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Selective inhibition of methoxyflavonoids on human CYP1B1 activity

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ABSTRACT

Cytochrome P450 (CYP) 1B1 catalyzes 17β-estradiol (E_2) to predominantly carcinogenic 4-hydroxy-E_2, whereas CYP1A1 and 1A2 convert E_2 to non-carcinogenic 2-hydroxy-E_2. Hence, selective inhibition of CYP1B1 is recognized to be beneficial for the prevention of E_2 related breast cancer. In this study, we first evaluated the structure–property relationship of 18 major flavonoids on inhibiting enzymatic activity of CYP1A1, 1A2 and 1B1 by using an ethoxyresorufin *O*-deethylation assay. Flavones and flavonols indicated relatively strong inhibitory effects on CYP1s compared with flavanone that does not have the double bond between C-positions 2 and 3 on the C-ring. Flavonoids used in this study selectively inhibited CYP1B1 activity. In particular, methoxy types of flavones and flavonols such as chrysoeriol and isorhamnetin showed strong and selective inhibition against CYP1B1. To understand why selective inhibition was observed, we carried out a molecular docking analysis of these methoxyflavonoids with the 2–3 double bond and CYP1s. The results suggested that chrysoeriol and isorhamnetin fit well into the active site of CYP1B1, but do not fit into the active site of CYP1A2 and 1A1 because of steric collisions between the methoxy substituent of these methoxyflavonoids and Ser-122 in CYP1A1 and Thr-124 in CYP1A2. In conclusion, our results demonstrate: (1) strong inhibitory effects of flavonoids on CYP1 activities require the 2–3 double bond on the C-ring; (2) methoxyflavonoids with the 2–3 double bond had strong and selective inhibition against CYP1B1, suggesting chemopreventive flavonoids for E_2 related breast cancer; and (3) binding specificity of these methoxyflavonoids is based on the interactions between the methoxy groups and specific CYP1s residues.

1. Introduction

The endogenous estrogen, 17β-estradiol (E_2), is recognized as a prime risk factor for the development of estrogen-related carcinogenesis, such as breast cancer.[1] The conceivable mechanisms that may play a part in the mutagenic effects of E_2 to the endogenous metabolites are shown in Figure 1. This pathway is promoted via the cytochrome P450 (CYP) families, especially CYP1A1 and 1B1, and catechol-*O*-methyltransferase (COMT). CYP1A1 exhibits catalytic activity on E_2, in which the C2-position of E_2 is oxidized and converted to 2-hydroxyestradiol (2-OHE_2).[2,3] 2-OHE_2 is rapidly methylated by COMT to 2-methoxyestradiol (2-$MeOE_2$). 2-$MeOE_2$ is known to be non-carcinogenic and have an inhibitory effect on cell proliferation.[4] Hence, CYP1A1 presents an important factor for detoxification of estrogen. Additionally, CYP1A1 is a key enzyme in the drug metabolizing pathway for the excretion of exogenous compounds absorbed into the body. In contrast, CYP1B1 catalyzes predominantly the C4-position of E_2 to 4-hydroxyestradiol (4-OHE_2). 4-OHE_2, but not 2-OHE_2, exerts a strong agonistic effect for the estrogen receptor (ER), and is therefore recognized to accelerate proliferation of estrogen-dependent cells.[5,6] Moreover, most of 4-OHE_2 is oxidized by peroxidase to produce estradiol-3,4-quinone (E_2-3,4-Q), and forms a quinone-DNA adduct, which generates highly mutagenic apurinic sites.[7,8] Conversely, a part of 4-OHE_2 is methylated by COMT to 4-$MeOE_2$, which is thought to be inactive and to obviate further conversion to genotoxic metabolites; however, the amount of this species produced is negligible.[9] Interestingly, ratios of 4-OHE_2/2-OHE_2 formation in neoplastic tissue were higher than found in normal breast tissue.[10] E_2 is also metabolized to 2-OHE_2 in the liver where constitutive and dominant CYP1A2 plays an important role in the detoxification of estrogen. These observations indicate that inhibition of hydroxylation of E_2 by the CYP family, especially CYP1B1 but not CYP1A1 and CYP1A2, has been postulated to be important for the estrogen-related carcinogenesis, such as breast cancer.

Flavonoids, which are ubiquitously present in fruits and vegetables,[11] are important constituents of the human diet and are actively investigated as possible chemopreventive agents against environmental mutagens and carcinogens.[12] Based on their

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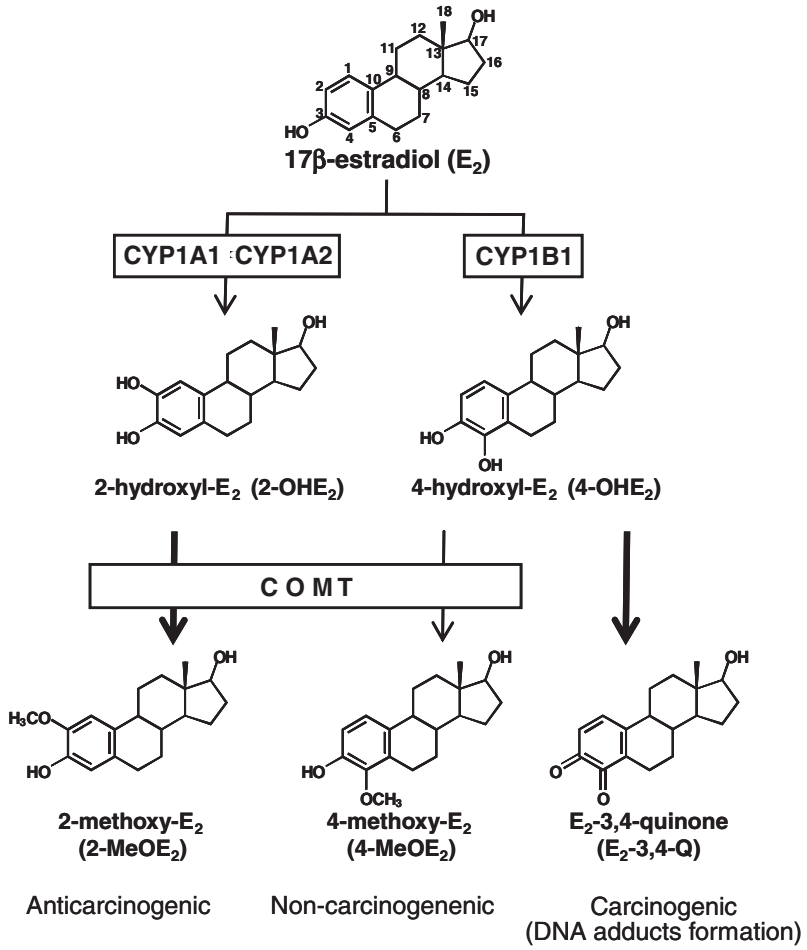


Figure 1. Scheme of estrogen metabolism in breast cells. COMT, catechol-*O*-methyltransferase.

structural scaffold, flavonoids are classified into several groups including flavones, flavonols and flavanones (Table 1). The large number of compounds arises from various combinations of multiple hydroxyl and methoxy substituents on the basic flavonoid scaffold. Many types of flavonoids have been reported as chemopreventive compounds that inhibit the activity of different CYP1 isozymes.[13]

The flavone skeleton has been reported to inhibit both activities of CYP1A1 and CYP1A2; however, the inhibitory effect on CYP1A1 is twofold stronger than CYP1A2. The hydroxyl derivatives of the flavone have been shown to differ in their inhibitory potency and isozyme selectivity.[14] We previously reported that chrysoeriol, a dietary methoxy flavone, selectively inhibited human recombinant CYP1B1 activity and prevented the formation of carcinogenic 4-OHE_2 from E_2.[15] The other methoxy flavone, acacetin, was also suggested to be a potent inhibitor of the CYP1 family.[16] These results indicate that flavonoids with methoxy substituents appear to be effective candidates for use as chemopreventive food ingredients against E_2 related breast cancer through inhibition of CYP1B1.

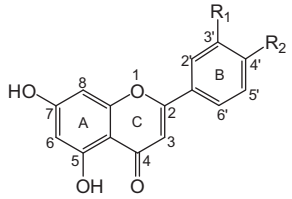
Inhibitory mechanisms of particular flavonoids on CYP enzymatic activities remain unresolved. We recently constructed three-dimensional structures of CYP1A1 and 1B1 by homology modeling using the crystal structure of CYP1A2, and studied the docking mode of E_2 with CYP1A1, 1A2 and 1B1, in order to understand why CYP1A1 and CYP1B1 predominantly metabolize E_2 to 2-OHE_2 and 4-OHE_2, respectively.[17] These results led us to hypothesize that inhibition selectivity of particular flavonoids, which strongly and selectively prevent the activities of CYP1 family members, may be resolved by the molecular docking method.

In this study, we investigated the biological evaluation of 18 major flavonoid aglycones with B-ring substitution of the hydroxyl and methoxy groups using an ethoxyresorufin *O*-deethylation (EROD) assay with recombinant human CYP1A1, 1A2 and 1B1. The inhibition selectivity of the flavonoids, which exerted strong and selective inhibition of CYP1B1 activity, was investigated using a molecular docking approach.

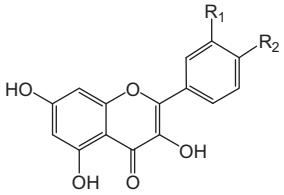
2. Results and discussion

The 18 major different flavonoid aglycones employed in this study were divided into three groups according to their basic structure, including flavones, flavonols and flavanones (Table 1). Within each group, the compounds contain different numbers of hydroxyl groups and/or methoxy groups on the B-ring. One of the most prominent features of these flavonoids is the strong antioxidative potency.[18–21] However, the potency of each flavonoid varies and this is dependent on the structure. The structures that conferred flavonoids with strong antioxidant activity have been reported to contain a 3′,4′-*O*-dihydroxyl group in the B-ring, a 2–3 double bond combined with a 4-keto group and a 3-hydroxyl group on the C-ring.[22–24] In the first step, we examined the structure–activity relationship of these 18 flavonoids on the inhibition of enzymatic activity of the CYP1 family to identify novel structure-dependent features of the flavonoids. The inhibitory effects of these 18 flavonoids on recombinant human CYP1A1, 1A2 and 1B1 activities were analyzed using the EROD assay. The 50% inhibitory concentration (IC_{50}) values are summarized in Table 1. The flavones and flavonols were observed to be relatively more potent inhibitors for three

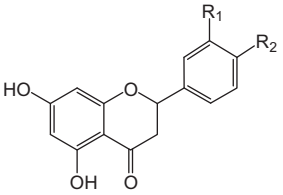
Table 1
IC_{50} values of flavonoids for CYP1A1, CYP1A2 and CYP1B1-catalyzed 7-ethoxyresorufin O-deethylation activities



Flavone



Flavonol



Flavanone

Class	Compound	R_1	R_2	IC_{50} (nM)		
				CYP1A1	CYP1A2	CYP1B1
Flavone	Chrysin	H	H	153 ± 22	84 ± 18	24 ± 6
	Apigenin	H	OH	427 ± 46	795 ± 95	25 ± 3
	Luteolin	OH	OH	1249 ± 37	3370 ± 144	79 ± 18
	Chrysoeriol	OCH_3	OH	95 ± 6	1118 ± 131	20 ± 2
	Diosmetin	OH	OCH_3	140 ± 5	2437 ± 169	29 ± 2
	Acacetin	H	OCH_3	86 ± 10	251 ± 23	12 ± 1
Flavonol	Galangin	H	H	77 ± 10	40 ± 4	25 ± 1
	Kaempferol	H	OH	632 ± 19	716 ± 173	47 ± 8
	Quercetin	OH	OH	1191 ± 169	4097 ± 409	77 ± 7
	Isorhamnetin	OCH_3	OH	56 ± 3	1261 ± 65	17 ± 3
	Tamarixetin	OH	OCH_3	120 ± 5	1215 ± 239	20 ± 4
	Kaempferide	H	OCH_3	88 ± 7	509 ± 18	25 ± 6
Flavanone	Pinocembrin	H	H	7906 ± 1278	1491 ± 191	1679 ± 478
	Naringenin	H	OH	15,167 ± 1795	26,339 ± 1034	3656 ± 459
	Eriodictyol	OH	OH	10,973 ± 1757	53,214 ± 4570	1284 ± 235
	Homoeriodictyol	OCH_3	OH	19,109 ± 3671	37,768 ± 18,128	1716 ± 478
	Hesperetin	OH	OCH_3	2786 ± 267	34,647 ± 3326	511 ± 115
	Isosakuranetin	H	OCH_3	2196 ± 69	3147 ± 300	1024 ± 140

Individual recombinant human CYP1A1, 1A2 and 1B1 supersomes were incubated with increasing concentrations of each flavonoid for 5 min, and the reaction was then initiated with the ethoxyresorufin substrate. The appearance of resorufin was measured over 10 min using a fluorescence plate reader (530 nm excitation and 590 nm emission) at 37 °C. The data are indicated as a percentage compared with controls that were treated with vehicle solvent but without a flavonoid (mean ± SD, $n = 3$). Each IC_{50} value was calculated by plotting the suppression of EROD activity against the dose, therefore providing a measure of the amount (nM) required for 50% suppression of EROD activity.

CYP1s than that of flavanones, which do not have a double bond between the 2 and 3 C-position of the C-ring, indicating that the 2–3 double bond might be an essential structural feature for strong inhibition of the activities of CYP1s. Previous studies have also showed that the flavones are better inhibitors of CYP1 activity than the flavanones.[16] The torsion angles of the B ring in flavones and flavonols are smaller than those of flavanones. This is due to the presence of the 2–3 double bond on the C-ring.[25] Therefore, the stereostructures of the flavone and flavonol molecules might easily maintain a largely planar conformation when compared with flavanones, and consequently more readily intercalate into the active site of CYP1.

All of the flavonoids used in this study showed a tendency to selectively inhibit CYP1B1 activity, although the inhibitory effect of the flavanones was considerably weaker than the activity of the flavones and flavonols (Table 1). The essential substituent on the B-ring for strong inhibition was found to follow this order: methoxy (–OCH_3) > hydrogen (–H) > hydroxyl (–OH). Luteolin (flavone group) and quercetin (flavonol group) have 3′,4′-*O*-dihydroxyl substituents; an essential structural feature for strong antioxidative potency. Removal of these hydroxyl substituents on the B-ring, for example methylation, has been recognized to diminish the antioxidant activity.[26] However, the hydroxyl group on the B-ring, even if *O*-dihydroxyl, was observed to be not a key structural feature that inhibits the activity of CYP1s. Additionally, the 3-hydroxyl group on the C-ring was also a non- or weak-essential substituent, because there was no difference between the inhibitory effects of flavones and flavonols. Among the flavones and flavonols with a methoxy substituent on the B-ring, acacetin was the most potent CYP1B1 inhibitor with an IC_{50} value of 12 ± 1 nM. Isorhamnetin, chrysoeriol, tamarixetin, kaempferide and diosmetin also have strong inhibitory effects on CYP1B1 activity with IC_{50} values of 17 ± 3, 20 ± 2, 20 ± 4, 25 ± 6 and 29 ± 2 nM, respectively. On the other hand, methoxy flavanones, homoeriodictyol, hesperetin and isosakuranetin, which do not have the 2–3 double bond on the B-ring, were very weak inhibitors of CYP1B1 activity with IC_{50} values of 1716 ± 478, 511 ± 115 and 1024 ± 140 nM, respectively.

In conclusion, the results indicate that the flavonoids structures conferring the 2–3 double bond in the C-ring were the strongest inhibitors of CYP1 proteins. Flavones and flavonols selectively inhibited CYP1B1. 3′,4′-*O*-Dihydroxyl-type flavonoids such as quercetin and luteolin exerted strong selective inhibition of CYP1B1 activity. However, such 3′,4′-*O*-dihydroxyl-type flavonoids are recognized to be a substrate of COMT,[27] indicating that dihydroxyl-type flavonoids might compete with *O*-methylation of 2-OHE_2, which represents a major detoxification pathway of E_2. Therefore, we considered that methoxy-type flavones and flavonols are strong and selective inhibitors on CYP1B1 activity under physiological conditions when compared with the hydroxyl and hydrogen substituents.

Recently, a kinetic analysis showed that the inhibitory mode of flavonoids on the activity of CYP1s was different among the individual flavonoids. For example, apigenin[28] and hesperetin[16] were competitive inhibitors of CYP1B1, whereas quercetin and kaempferol showed a mixed type of inhibition, and that isorhamnetin was a noncompetitive inhibitor.[29] Additionally, we have previously investigated the mode of action of CYP1B1 inhibition by chrysoeri-

ol. In this study, chrysoeriol was found to be a competitive inhibitor of CYP1B1 with a K_i value of 8.3 nM.[15] However, an inhibitory mechanism for selective inhibition of methoxyflavonoids on CYP1B1 remains unresolved. Therefore, we further extended the investigation, in order to elucidate the selectivity of inhibition of CYP1B1 activity using a molecular docking approach.

Using multiple alignments of CYP1A1, 1A2 and 1B1 indicated that the identity of 1B1 has been found to be 39% with 1A1 and 37% with 1A2, whereas 1A1 and 1A2 have 72% sequence identity.[17] The models of CYP1A1 and CYP1B1 were obtained by homology modeling based on the crystal structure of the CYP1A2 (Fig. 2). The models indicate that these enzymes have a planar pocket in the human CYP1 active site. The pocket fits closely with planar compounds such as α-naphthoflavone, which is reported to be a potent, competitive inhibitor of CYP1A2 (Fig. 2),[30] and typical CYP1A2 substrates such as caffeine, melatonin and alkoxyresorufins. Flavones and flavonols have a planar polycyclic structure, which is a key criterion in defining CYP1 specificity.

Human CYP1 isozymes show overlapping specificities for the molecular planarity of substrates and inhibitors. The lipophilicity in the active site cavity is shown in Figure 3. The high lipophilicity in the cavity, which places the phenyl ring close to the heme iron, suggests that the site of CYP1 limits the binding of quercetin and luteolin, as shown in Table 1. In contrast, the flavonoids with a methoxy group on the B-ring fit suitably into the pocket of CYP1, since the methoxy group shows more lipophilic than two hydroxyl groups. In addition, these factors are likely to aid release of the oxidized products from CYP1 enzymes.

However, the 3D structures of CYP1s demonstrate that substrate binding specificity is based on specific amino acid residues, that is,

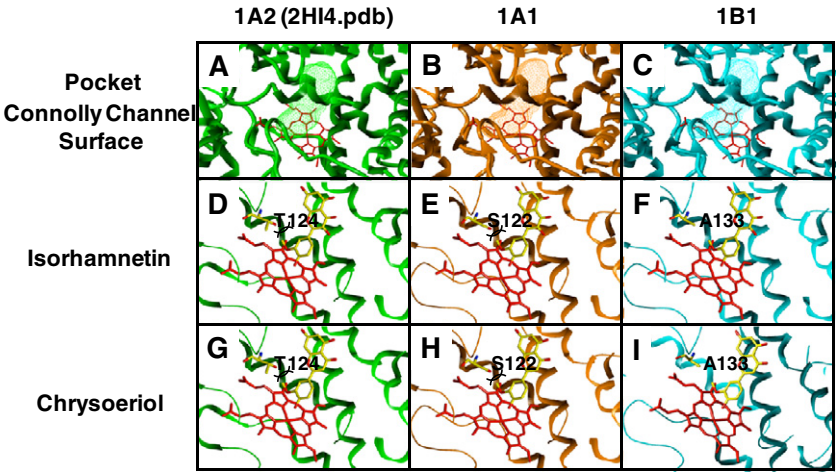


Figure 2. Comparison between the active site cavity volumes for human CYP1 (A–C) and the corresponding docked conformation of isorhamnetin (D–F) or chrysoeriol (G–I) in the human CYP1 active site. The substrate binding cavity is illustrated as a mesh surface. The heme prosthetic group is represented as a stick model and is colored red, with portions of the protein backbone represented as a ribbon schematic colored as follows: 1A2 (PDB: 2HI4), green (A, D, G); CYP1A1, brown (B, E, H); and CYP1B1, dark blue (C, F, I). Isorhamnetin and chrysoeriol are shown as stick models, with carbon and oxygen atoms colored yellow and red, respectively.

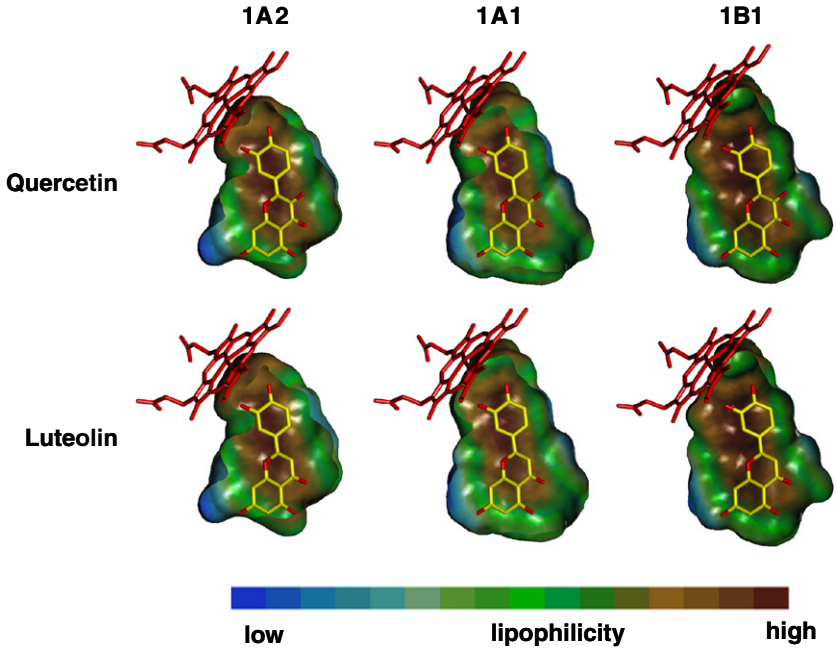


Figure 3. Lipophilicity of the human CYP1 active site docked with quercetin or luteolin. The heme prosthetic group is represented in red sticks. Quercetin and luteolin are shown as stick models, with carbon and oxygen atoms colored yellow and red, respectively.

Thr-124 of CYP1A2, Ser-122 of CYP1A1 and Ala-133 of CYP1B1. Focusing on these residues, the differences in the size and polarity profile of these residues were perceived to be crucial in ligand recognition. The rank order of size is threonine > serine > alanine, and this shows the possible role size and shape has in substrate binding recognition and the actual size of the binding cavity. The predicted less compact CYP1B1 active site topology has less of an influence on substrate binding and orientation. Our data indicate that CYP1B1 prefers substrates that are slightly bulkier than CYP1A1 and CYP1A2 substrates and may explain the differences in catalytic activities towards overlapping substrates observed with these enzymes. Isorhamnetin and chrysoeriol preferentially inhibited CYP1B1 with higher IC_{50} ratios for CYP1A2/1B1 and CYP1A1/1B1. Therefore, the docking study was carried out using CYP1A2, CYP1A1 or CYP1B1 and isorhamnetin or chrysoeriol, respectively (Figs. 2 and 4). Isorhamnetin and chrysoeriol were docked into the CYP1B1 binding pocket, avoiding the steric repulsion with the hydroxyl group to the heme. However, difficulties were encountered with the docking of the compounds in the binding pockets of CYP1A2 and 1A1. This was due to collisions between the methoxy substituent of the compounds and the methyl group of Thr-124 of CYP1A2 or hydroxyl group of Ser-122 of CYP1A1.

The docking study demonstrated that specific residues for each CYP1s, Thr-124 of CYP1A2, Ser-122 of CYP1A1 and Ala-133 of CYP1B1, play an important role in the selectivity of methoxyflavonoids. Our previous docking study also indicated that Thr-124 and Phr-260 of CYP1A2, Ser-122 and Phe-258 of CYP1A1, and Ala-133 and Asn-265 of CYP1B1 are critical residues for E_2 recognition.[17] It was previously revealed that the conserved residues in the active site are involved in selective inhibition and E_2 recognition on CYP1. Our study suggests that Ala-133 of CYP1B1 also plays a critical role in binding competitively to methoxyflavonoids and E_2.

3. Conclusions

When analyzing the preventive potencies of 18 flavonoid aglycones on CYP1A1, 1A2, and 1B1 activities using an EROD assay, all flavonoids tested showed a preference to selectively inhibit CYP1B1 activity. However, the flavonoid structures with the strongest inhibition of CYP1 family members were compounds with a 2–3 double bond in the C-ring and with or without a 3-hydroxyl substitution. Interestingly methoxy types of flavones and flavonols such as chrysoeriol and isorhamnetin were observed to selectively and strongly inhibit CYP1B1. Consequently, these methoxyflavonoids might represent strong candidates as chemopreventive food ingredients against CYP1B1-related carcinogenesis. The molecular docking study suggested that such methoxyflavonoids fit well into the active site pocket of CYP1B1, but do not fit into the pockets of CYP1A1 and 1A2 due to collisions between both a methoxy substituent of these methoxyflavonoids and Ser-122 of CYP1A1 and Thr-124 of CYP1A2.

These results explain why methoxyflavonoids with the 2–3 double bond in the C-ring can act as selective inhibitors against human CYP1B1.

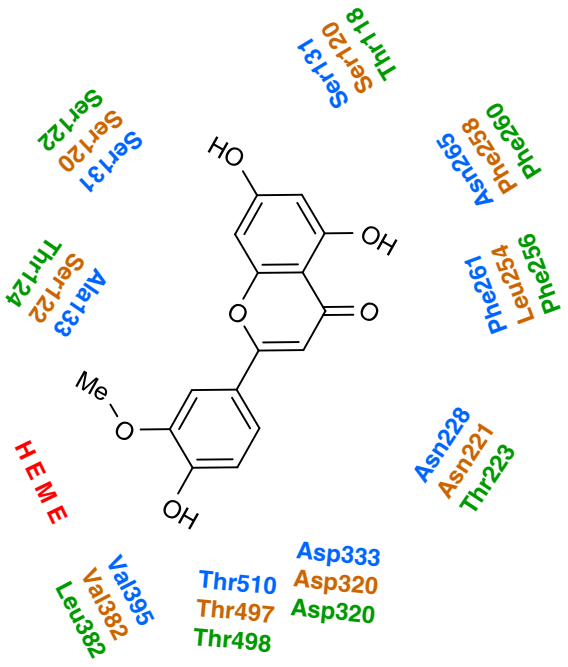


Figure 4. The amino acid residues within a 4 Å radius surrounding chrysoeriol docked with CYP1B1 (upper; blue), 1A1 (middle; brown) and 1A2 (lower; green).

4. Experimental

4.1. Chemicals

The 18 standard flavonoids used in this study are summarized in Table 1. Every flavonoid was HPLC grade and purchased from Extrasynthèse (Genay, France). These flavonoids were dissolved in dimethyl sulfoxide (DMSO) at a concentration 20 mM and stored at −20 °C in the dark for up to 3 months. The flavonoid solutions were diluted to a range between 1 nM and 200 μM with DMSO, and then immediately used in experiments. Ethoxyresorufin and resorufin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). β-NADPH, β-NADH and glucose-6-phosphate (Oriental Yeast Co., Ltd, Osaka, Japan) and recombinant human CYP1A1, CYP1A2 and CYP1B1 supersomes (BD Biosciences, San Jose, CA, USA) were used in this study. All the other chemicals and reagents were of the highest grade available.

4.2. 7-Ethoxyresorufin *O*-deethylation (EROD) enzyme assay

EROD activity was assayed as previously described[28] with slight modifications and determined for the measurement of CYP1A1, 1A2 and 1B1 activities. Briefly, the reaction mixture contained 4 mM NADPH, 4 mM NADH, 20 mM $MgCl_2$ and 0.1 M potassium phosphate buffer (pH 7.4), recombinant CYP microsome and various concentrations of individual flavonoids in a total volume of 200 μL. After pre-incubation at 37 °C for 5 min, the reactions were initiated by the addition of 40 μL of ethoxyresorufin and incubated at 37 °C for 10 min. In our preliminary experiments, the apparent K_m values were 0.14, 0.28 and 0.20 μM for CYP1A1, 1A2 and 1B1, respectively (data not shown). The inhibition studies were performed using concentrations approximately two times the K_m values.[14] Therefore, 0.25, 0.50 and 0.35 μM ethoxyresorufin was used for the analysis of CYP1A1, 1A2 and 1B1 activities, respectively. The formation of resorufin was determined fluorometrically (530 nm excitation and 590 nm emission) using a spectrofluorometer (Varioskan Flash, Thermo Fisher Scientific Inc., Worcester, MA, USA). The IC_{50} values for the activity–concentration curves from individual experiments were calculated with GraphPad Prism 4 (San Diego, CA, USA).

4.3. Homology modeling and docking analysis

4.3.1. Modeling

Based on the alignment, we constructed a 3D model of CYP1B1 and CYP1A1 by sequence replacement using the SYBYL Biopolymer program[31] (Tripos Inc., St. Louis, MO, USA) and the atomic coordinates (Arg34-Ser513) of the crystal structure of CYP1A2 (PDB; 2HI4) as a template.[30] Details of procedures are described in a previous paper.[17]

4.3.2. Docking

Docking was performed using Surflex Dock in SYBYL 8.0 (Tripos Inc., St. Louis, MO, USA). The crystal structure of CYP1A2 (PDB: 2HI4) and the CYP1A1 and CYP1B1 models constructed above were used as the CYP protein structure. Details of procedures are described in the previous paper.[17] Substrate structures having top 20 scores were selected and then structures whose B-ring was oriented to the iron of heme of CYP were extracted. From among them, the structure having best score was finally chosen.

Acknowledgments

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