

Enantiomers of Naringenin as Pleiotropic, Stereoselective Inhibitors of Cytochrome P450 Isoforms

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ABSTRACT Interactions between naringenin and the cytochrome P450 (CYP) system have been of interest since the first demonstration that grapefruit juice reduced CYP3A activity. The effects of naringenin on other CYP isoforms have been less investigated. In addition, it is well known that interactions with enzymes are often stereospecific, but due to the lack of readily available pure naringenin enantiomers, the enantioselectivity of its effects has not been characterized. We isolated pure naringenin enantiomers by chiral high-performance liquid chromatography and tested the ability of (*R*)-, (*S*)- and *rac*-naringenin to inhibit several important drug-metabolizing CYP isoforms using recombinant enzymes and pooled human liver microsomes. Naringenin was able to inhibit CYP19, CYP2C9, and CYP2C19 with IC₅₀ values below 5 μ M. No appreciable inhibition of CYP2B6 or CYP2D6 was observed at concentrations up to 10 μ M. Whereas (*S*)-naringenin was 2-fold more potent as an inhibitor of CYP19 and CYP2C19 than (*R*)-naringenin, (*R*)-naringenin was 2-fold more potent for CYP2C9 and CYP3A. Chiral flavanones like naringenin are difficult to separate into their enantiomeric forms, but enantioselective effects may be observed that ultimately impact clinical effects. Inhibition of specific drug-metabolizing enzymes by naringenin observed *in vitro* may be exploited to understand pharmacokinetic changes seen *in vivo*. *Chirality* 23:891–896, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: flavonoid; drug metabolism; CYP19; CYP2C9; CYP2C19; CYP3A; enantioselectivity; food–drug interaction

INTRODUCTION

Naringenin (Fig. 1, Structure 1) is a chiral flavanone belonging to the flavonoid class that has potential health related properties. It is contained in some *Citrus* species, in particular grapefruit, that contain large amounts of its 7-*O*-neohesperidoside, naringin (Fig. 1, Structure 2). Upon ingestion, naringin undergoes cleavage of the sugar moiety, leaving the free aglycone naringenin in the gastrointestinal tract.¹ In a seminal article, naringenin was found to inhibit the cytochrome P450 (CYP) 3A4-mediated oxidation of two dihydropyridine cardiodepressive drugs, more markedly than naringin.² Later, naringenin was found *inter alia* to inhibit the activity of CYP isoforms that activate the potent environmental carcinogen, nicotine-derived nitrosamine ketone.³ Naringenin also inhibited quinine 3-hydroxylation mediated by CYP3A4, although it is recognized that other components of grapefruit juice are more active.⁴ It may also act as a potent immunomodulator in mice with pulmonary fibrosis,⁵ and the antioxidant properties of naringenin and other polyphenols have been well studied.⁶

Despite a volume of research over many years, the relevance of stereochemistry at the C-2 stereogenic center of naringenin has not been carefully evaluated. It is well known that interactions between an enzyme system and a substrate are frequently stereospecific,⁷ and often influence the potency and the response to single enantiomer.⁸ For example, quinidine is a clinically relevant and potent inhibitor of CYP2D6, while its diastereomer quinine is not,⁹ (*S*)-lansoprazole is a more potent inhibitor of CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4-mediated hydroxylation than (*R*)-lansoprazole,¹⁰ and many other chiral drugs have been

well documented to exhibit enantioselective inhibition of P450-mediated metabolism.^{11–13} Difficulties in separating enantiomers and the resulting absence of readily available pure enantiomers have limited research in this area, until now. In one report, inhibition of recombinant CYP3A4 by specific components of grapefruit juice was described for single enantiomers of naringenin (50 μ M), but details on the separation of the enantiomers and their purity were not given.¹⁴

The inhibition of human drug metabolism by flavonoids like naringenin may be of medical importance. Since the demonstration that grapefruit juice can inhibit the metabolism of the CYP3A substrate felodipine,¹⁵ it has become widely appreciated that the ingestion of a number of fruit juices can slow drug metabolism,¹⁶ and thereby increase the concentrations of important medications such as cyclosporine¹⁷ and methadone.¹⁸ The biochemical basis for these interactions involves the interaction of flavonoids with specific CYP isoforms. Although CYP3A has been most frequently studied, a number of different isoforms may interact

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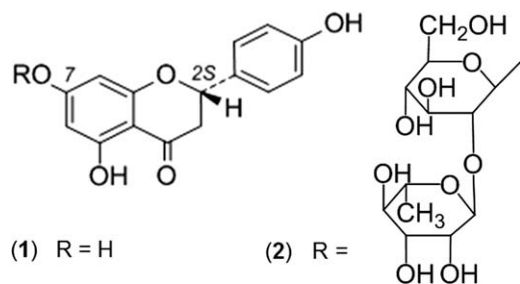


Fig. 1. Structures of naringenin (1, R = H) and naringin (2, R = neohesperidosyl).

in this way. It is therefore important to study interactions of naringenin with multiple CYP isoforms to be aware of any potential clinically relevant interactions between naringenin and medications.

For these reasons, we set out to develop an efficient procedure for the isolation of single naringenin enantiomers (*R* and *S*) in sizeable amounts. We used *rac*-naringenin and the purified (*R*)- and (*S*)-naringenin preparations to test the ability of naringenin to inhibit a series of key drug metabolizing CYP isoforms *in vitro*, with a focus on the enantioselectivity of the interaction.

MATERIALS AND METHODS

Chemicals and Reagents

Rac-naringenin, testosterone, 6- β hydroxytestosterone, desmethyldiazepam, β -NADP, glucose-6-phosphate dehydrogenase, and glucose-6-phosphate were purchased from Sigma-Aldrich (St. Louis, MO). Bupropion, 4-hydroxybupropion, and nevirapine were purchased from Toronto Research Chemicals (North York, ON, Canada). Magnesium chloride was purchased from Fisher Scientific (Pittsburgh, PA). All drug solutions were prepared by dissolving each compound in methanol or acetonitrile, and were stored at -20°C . All high-performance liquid chromatography (HPLC)-grade reagents and chemicals used for mobile phase and buffers were obtained as previously described.¹⁹ Pooled human liver microsomes (HLMs) and the CYP inhibitor screening kits for CYP19, 2C9, 2C19, and 2D6 were purchased from BD Biosciences (San Jose, CA). All microsomal preparations were stored at -80°C .

Separation, Isolation, and Purification of Individual Enantiomers of Naringenin

The HPLC apparatus used for separation of the (*R*)- and (*S*)-enantiomers consisted of a Jasco pump PU 980 with Rheodyne 20 or 200- μl sample loops, a low pressure mixer LG-1580-02 and a line degasser 1550-54, a Uvidec 100-III spectrophotometric detector operating at 292 nm (Jasco, Tokyo, Japan). Chromatograms were acquired and processed using computer-based Jasco Borwin 2 software. Circular dichroism (CD) spectra were recorded on a Jasco 810 spectropolarimeter using a 1-mm cell. Chromatographic separations were performed on a Lux Cellulose 2 column, 250 mm \times 4.6 mm i.d., whose chiral selector was tris-3-chloro-4-methylphenylcarbamate coated on 5- μm silica gel (Phenomenex Italia, Bologna). A column in line filter with 0.5- μm stainless steel frit of 3 mm diameter from Rheodyne was used to protect the HPLC column. Disposable polytetrafluoroethylene (PTFE) filters of 0.2 μm pore size were used for filtration of sample solutions. HPLC chromatographic parameters α and R_s were calculated according to the usual procedure.²⁰

Inhibition of Recombinant Human CYP450 Isoforms

The activity of each recombinant human CYP450 isoform was determined by measuring the conversion rate of a fluorometric substrate to its fluorescent metabolite. CYP19 and CYP2C9 activities were determined using the metabolism of 7-methoxy-4-trifluoromethylcoumarin (MFC) to 7-hydroxytrifluoromethylcoumarin. CYP2C19 activity was determined using the metabolism of 3-cyano-7-ethoxycoumarin (CEC) to

3-cyano-7-hydroxycoumarin. CYP2D6 activity was determined using the metabolism of 3-[2-(*N,N*-diethyl-*N*-methylamino)ethyl]-7-methoxy-4-methylcoumarin (AMMC) to 3-[2-(*N,N*-diethylamino)ethyl]-7-methoxy-4-methylcoumarin. Experimental procedures were consistent with the published methodology.²¹ All incubations were performed using incubation times and protein concentrations that were within the linear range for reaction velocity. Substrates and inhibitors were prepared in acetonitrile. A series of concentrations of inhibitor in a volume of 4 μl were mixed with 96 μl of NADPH-Cofactor Mix (16.3 μM NADP, 828 μM glucose-6-phosphate, 828 μM MgCl_2 , and 0.4 U/ml glucose 6-phosphate dehydrogenase), and prewarmed for 10 min at 37°C . Enzyme/Substrate Mix was prepared with fluorometric substrate, recombinant human CYP450 isoform and 0.1 M potassium phosphate buffer (pH 7.4). Reactions were initiated by adding 100 μl of Enzyme/Substrate Mix to bring the incubation volume to 200 μl . The optimal final recombinant enzyme concentrations, substrate concentrations, and incubation times were: 7.5 nM CYP19 + 25 μM MFC for 30 min, 15 nM CYP2C9 + 150 μM MFC for 45 min, 7.5 nM CYP2C19 + 25 μM CEC for 30 min, and 7.5 nM CYP2D6 + 1.5 μM AMMC for 30 min. All reactions were stopped by adding 75 μl of acetonitrile/0.1 M tris base. The generation of fluorescent metabolites was determined immediately by measuring fluorescent response using a Bio-Tek (Winooski, VT) Synergy 2 fluorometric plate reader. Excitation-emission wavelengths were 400–540 nm for the MFC metabolite or 400–460 nm for the CEC and AMMC metabolites. Standard curves were constructed using the appropriate fluorescent metabolite standards. Quantification of samples was performed by applying the linear regression equation of the standard curve to the fluorescence response. The limits of quantification for the metabolites of MFC, CEC, and AMMC were 4 pmol, 0.1 pmol, and 0.8 pmol in a final volume of 200 μl , respectively, with intra- and inter-assay coefficients of variation of less than 10%.

Inhibition of Specific CYP450 Isoforms Using Pooled HLMs

A single isoform-specific substrate concentration at the respective K_m value (10 μM testosterone or 100 μM bupropion) was incubated at 37°C in duplicate with pooled HLMs and the NADPH-generating system (1.3 mM NADP, 3.3 mM glucose-6-phosphate, 3.3 mM MgCl_2 , and 0.4 U/ml glucose 6-phosphate dehydrogenase) in the absence or the presence of a range of (*R*)-, (*S*)- or *rac*-naringenin (up to 50 μM). The activity of CYP2B6 was determined by measuring the formation rate of 4-hydroxybupropion from bupropion in pooled HLMs, while the activity of CYP3A was determined by measuring the formation rate of 6 β -hydroxytestosterone from testosterone. All incubations were performed using incubation time and protein concentrations that were within the linear range for reaction velocity. An incubation mixture that consisted of substrate probes, HLMs, and 100 mM phosphate reaction buffer (pH 7.4) was prewarmed for 5 min at 37°C . The reaction was initiated by the addition of the NADPH-generating system, and incubated at 37°C for 15 min. The final protein concentration of pooled HLMs was 0.25 mg/ml. All reactions were terminated by the addition of 500 μl of acetonitrile, followed by immediate vortex and placement of the tubes on ice.

Quantification of 4-Hydroxybupropion and 6 β -Hydroxytestosterone Formation

All samples were extracted immediately after the incubations were performed. First, an internal standard was added to each sample. The incubation mixture was then centrifuged at 14,000 rpm for 5 min at room temperature. The supernatant layer was made alkaline by adding 500 μl of 1 M glycine-NaOH buffer (pH 11.3) and extracted by adding 6 ml of ethyl acetate. This mixture was vortex-mixed for 10 min and then centrifuged at 36,000 rpm for 15 min. The organic layer was transferred to 13 \times 100-mm glass culture tubes and evaporated to dryness. The resulting residue was reconstituted in mobile phase. HPLC assays with ultraviolet (UV) detection were developed for the quantification of 4-hydroxybupropion and 6 β -hydroxytestosterone. The HPLC system consisted of a Shimadzu LC-10AT pump, SIL-10AD auto-sampler, SCL-10A system controller, and SPD-10A UV-vis detector (Shimadzu Scientific Instruments, Columbia, MD). (1) 4-hydroxybupropion and nevirapine (internal standard)

were separated using a Zorbax SB-C₁₈ column (150 mm × 4.6 mm, 3.5 µm particle size; Phenomenex, Torrance, CA), a Luna C₁₈ Guard column (30 mm × 4.6 mm, 5 µm; Phenomenex) and mobile phase consisting of 85% (v/v) 10 mM KH₂PO₄ (pH adjusted to 3) and 15% acetonitrile (flow rate, 1 ml/min). UV detection was set at 214 nm for 4-hydroxybupropion (retention time: 14.2 min) and 282 nm for nevirapine (retention time: 29 min). (2) 6β-hydroxytestosterone and desmethyldiazepam (internal standard) were separated using the same columns, but with a mobile phase consisting of 40% 30 mM ammonium acetate (pH adjusted to 6.3) and 60% methanol (flow rate, 1 ml/min). UV detection was set at 254 nm for 6β-hydroxytestosterone (retention time: 5 min) and desmethyldiazepam (retention time: 10.4 min). Peak areas for each peak were obtained from an integrator, and peak area ratios with internal standard were calculated. Formation rate of metabolite from the respective probe substrate was quantified by using the appropriate standard curve. Intra- and inter-day coefficients of variation of the assays were less than 15%.

Kinetic Analysis

The rates of metabolite formation from substrate probes in the presence of the test inhibitors were compared with those in control in which the inhibitor was replaced with vehicle. The extent of CYP450 inhibition was expressed as percent enzyme activity remaining compared to control. IC₅₀ values were determined as the inhibitor concentrations that brought about a 50% reduction in enzyme activity by fitting all the data to a one-site competition equation using Prism version 5.01 for Windows (GraphPad Software, San Diego, CA).

RESULTS

Separation, Isolation, and Purification of Naringenin Enantiomers

An improved separation of the enantiomers of naringenin was accomplished by chiral HPLC on a polysaccharide-derived²² chiral stationary phase (Lux Cellulose 2) using a mobile phase *n*-hexane/ethanol with 0.5% of trifluoroacetic acid (TFA) 80:20 at 1.2 ml/min. We obtained good values for enantioselectivity (α) and resolution factor (R_s), of 1.97 and 8.3, respectively. This is a large improvement in the resolution factor with respect to previous enantioseparation reported on a different chiral stationary phase.²³ A typical chromatogram is shown in Figure 2A with retention times of 6.87 and 11.19 min for the (*S*)- and (*R*)-enantiomers, respectively. Isolation of the single enantiomers of naringenin was performed using a composition 70:30 of the same mobile phase without TFA, at a flow rate 1.5 ml/min. The absence of TFA avoided problems in the solvent elimination from the eluates although some tailing of the peaks was present. These conditions are a compromise between short elution times ($t_1 = 3.38$, $t_2 = 4.18$ min) and adequate resolution factors. The isolation was accomplished by repeated injections (about 160) of 70 µl of a solution (5 mg/ml) of *rac*-naringenin in *n*-hexane/ethanol (1:1). Collection of the eluates after filtration and rotoevaporation yielded ~20 mg of each enantiomer. The enantiomeric purity (e.p.) was checked using the same analytical conditions and it was 98.0 and 96.5% for the first and the second eluted enantiomer, respectively. Because of the high values of α and R_s , the method can be scaled up using a larger column with the same chiral selector (not available to us) for isolating a greater quantity of single enantiomers with a much minor number of injections.

The CD spectra of the enantiomers were mirror images of each other indicating the enantiomeric nature of the collected peaks, as shown in Figure 2B. The absolute configuration of the enantiomers was established taking into account that the negative CD band centred at 288 nm corresponds to the (*S*)-configuration.^{23,24} Thus, the first eluted enantiomer,

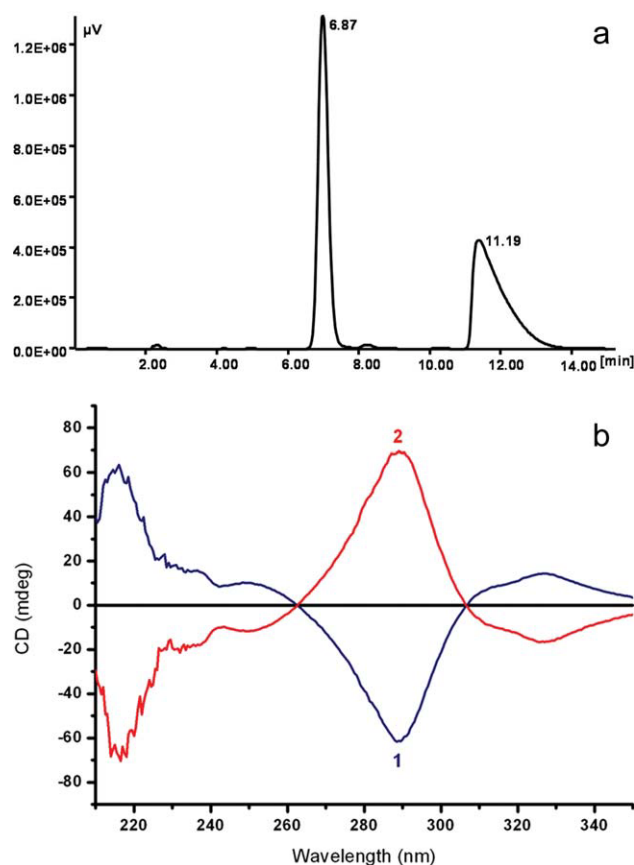


Fig. 2. Separation of naringenin enantiomers. (A) Typical HPLC separation of the (*S*) and (*R*) enantiomers of naringenin with retention times of 6.87 and 11.19 min, respectively. Conditions: chiral stationary phase Lux Cellulose 2; mobile phase: *n*-hexane/ethanol with 0.5% TFA, 80:20 at 1.2 ml/min. (B) CD spectra (ethanol, 22°C) of the enantiomers of naringenin obtained from the first (1) and the second (2) HPLC eluted peaks. The negative CD band centred at 288 nm corresponds to the (*S*)-configuration. [Color figure can be viewed in the online issue, which is available at [wileyonlinelibrary.com](http://www.interscience.wiley.com).]

shown in Figure 2A, is the (*S*)-enantiomer and the second eluted enantiomer is the (*R*)-enantiomer. Remarkably, the elution order of the enantiomers is reversed with respect to the elution order observed using different polysaccharide-based chiral stationary phases.^{23,24}

Using this efficient procedure the (*R*)- and (*S*)-enantiomers of naringenin were isolated in sizeable amounts (15 mg) and used for subsequent experiments.

Inhibition of Cytochrome P450 Isoform Activity

Naringenin was able to inhibit a number of CYP isoforms. Specifically, dose-dependent inhibition of CYP19, CYP2C9, CYP2C19, and CYP3A was demonstrated (Figs. 3–6), while no appreciable inhibition of CYP2B6 or CYP2D6 was observed at concentrations up to 10 µM (data not shown). When the enantiomers of naringenin were tested, enantioselective inhibition of CYP19, CYP2C9, CYP219, and CYP3A was shown. While the (*S*)-enantiomer was approximately 2-fold more potent than the (*R*)-enantiomer as an inhibitor of CYP19 and CYP2C19, the (*R*)-enantiomer was about 2-fold more potent as an inhibitor of CYP2C9 and CYP3A (Figs. 3–6, Table 1). In each case, the IC₅₀ value for *rac*-naringenin was between that for the (*R*)-enantiomer and that for the (*S*)-enantiomer.

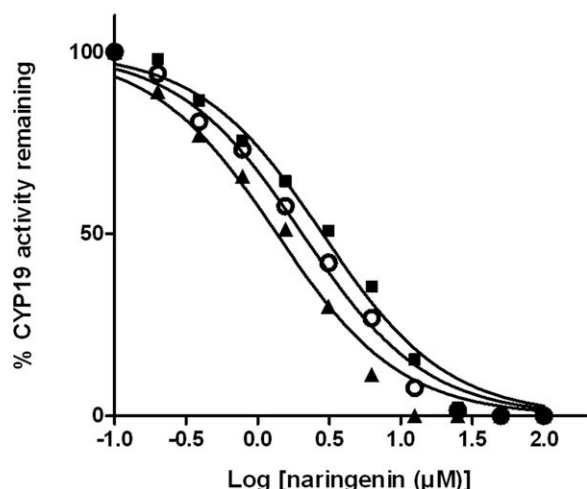


Fig. 3. Enantioselective inhibition of human CYP19 by naringenin. A range of concentrations of *rac*-naringenin (open circle), (*R*)-naringenin (dark square), or (*S*)-naringenin (dark triangle) was incubated with recombinant human CYP19 (7.5 nM) and substrate MFC at 37°C for 30 min and enzyme activity was determined in three independent experiments. Individual points represent the mean of duplicate incubations.

DISCUSSION

In this study we describe an efficient and effective separation of the chiral enantiomers of naringenin. This has allowed the testing of these enantiomers as inhibitors of CYP isoforms, but their use need not be limited to this study. Many other naturally occurring flavonoids and isoflavonoids exist as stereoisomeric mixtures, in which one enantiomer predominates. The enantioselective separation and purification of chiral flavonoids such as that described here should allow researchers to address biological questions with clarity, and to understand biochemical mechanisms with precision.

The data presented here demonstrate the ability of naringenin to inhibit multiple CYP450 isoforms *in vitro*. The IC_{50}

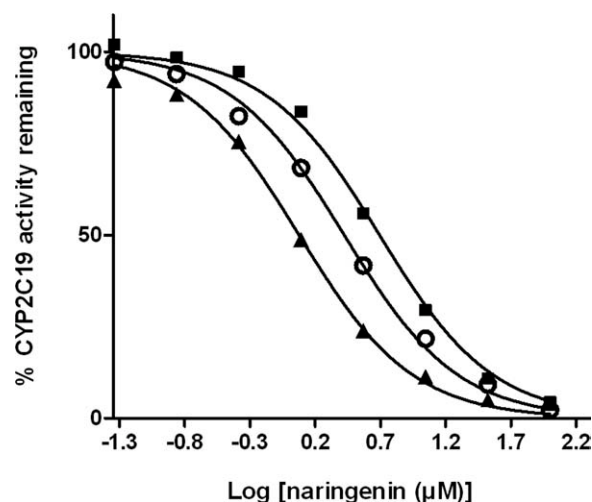


Fig. 5. Enantioselective inhibition of human CYP2C19 by naringenin. A range of concentrations of *rac*-naringenin (open circle), (*R*)-naringenin (dark square), or (*S*)-naringenin (dark triangle) was incubated with recombinant human CYP2C19 (7.5 nM) and substrate CEC at 37°C for 30 min and enzyme activity was determined in three independent experiments. Individual points represent the mean of duplicate incubations.

concentrations of naringenin we observed all fall in the low micromolar range (Table 1), consistent with previous studies that have shown maximal plasma concentrations of naringenin after ingestion of grapefruit juice to be $\sim 6 \mu\text{M}$.²⁵ These data suggest that naringenin may have effects *in vivo* on the activity of multiple enzymes. Such inhibitory effects may contribute to the clinically observed food–drug interactions brought about by grapefruit juice and other fruit products.²⁶ These data are consistent with those reported on inhibition of CYP3A, but whether interactions with CYP19, CYP2C9, or CYP2C19 carry similar clinical relevance must await further research.

Studies presented here with purified (*R*)- and (*S*)-naringenin showed that the individual enantiomers were selective.

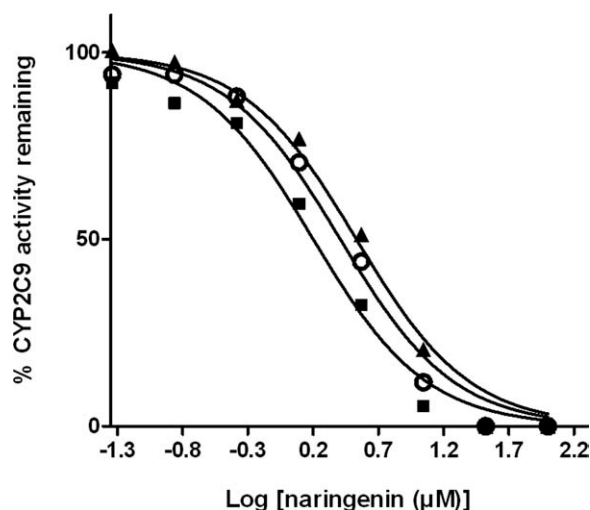


Fig. 4. Enantioselective inhibition of human CYP2C9 by naringenin. A range of concentrations of *rac*-naringenin (open circle), (*R*)-naringenin (dark square), or (*S*)-naringenin (dark triangle) was incubated with recombinant human CYP2C9 (15 nM) and substrate MFC at 37°C for 45 min and enzyme activity was determined in three independent experiments. Individual points represent the mean of duplicate incubations.

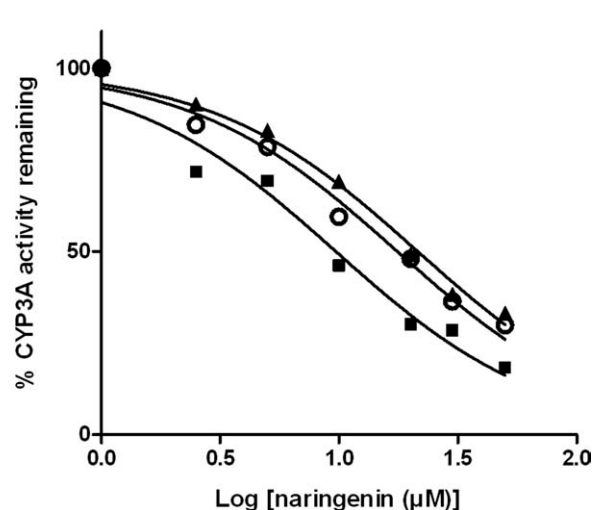


Fig. 6. Enantioselective inhibition of human CYP3A by naringenin. A range of concentrations of *rac*-naringenin (open circle), (*R*)-naringenin (dark square), or (*S*)-naringenin (dark triangle) was incubated with pooled HLMs (protein concentration 0.25 mg/ml) and probe substrate testosterone at 37°C for 15 min and enzyme activity was determined in two independent experiments. Individual points represent the mean of duplicate incubations.

TABLE 1. IC₅₀ values for enantioselective inhibition of multiple human CYP450 isoforms by naringenin

μM	CYP19	CYP2C9	CYP2C19	CYP3A
<i>rac</i> -Naringenin	2.0 (1.7, 2.4)	2.6 (2.0, 3.4)	2.6 (2.4, 2.9)	17.6 (14.8, 20.8)
(<i>R</i>)-Naringenin	2.8 (2.4, 3.4)	1.6 (1.2, 2.1)	4.9 (4.4, 5.5)	9.7 (7.3, 12.8)
(<i>S</i>)-Naringenin	1.4 (1.1, 1.7)	3.4 (2.6, 4.5)	1.2 (1.1, 1.4)	21.4 (18.5, 24.7)

IC₅₀ values are expressed as mean (95% confidence interval).

While the (*R*)-enantiomer was a more potent inhibitor of some CYP isoforms (e.g., CYP2C9 and CYP3A), remarkably the (*S*)-enantiomer was more potent on others (CYP2C19 and CYP19). These data shed light on the enantioselective preferences of specific CYP isoforms. While this enantioselective inhibition is clear in vitro, the predominant enantiomer of naringenin in natural fruits is unknown and so the vulnerability of specific enzymes to naringenin-related drug interactions in vivo cannot be determined. While a large literature exists on the effects of grapefruit and other juices on CYP3A,^{16,27} few attempts have been made to study effects on other isoforms. The data presented here now suggest that interactions with substrates of CYP2C9 such as (*S*)-warfarin,²⁸ of CYP2C19, such as clopidogrel,²⁹ and of CYP19, such as methadone¹⁹ may also be important.

Of note, naringin, the natural precursor of naringenin, is present as (*2S*)- and (*2R*)-diastereomers in grapefruit, sour orange, and pummelo with the (*2S*)-naringin being predominant. Naringin is responsible for the bitter taste of juice and marmalade and the ratio (*2S*)/(*2R*) of the concentrations of the diastereomers markedly decreases during maturation due to a nonenzymatic racemization at the C-2 position via ring opening to a chalcone.^{30–32} As our data show that the inhibitory effects of naringenin on drug-metabolizing enzymes are pleiotropic and enantioselective, the well known grapefruit juice–drug interaction³³ may be affected by the degree of maturation of the fruits. In addition, other chiral flavonoids contained in fruits may also possess enantioselective pharmacologic and biological effects.

A limitation of this study is that inhibitory potencies for different CYP isoforms were obtained using different microsomal systems. Four microsomal preparations of different recombinant CYP isoforms and one preparation of pooled HLMs were used. As a result the observed IC₅₀ values in any individual enzyme system should not be directly compared with those obtained in any other system. The question of whether or not naringenin inhibits CYP19, CYP2C9, or CYP2C19 clinically, as has been reported for CYP3A, requires further investigation. Despite this limitation, it is clear that the inhibition of these CYP enzymes by naringenin is enantioselective in every case.

CONCLUSIONS

These studies have characterized for the first time the different pharmacologic properties of the enantiomers of naringenin, specifically the enantioselective inhibition of CYP19, CYP2C9, CYP2C19, and CYP3A. The data indicate that inhibition of these enzymes by naringenin can occur with potency in the physiologic range. These findings allow more precise estimates of the potential risks for food–drug interactions to be made and identify interactions between specific CYP isoforms and naringenin that deserve further study.

Lastly, the enantioselective properties exhibited by naringenin here could provide insight into the use of naringenin and other flavonoids as novel, selective therapeutic agents.

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