 2007). Nakai et al. (2003) demonstrated that sesamin

ABBREVIATIONS: P450, cytochrome P450; UGT, UDP-glucuronosyltransferase; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; DMSO, dimethyl sulfoxide; COMT, catechol-O-methyltransferase; SAM, (S)-adenosylmethionine; AZT, 3'-azido-3'-deoxythymidine; MBI, mechanism-based inhibition; GlcUA, glucuronic acid.
was firstly metabolized to monocatechol and dicatechol metabolites in rat liver. They assumed that some cytochromes P450 (P450s) were involved in sesamin catecholization, because methylenedioxyphenyl compounds were demethylated by P450-dependent oxidation (Kumagai et al., 1991; Murray, 2000). Furthermore, Tomimori et al. (2012) demonstrated that episesamin was metabolized to two epimers of monocatechol and one dicatechol by P450s in human and rat liver microsomes. Thus, P450s would be essential for the metabolism of both sesamin and episesamin. We demonstrated previously that sesamin was catecholized in human liver microsomes predominately by CYP2C9, and sesamin was a mechanism-based inhibitor of CYP2C9 (Yasuda et al., 2010). Furthermore, we also demonstrated the species-specific differences in sesamin metabolism between humans and rats (Yasuda et al., 2011). In this article, we reveal P450 species responsible for episesamin metabolism and compared the sequential metabolism of episesamin by drug-metabolizing enzymes with that of sesamin in human and rat liver.

Materials and Methods

Materials. Sesamin was purchased from Sigma-Aldrich (St. Louis, MO), and episesamin was purchased from Nagara Science Co., Ltd. (Gifu, Japan). NADPH was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). Human single-donor liver microsomes (HG43, HH47, HH18, HH74, HH77, HG95, HH175, HH581, HG3, and HH741), a 50-donor human liver microsomes pool, human liver cytosol pool, male Sprague-Dawley male rat liver microsomes and cytosol, and recombinant human UDP-glucuronosyltransferases (UGT5; UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, and UGT2B17) expressed in Saccharomyces cerevisiae AH22 cells expressing human P450s (CYP2C9, CYP1A2, CYP2C19, CYP3A4, CYP2E1, and CYP2D6) with the vector pGyr were kindly provided by Sumitomo Chemical Co., Ltd. (Osaka, Japan). Polyclonal antibodies against each of the human P450s (CYP1A2 and CYP2C9) were purchased from Nihon Nosan Kogyo (Yokohama, Japan). Sesamin monocatechol, episesamin S- and R-monocatechols, sesamin dicatechol, and episesamin dicatechol were kindly provided by Suntory Wellness Ltd. (Tokyo, Japan) (Urata et al., 2008; Tomimori et al., 2012). All other chemicals were purchased from standard commercial sources and were of the highest available quality.

Metabolism of Episesamin, Phenacetin, and Diclofenac in Human Liver Microsomes. To measure enzyme activities and determine kinetic parameters, time and protein linearity studies were performed initially. The reaction mixture (200 μL) containing 0.5 mg protein/ml of human liver microsomes, 1 mM NADPH, and 5 μM episesamin or phenacetin or diclofenac in 100 mM potassium-phosphate buffer (pH 7.4) was incubated at 37°C for 15 min. The reaction mixture was extracted with 4 volumes of chloroform/methanol (3:1, v/v). The organic phase was recovered and dried in a vacuum evaporator centrifuge (Sakuma Seisakusyo, Tokyo, Japan). The resultant residue was solubilized with methanol and applied to high-performance liquid chromatography (HPLC) under the following conditions for episesamin: column, YMC-pack ODS-AM (4.6 × 300 mm) (YMC Co., Tokyo, Japan); UV detection, 280 nm; flow rate, 1.0 mL/min; column temperature, 40°C; and linear gradients of 10 to 90% methanol aqueous solution per 30 min and 90% to 100% per 5 min. For diclofenac and phenacetin, we used the same methods as those for episesamin except for the following conditions: UV detection, 267 nm; linear gradients of 20 to 95% acetonitrile aqueous solution per 25 min, containing 0.05% of trifluoroacetate acid as a buffer for diclofenac and UV detection, 245 nm; and linear gradients of 10 to 35% methanol aqueous solution per 15 min, containing 0.05% of TFA as a buffer for phenacetin.

Episesamin monocatechol had two possible epimers, R-catechol and S-catechol, as shown in Fig. 1. Thus, the metabolites, which were recovered from the HPLC conditions as described above, were further analyzed by HPLC using a chiral β-cyclodextrin column under the following conditions: column, SUMICHIRAL OA-7000 (4.6 × 250 mm) (Sumika Chemical Analysis Service, Ltd., Tokyo, Japan); UV detection, 280 nm; flow rate, 0.7 mL/min; column temperature, 25°C; mobile phase, methanol/water (75:25, v/v) containing 0.01% TFA.

Liquid Chromatography/Mass Spectrometric Analysis of a Metabolite of Episesamin. The metabolite of episesamin produced in the human liver microsomes was isolated by HPLC and subjected to mass spectrometric analysis, using a Finnigan LCQ Advantage Max (Thermo Fisher Scientific, Waltham, MA) with atmospheric pressure chemical ionization, positive mode. The conditions of liquid chromatography were as follows: reverse-phase ODS column (2 × 150 mm, Develosil ODS-HG-3; Nomura Chemical Co. Ltd., Aichi, Japan); mobile phase, acetonitrile/methanol/water, 3:4:3; flow rate, 0.2 mL/min; and UV detection, 280 nm.

Kinetic Analysis of Episesamin Catecholization Using Human Liver Microsomes or Microsomal Fraction of Recombinant Yeast Cells. The reaction mixture (200 μL) contained 0.5 mg protein/ml of human liver microsomes or recombinant yeast microsomes containing CYP2C9, CYP1A2, or CYP2C19, 1 mM NADPH, and various concentrations of episesamin (2, 5, 10, 25, 50, and 75 μM) in 100 mM potassium-phosphate buffer (pH 7.4). The reaction was started by the addition of 1 mM NADPH and continued at 37°C for 15 min. The reaction mixture also contained 0.5% dimethyl sulfoxide (DMSO), because 1 μL of sesamin DMSO solution was added as the substrate. As a control, the same reaction was performed using the microsomes prepared from AH22 cells harboring the control vector.

An HPLC calibration curve of authentic standards of sesamin, episesamin, and their catechols was used to determine their concentrations. In contrast, we have no authentic standards of monocatechol-glucuronide and monocatechol-methylated metabolites. We assumed that UV absorption at approximately 280
nm of monocatechol-glucuronide and monocatechol-methylated metabolites is not different from those of corresponding monocatechols. Kinetic parameters, \(k_{\text{inact}}\) and \(k_{\text{act}}\), were calculated by the nonlinear regression analysis using KaleidaGraph (Synergy Software, Reading, PA). The equation was applied for Michaelis–Menten kinetics.

Inhibition of Episesamin Catecholization in Human Liver Microsomes by Polyonal Antibodies against Human P450s. The pooled human liver microsomes (50 \(\mu\)g of protein) and polyclonal antibodies against human CYP2C9 or CYP1A2 (0–200 \(\mu\)g of protein) were incubated at 4°C for 1 h, and the reaction mixture containing 1 mM NADPH and 5 \(\mu\)M episesamin in 100 mM potassium-phosphate buffer (pH 7.4) was added at a final volume of 200 \(\mu\)l and then incubated at 37°C for 15 min. The metabolite was analyzed as described under Metabolism of Episesamin, Phenacetin, and Diclofenac in Human Liver Microsomes.

Inhibition of Episesamin Catecholization in Human Liver Microsomes by P450 Enzyme-Specific Inhibitors. The reaction mixture (100 \(\mu\)l) containing 0.5 mg protein/ml of pooled human liver microsomes and 1 to 5 \(\mu\)M sulfaphenazole (CYP2C9-specific inhibitor) or (+)-\(R\)-3-benzylirinanol (CYP2C19-specific inhibitor) in 100 mM potassium-phosphate buffer (pH 7.4) were preincubated at 37°C for 5 min. After the preincubation, 5 \(\mu\)M episesamin and 1 mM NADPH were added, and incubation was continued for 15 min. The metabolite was analyzed as described under Metabolism of Episesamin, Phenacetin, and Diclofenac in Human Liver Microsomes.

Mechanism-Based Inhibition of P450. The reaction mixture containing 0.5 mg protein/ml of recombinant yeast microsomes, 1 mM NADPH, and various concentrations of episesamin (0–50 \(\mu\)M) in 100 mM potassium-phosphate buffer (pH 7.4) was preincubated at 37°C for 5, 10, and 30 min. After the preincubation, substrates for each P450-specific activity were added at the final concentration of 100 \(\mu\)M, and incubation was continued for 15 min under the same conditions. Their metabolites were analyzed as described under Metabolism of Episesamin, Phenacetin, and Diclofenac in Human Liver Microsomes. Kinetic parameters of the inactivation process were calculated according to the method of Waley (1980, 1985). The observed rate constant of inactivation \(k_{\text{obs}}\) was calculated from the initial slopes of the linear regression line of the “residual activity” versus “preincubation time” profile plotted on a semilogarithmic scale. The inactivation rate constant at infinite concentration of inhibitor \(k_{\text{mact}}\) and the apparent inhibitor constants \(K_i\) were calculated from the double-reciprocal plots of \(k_{\text{obs}}\) versus episesamin concentration, according to the following equation (eq. 1), where \([I]\) indicates the concentration of inhibitor, episesamin:

\[
k_{\text{mact}} = \frac{k_{\text{mact}}[I]}{[I] + K_i}
\]

Preparation of Each of Two Epimers of Episesamin Monocatehol. The mixture of 0.25 mg/ml rat liver microsomal fraction, 1 mM NADPH, and 30 \(\mu\)M of episesamin in 100 mM phosphate buffer was incubated for 10 min at 37°C. The metabolites were extracted and isolated by HPLC using an ODS column for the isolation of the mixture of M1 and M2 and chiral cyclodextrin column for the separation of M1 and M2 from the mixture. Details of the methods were described under Metabolism of Episesamin, Phenacetin, and Diclofenac in Human Liver Microsomes. Separated M1 and M2 were recovered, dried, and then dissolved in DMSO to be used as substrates as described in the following sections.

Metabolism of Episesamin Monocatehol by P450 or UGT or Catechol-O-methyltransferase in the Liver Microsomes or Liver Cytosol. Each of P450-dependent catecholization, UGT-dependent glucuronidation, and catechol-O-methyltransferase (COMT)-dependent methylation of M1 and M2 were analyzed by the same methods described in our previous study (Yasuda et al., 2011). In brief, the conditions of each reaction were described as follows. In P450-dependent catecholization, the reaction mixture containing 0.5 mg protein/ml of the human liver microsomes or 0.25 mg protein/ml of the rat liver microsomes, 1 to 30 \(\mu\)M episesamin monocatehol, 1 mM NADPH in 100 mM potassium-phosphate buffer (pH 7.4) was incubated for 10 to 30 min at 37°C. In UGT-dependent glucuronidation, the reaction mixture containing 0.5 mg protein/ml of the human or rat liver cytosol, 1 to 30 \(\mu\)M episesamin monocatehol, 200 \(\mu\)M (S)-adenosylmethionine (SAM), 2 mM MgCl\(_2\), and 1 mM dithiothreitol in 100 mM potassium-phosphate buffer (pH 7.4) was incubated for 10 to 30 min at 37°C. Each metabolite was analyzed by HPLC.

Metabolism of Episesamin Monocatehol by Recombinant Human UGTs. The reaction mixture containing 0.1 mg protein/ml of the recombinant human UGT (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B15, or UGT2B17) microsomes, 1 to 30 \(\mu\)M episesamin monocatehol, 2 mM UDP-GlcUA, and 1 mM MgCl\(_2\) in 100 mM potassium-phosphate buffer (pH 7.4) was incubated for 10 to 30 min at 37°C. The metabolites were analyzed by HPLC as described under Metabolism of Episesamin, Phenacetin, and Diclofenac in Human Liver Microsomes.

Metabolism of 3′-Azido-3′-deoxythymidine in Human Liver Microsomes. The 3′-azido-3′-deoxythymidine (AZT) glucuronidation activity in pooled human liver microsomes was analyzed as described in our previous study (Yasuda et al., 2011). In brief, the reaction mixture containing 0.5 mg protein/ml of human liver microsomes, 1 mM AZT, 2 mM UDP-GlcUA, and 1 mM MgCl\(_2\) in 100 mM potassium-phosphate buffer (pH 7.4) was incubated for 15 min at 37°C. Each metabolite was analyzed by HPLC.

Substrate Docking. Docking was performed using Surflex Dock program in SYBYL 8.1.1 (Tripos, St. Louis, MO). The coordinate file 2H14.pdb was used for CYP1A2 as protein structure. The Surflex Dock program uses an empirical scoring function to rank putative protein-ligand interactions by flexible docking of small molecules to protein structures.

Results

Identification of the Metabolites of Episesamin. Figure 1A shows the HPLC profiles of sesamin (a) or episesamin (b) and their metabolites in human liver microsomes. As described previously (Yasuda et al., 2010), sesamin was converted to one metabolite: sesamin monocatechol. Meanwhile, episesamin was converted to two metabolites designated M1 and M2 (Fig. 1A, b). Further HPLC analysis using the chiral column clearly separated both metabolites (Fig. 1A, c). These results are consistent with those reported by Tomimori et al. (2012). Cochromatography of M1 and M2 with authentic standard of each epimer of episesamin monocatechol strongly suggests that M1 and M2 were S- and R-monocatechol, respectively (Fig. 1B). To confirm the chemical structures of the metabolites, we collected the metabolites containing M1 and M2 in the effluents from HPLC and subjected them to mass spectrometric analysis. Relative intensities (percentages) of major ion fragments of the authentic standard of episesamin and its metabolites were as follows: episesamin: \(m/z\) 319 (M+H–2H\(_2\)O, 6%); \(m/z\) 325 (M+H–HO, 100%); \(m/z\) 337 (M+H–HO, 42%); M1: \(m/z\) 307 (M+H–2H\(_2\)O, 10%); \(m/z\) 325 (M+H–HO, 100%); \(m/z\) 343 (M+H, 40%); M2: \(m/z\) 307 (M+H–2H\(_2\)O, 10%); \(m/z\) 325 (M+H–HO, 100%); and \(m/z\) 343 (M+H, 36%).

Episesamin Metabolism by Recombinant Human P450s. In our previous study (Yasuda et al., 2010), we revealed that some P450 enzyme had the sesamin catecholization activity, and their activity was high in order of CYP2C19, CYP2D6, CYP2C9, and CYP1A2, although CYP2C9 was the most important in human liver. In this study, we examined the episesamin catecholization by P450 enzymes including these P450s. Although no metabolites were observed in CYP2D6, CYP3A4, and CYP2E1 as well as the control (Supplemental Fig. 1A), the metabolites were detected in CYP1A2, CYP2C9, and CYP2C19 (Fig. 2). Each of the three P450 enzymes showed different metabolic patterns. CYP1A2 showed only one metabolite, M2 (Fig. 2, a), whereas the major metabolite of CYP2C9 was M1. The ratio of M1/M2 in CYP2C9 was approximately 5:1 (Fig. 2, b). On the other hand, the ratio of M1/M2 in CYP2C19 was approximately 1:1 (Fig. 2, c). Another polar metabolite designated M3, which was not observed in both CYP1A2 and CYP2C9, was observed in CYP2C19 (Fig. 2, c). Cochromatography of M3 with the authentic episesamin dicatechol strongly suggested that M3 was episesamin dicatechol. It is noted that
episesamin dicatechol was observed in the presence of a high amount of episesamin. The ratio of sesamin dicatechol/monocatechol in 20 μM sesamin was reduced to 8.6% of that in 2 μM sesamin, whereas the ratio of episesamin dicatechol in 20 μM episesamin was retained at 46% of that in 2 μM episesamin (Supplemental Fig. 2). These results suggest that the mechanism of dicatechol formation by CYP2C19 could be different between sesamin and episesamin. It is possible that, at least in part, episesamin is converted to dicatechol via monocatechol without a release of monocatechol from the substrate-binding pocket of CYP2C19.

**Correlation between Episesamin Catecholization Activity and Each P450-Specific Activity in Human Liver Microsomes.** Different metabolic patterns of episesamin by the three P450 enzymes in Fig. 2 suggest interindividual differences in episesamin metabolism. Comparison of metabolic patterns of episesamin among 10 human single-donor liver microsomes confirmed that this hypothesis is correct. Figure 3A shows HPLC profiles of episesamin metabolites by three donors, HH741, HH581, and HG43. The metabolite M1 is predominant in HH741, whereas M2 is predominant in HH581 and HG43. Only HG43 showed a remarkable peak of the metabolite M3 among 10 human single-donor liver microsomes.

Although M1 and M2 were detected in all donors, the ratios of M1/M2 were different among 10 donors, as shown in Fig. 3B. To determine the P450 enzymes responsible for M1 and M2 production, we examined the correlation between each epimer production and each P450-specific activity among 10 donors. As shown in Fig. 4A, a high correlation was observed between M1 production and diclofenac 4′-hydroxylation activity, which is known to be a CYP2C9-specific activity ($R^2 = 0.83, p < 0.01$). On the other hand, significant correlation was not observed between M2 production and diclofenac 4′-hydroxylation activity ($R^2 = 0.19, p = 0.2$), as shown in Fig. 4B. However, a high correlation was observed between M2 production and phenacetin O-deethylation activity, which is known to be a CYP1A2-specific activity ($R^2 = 0.52, p = 0.018$). HH581 has much higher CYP1A2-specific activity than any other donors. When HH581 was omitted from the 10 donors, a marginally significant correlation was observed ($R^2 = 0.39, p = 0.074$). However, no correlation was observed between M1 or M2 formation and (S)-mephenytoin 4′-hydroxylation activity, which is known to be the CYP2C19-specific activity. On the basis of these results, the P450 enzymes responsible for M1 and M2 production were considered to be CYP2C9 and CYP1A2, respectively.

A significantly large amount of episesamin dicatechol was produced in HG43 (Fig. 3A, c), whereas no metabolite was detected in the heat-inactivated HG43 microsomes (Supplemental Fig. 1B). The CYP2C19-specific activity of this donor was approximately 10 times higher than that of the other donors, although other P450 enzyme-specific activities were not so different. These results are consistent with those using recombinant CYP2C19, shown in Fig. 2, c, and suggest that dicatechol production should be considered in people who have high CYP2C19-dependent activity.

**Inhibition of Episesamin Catecholization in Human Liver Microsomes by Anti-P450 Antibody or CYP2C9-Specific Inhibitor.** To confirm that CYP2C9 and CYP1A2 are responsible for M1 and M2 formation, respectively, we examined the effects of antibodies against CYP1A2 and CYP2C9 on episesamin catecholization in the pooled human liver microsomes (Fig. 5). As expected, M1 production was significantly inhibited by anti-CYP2C9 antibody but was not inhibited by anti-CYP1A2 antibody.
Inhibited by anti-CYP1A2 at all (Fig. 5A). On the other hand, M2 production was significantly inhibited by anti-CYP1A2 antibody and was also inhibited by anti-CYP2C9 antibody (Fig. 5B). The activity was not inhibited by addition of control antibody. These results are consistent with those in Fig. 4B, suggesting a small contribution of CYP2C9 to M2 production. Figure 5C shows inhibitory effects of both antibodies on the total production in M1 and M2 and indicates that CYP2C9 is the most important P450 enzyme for production of episesamin monocatechol, but CYP2C19 may be essential for the dicatechol production.

Comparison of the Kinetic Parameters between Episesamin and Sesamin Monocatecholization in Recombinant Human CYP2C9 and CYP1A2. As described in the previous section, both CYP2C9 and CYP1A2 were important for the episesamin metabolism. On the other hand, for sesamin metabolism, the contribution of CYP2C9 to the monocatechol production was less significant compared to episesamin.

Inhibition of Episesamin Catecholization in Human Liver Microsomes by CYP2C9- or CYP2C19-Specific Inhibitor. To confirm that CYP2C9 and CYP1A2 are most essential P450 enzymes for episesamin catecholization, but CYP2C19 is not, we examined the effect of chemical inhibitors for human CYP2C9 (sulfaphenazole) (Miners et al., 1988) and CYP2C19 [(+)-N-3-benzylirvinanol] (Suzuki et al., 2002). Sulfaphenazole showed a significant inhibitory effect of episesamin monocatecholization activity in human liver microsomes, whereas (+)-N-3-benzylirvinanol did not; the activity in the presence of 5 μM sulfaphenazole was approximately 50% of that in the absence of inhibitor, whereas the activity did not change in the presence of 5 μM (+)-N-3-benzylirvinanol. These results strongly suggest only a small contribution of CYP2C19 for the episesamin monocatechol production in human liver microsomes, although CYP2C19 may be essential for the dicatechol production.

Comparison of the Kinetic Parameters between Episesamin and Sesamin Monocatecholization in Recombinant Human CYP2C9 and CYP1A2. As described in the previous section, both CYP2C9 and CYP1A2 were important for the episesamin metabolism. On the other hand, for sesamin metabolism, the contribution of CYP2C9 to the monocatechol production was less significant compared to episesamin.
CYP2C9 was much larger than that of CYP1A2 (Yasuda et al., 2010). Table 1 shows the comparison of kinetic parameters of CYP2C9 and CYP1A2 between sesamin and episesamin. The $k_{cat}$ value of CYP2C9 for episesamin was half that for sesamin, whereas the $K_m$ value was 3 times larger. On the other hand, the $k_{cat}/K_m$ value of CYP1A2 for episesamin was not so different from that for sesamin. Thus, episesamin appears to be a significantly poorer substrate of CYP2C9 than sesamin, whereas CYP1A2 equally catalyzes catecholization for both sesamin and episesamin.

**Docking of Episesamin into CYP1A2.** To interpret the stereoselective catecholization of episesamin by CYP1A2, we performed a docking study of episesamin. We obtained 47 diverse conformations of episesamin docked in CYP1A2, from which we selected nine conformations of episesamin whose acetyl moiety was located close enough to iron atom—less than 6 Å—and faced to the iron. All of these nine conformations have $(R)$-1,3-benzodioxole toward the heme iron (Fig. 6). $(S)$-1,3-Benzodioxole seems to be not accommodated into the cavity with proper conformation because it would cause steric crash with Ala317. On the other hand, Ala317 guides $(R)$-1,3-benzodioxole toward the heme iron atom so that $(R)$-1,3-benzodioxole is able to be oxidized. This result is consistent with the fact that the major metabolite of episesamin by CYP1A2 was $R$-monocatechol, as shown in Fig. 1.

**Mechanism-Based Inhibition of P450s.** Our previous study demonstrated that sesamin caused MBI of CYP2C9 (Yasuda et al., 2010), which was the most responsible enzyme for sesamin metabolism. As described above, for the metabolism of episesamin, both CYP2C9 and CYP1A2 play a key role almost equally. To examine MBI of each P450 enzyme by episesamin, we used the microsomal fraction of the recombinant yeast expressing each of CYP2C9, CYP1A2, and CYP3A4. Although CYP1A2 and CYP3A4 showed no MBI [Δ(log activity % of control) per min were almost the same between 0 and 50 μM episesamin], CYP2C9 showed time- and concentration-dependent MBI (Fig. 7). It is noted that these results were the same as those of sesamin. Table 2 shows a comparison of MBI parameters between sesamin and episesamin for the recombinant CYP2C9. The apparent $K_i$ and $k_{inact}$ values of episesamin in CYP2C9-dependent diclofenac 4′-hydroxylation were estimated to be 6.8 μM and 0.17 min$^{-1}$, respectively. The $k_{inact}/K_i$ value of episesamin was 18% of that of sesamin. This result demonstrated that episesamin was also a MBI of CYP2C9 as well as sesamin.

**Comparison of Sequential Metabolism of Sesamin and Episesamin in Rat or Human Liver Microsomes.** In our previous study, we clarified the metabolism of sesamin monocatechol, which is the first metabolite of sesamin, and found that oxidation by P450, glucuronidation by UGT, and methylation by COMT played essential roles for its metabolism in human liver. To examine whether episesamin monocatechol is metabolized like sesamin monocatechol, each epimer of episesamin monocatechol, M1 or M2, was metabolized in human liver microsomes in the presence of NADPH or UDP-GlcUA or in human liver cytosols in the presence of SAM. Each coenzyme-dependent
metabolites were detected (data not shown), and metabolic enzymes of episesamin monocatechol were similar to those of sesamin monocatechol (Yasuda et al., 2011). These results strongly suggest that oxidation, glucuronidation, and methylation of episesamin monocatechol would occur simultaneously in human liver.

To compare the sequential metabolism of sesamin and episesamin in human liver, we performed kinetic analysis of P450-dependent metabolism of episesamin and then P450-, UGT-, or COMT-dependent metabolism of episesamin monocatechol (Yasuda et al., 2011). These results strongly suggest that oxidation, glucuronidation, and methylation of episesamin monocatechol were similar to those of sesamin monocatecholization. Thus, episesamin was metabolized by P450 and UGT in human liver. Episesamin was metabolized to M1 and M2 at a ratio of 58% of that of sesamin monocatecholization. Thus, episesamin was a poorer substrate than sesamin in human liver (Table 3). Comparing the metabolism of sesamin monocatechol and episesamin monocatechol, the V_{max}/K_{m} values of glucuronidation reaction were significantly different, although those values of oxidation and methylation were not so different. It should be noted that glucuronidation by UGT was predominant over catecholization by P450 in the metabolism of sesamin monocatechol. Because P450 and UGT reactions occur in microsomes and the COMT reaction occurs in cytosol, we cannot compare the kinetic parameter of COMT with that of P450 or UGT. However, the metabolism of sesamin and episesamin was significantly different; UGT was the most important for the sesamin metabolism as the second-step reaction, whereas COMT would be the most important, and the contribution of UGT was not so large for the episesamin metabolism.

We further performed the same analysis using rat liver microsomes and cytosol. The V_{max}/K_{m} value of episesamin monocatecholization was 58% of that of sesamin monocatecholization. Thus, episesamin was a somewhat poorer substrate compared with sesamin in rat liver microsomes. Focused on the second-step metabolism, it is noted that the oxidation and glucuronidation activities toward M1 were significantly different from those toward M2 in rat microsomes. M1 was predominantly metabolized by P450 and UGT over M2 in rat liver, whereas M1 and M2 were equally metabolized by P450 and UGT in human liver. Episesamin was metabolized to M1 and M2 at a ratio of approximately 1:1 in rat liver microsomes (data not shown) similarly to human liver microsomes. On the basis of the relative V_{max}/K_{m} of each reaction in Table 3, metabolic profiles of sesamin and episesamin in human or rat liver are summarized in Fig. 8. These results demonstrated that the difference of the sequential metabolism between sesamin and episesamin in rat liver was smaller than that in human liver.

### Table 3: Comparison of kinetic parameters between episesamin and sesamin monocatecholization in human and rat liver microsomes

<table>
<thead>
<tr>
<th>Species</th>
<th>Substrate</th>
<th>Reaction</th>
<th>V_{max}/K_{m} (nmol/mg protein/min)</th>
<th>V_{max}/K_{m} (μM)</th>
<th>Relative V_{max}/K_{m}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Sesamin</td>
<td>Cat (P450)</td>
<td>0.81 ± 0.07</td>
<td>7.7 ± 1.7</td>
<td>0.11 ± 0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glu (UGT)</td>
<td>0.44 ± 0.09</td>
<td>18.8 ± 5.6</td>
<td>0.43 ± 0.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Met (COMT)</td>
<td>6.0 ± 0.9</td>
<td>18.0 ± 5.3</td>
<td>0.33 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Episesamin</td>
<td>Cat (P450)</td>
<td>0.40 ± 0.04</td>
<td>13.0 ± 3.6</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Episesamin S-monocatechol (M1)</td>
<td>Cat (P450)</td>
<td>0.28 ± 0.02</td>
<td>4.1 ± 1.1</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Episesamin R-monocatechol (M2)</td>
<td>Cat (P450)</td>
<td>0.15 ± 0.01</td>
<td>1.8 ± 0.8</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glu (UGT)</td>
<td>0.60 ± 0.04</td>
<td>8.6 ± 1.3</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Met (COMT)</td>
<td>0.71 ± 0.03</td>
<td>2.5 ± 0.3</td>
<td>0.28 ± 0.02</td>
</tr>
<tr>
<td>Rat</td>
<td>Sesamin</td>
<td>Cat (P450)</td>
<td>4.8 ± 0.2</td>
<td>7.1 ± 1.8</td>
<td>0.68 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glu (UGT)</td>
<td>3.7 ± 0.1</td>
<td>5.3 ± 1.4</td>
<td>0.70 ± 0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Met (COMT)</td>
<td>1.1 ± 0.2</td>
<td>20.3 ± 6.8</td>
<td>0.54 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Episesamin</td>
<td>Cat (P450)</td>
<td>6.3 ± 0.7</td>
<td>16.0 ± 3.9</td>
<td>0.39 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Episesamin S-monocatechol (M1)</td>
<td>Cat (P450)</td>
<td>1.9 ± 0.2</td>
<td>2.9 ± 0.7</td>
<td>0.66 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Episesamin R-monocatechol (M2)</td>
<td>Cat (P450)</td>
<td>4.4 ± 0.5</td>
<td>16.8 ± 3.6</td>
<td>0.26 ± 0.02</td>
</tr>
</tbody>
</table>

Cat, catecholization; Glu, glucuronidation; Met, methylation.
the most important UGT enzyme for the sesamin monocatechol was UGT2B7. To identify the UGT enzyme responsible for the episesamin metabolism, we examined the metabolism of episesamin monocatechol by recombinant human UGTs expressed in baculovirus-infected insect cells. Among the 12 UGT enzymes, only UGT2B7 showed significant glucuronidation activity. Further study demonstrated that the ratio of the two epimers was not so good between episesamin monocatechol glucuronidation activity and AZT glucuronidation activity, which is known to be UGT2B7-specific activity ($R^2 = 0.28$, $p = 0.17$) as shown in Supplemental Fig. 3. However, it is obvious that UGT2B7 plays an important role in the glucuronidation of episesamin monocatechol, as was the case of sesamin monocatechol.

**Discussion**

Sesamin and episesamin are epimers of each other, and commercially available sesamin supplements contain both epimers. There are some reports that when taken at the same amount, the body concentrations and the extent of the gene expression of the hepatic enzymes involved in fatty acid β oxidation were different between sesamin and episesamin in rats (Umeda-Sawada et al., 1999; Kushiro et al., 2002; Ide et al., 2009). Tomimori et al. (2012) reported that episesamin is metabolized to catechol metabolites by P450s such as sesamin. However, to the best of our knowledge, there are no reports that compare sesamin and episesamin metabolism in detail. In this study, we compared the sequential metabolism of episesamin with that of sesamin in human liver.

As shown in Fig. 6, the tertiary structures of sesamin and episesamin are quite different. Thus, we predicted that the enzymes responsible for the metabolism of each would be significantly different between sesamin and episesamin; unexpectedly, the major enzymes involved in the metabolism of episesamin in human liver microsomes were CYP2C9 and CYP1A2, as was the case for sesamin, yet the contribution ratio of CYP2C9 to CYP1A2 was quite different between the epimers. The contribution of CYP2C9 was much larger than that of CYP1A2 in sesamin metabolism, whereas the contribution of CYP2C9 was almost equal to that of CYP1A2 in episesamin metabolism. CYP1A2 and CYP2C9 produced different epimers of episesamin monocatechol, S- and R-monocatechols (Fig. 1B). CYP1A2 catalyzed R-monocatechol formation stereoselectively (Fig. 2, a), and the docking study accords with this result (Fig. 6). The ratio of the two epimers was significantly different among 10 individual single human liver microsomes, probably because of the different expression levels of CYP2C9 and CYP1A2 in each liver microsome.

The $k_{cat}/K_m$ value of the CYP2C9-dependent episesamin catecholization was 16% of that for sesamin, suggesting that episesamin is a significantly poorer substrate for CYP2C9 than sesamin. The fact that we have failed to perform the molecular docking of episesamin using the "auto-docking" method (data not shown) suggests that episesamin is an inferior substrate for CYP2C9. A clearly different binding mode of the substrate-binding pocket of CYP2C9 between episesamin and sesamin may affect both the $k_{cat}$ and $K_m$ values. However, the $k_{cat}/K_m$ value of the CYP1A2-dependent episesamin catecholization was almost the same as that of sesamin.

Because episesamin contains two methylenedioxyphenyl groups, it is likely that episesamin displays an MBI of CYP2C9, and episesamin, indeed, exhibited an MBI of CYP2C9. After the first monooxygenation by CYP2C9, a part of the metabolite could be spontaneously converted to form a reactive carbene compound that probably covalently binds to the heme iron of CYP2C9, as described in our previous report (Yasuda et al., 2010). The $k_{cat}/K_m$ value of episesamin for CYP2C9 and the $k_{cat}/K_m$ value of CYP2C9-dependent episesamin catecholization were 18 and 16% of those of sesamin, respectively. Furthermore, the $k_{cat}/K_m$ value of each monocatechol was approximately half that of sesamin and episesamin, respectively, and catecholization activity by CYP2C9 toward each monocatechol was also approximately half that of sesamin and episesamin, respectively (Supplemental Table 2). These results strongly suggest that the inactivation of CYP2C9 by carbene formation occurs proportionally to the CYP2C9-
dependent monoxygenation at the same ratio for sesamin, episesamin, and their monocatechols.

We further examined the metabolism of episesamin monocatechol and found that, as is the case for sesamin monocatechol, the P450-dependent oxidation, UGT-dependent glucuronidation, and COMT-dependent methylation were important. However, the contributions of each reaction for sesamin and episesamin were significantly different. Although the UGT-dependent glucuronidation was predominant in sesamin metabolism, the contribution of the UGT-dependent glucuronide was not so large in episesamin metabolism (Fig. 8). Similar to sesamin monocatechol, the UGT enzyme responsible for episesamin monocatechol glucuronidation was UGT2B7, but the episesamin monocatechol glucuronidation activity and the UGT2B7-specific activity were not well correlated. These results might suggest the contribution of the other UGT enzymes, which are not commercially available, such as UGT1A5, UGT2B10, UGT2B11, UGT2B28, UGT2A1, UGT2A2, and UGT2A3. As shown in Fig. 8, the contribution of COMT would become significant in episesamin metabolism. The P450-dependent oxidation of sesamin monocatechol and episesamin monocatechol to yield their dicatechols in human liver microsomes was much weaker than that in rat microsomes (Fig. 8). However, we obtained rather important results in the dicatechol production of episesamin (Figs. 2 and 3). HG43 is derived from a CYP2C19 ultra-rapid metabolizer who has a CYP2C19*17 allele. Sim et al. (2006) reported that some nuclear proteins specifically bind to the 5′-flanking region of this allele to induce gene transcription. Thus, the heterozygotes (*1/*17) would be ultra-rapid metabolizers (Wang et al., 2009). The frequency of the CYP2C19*17 allele is high (18%) in Ethiopians and Swedes (Sim et al., 2006), 0.64% in Chinese (Wang et al., 2009), and 1.3% in Japanese (Sugimoto et al., 2008). The antioxidant effects of episesamin in ultra-rapid metabolizers might be larger than those in normal metabolizers because of a high ability of dicatechol formation (Nakai et al., 2003; Miyake et al., 2005). However, CYP2C19 poor metabolizers, whose frequency is approximately 20% in Japanese and Koreans, appear to have a low ability to form episesamin dicatechol. It is notable that CYP2C19 might catalyze a two-step catecholization from episesamin to its dicatechol without a release of the first product episesamin monocatechol. However, such reaction was observed only in the CYP2C19-dependent episesamin metabolism and not in sesamin metabolism. Further studies are needed to reveal the substrate recognition and reaction mechanism of the CYP2C19-dependent metabolism of the conversion of episesamin to its dicatechol.

In our previous study, we revealed the species-based differences of sesamin metabolism between humans and rats. As shown in Fig. 8 and Table 3, the remarkable species-based differences between humans and rats were also observed in the episesamin metabolism. In rats, P450-dependent oxidation was predominant over UGT-dependent glucuronidation in the metabolism of both sesamin and episesamin. Comparing the monocatecholization between sesamin and episesamin in rat liver microsomes, the \( V_{\text{max}}/K_m \) value of episesamin monocatecholization was 58% of that of sesamin monocatecholization. Thus, episesamin was also a somewhat poorer substrate than sesamin in rat liver microsomes. In vivo rat studies have demonstrated the differences between sesamin and episesamin: the serum level of episesamin was higher than that of sesamin in rats (Umeda-Sawada et al., 1999), and episesamin was much stronger in increasing the activity and gene expression of the enzymes involved in fatty acid oxidation in rats taking almost the same amount of sesamin and episesamin (Kushiro et al., 2002; Ide et al., 2009). These results suggest that episesamin is more resistant to metabolism and that the resulting higher serum level of episesamin induces fatty acid oxidation. The difference of the \( V_{\text{max}}/K_m \) values of monocatecholization between sesamin and episesamin in this study appears to be consistent with the in vivo rat studies. It is notable that the \( V_{\text{max}}/K_m \) value of episesamin monocatecholization in human liver microsomes was 29% of that of sesamin, and this difference was greater than that in rat liver microsomes. Furthermore, the differences of monocatechol metabolism between sesamin and episesamin in rat liver were smaller than that in human liver. On the basis of the in vivo rat studies, the efficacy of episesamin might be stronger than sesamin in human liver.

In conclusion, we have revealed that sesamin and episesamin have significantly different metabolic patterns by P450, UGT, and COMT, and this difference might affect their biological effects in the human body. Commercially available sesamin supplements contain an equal amount of sesamin and episesamin. Although the separation of sesamin and episesamin would not be easy at the industrial level, we propose that they should be isolated from each other to maximize their usefulness.

Acknowledgments
We thank Suntory Wellness Ltd. (Tokyo, Japan) for providing authentic standards of mono- and dicatechols of sesamin and episesamin.

Authorship Contributions
Participated in research design: Yasuda, Ikushiro, Yamamoto, and Sakaki.
Conducted experiments: Yasuda and Ohta.
Contributed new reagents or analytic tools: Wakayama, Itoh, Kamakura, and Ohta.
Performed data analysis: Yasuda, Itoh, Munetsuna, and Ohta.
Wrote or contributed to the writing of the manuscript: Yasuda, Sakaki, and Sakaki.

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