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Oxidative *In Vitro* Metabolism of Liquiritigenin, a Bioactive Compound Isolated from the Chinese Herbal Selective Estrogen β-Receptor Agonist MF101

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Running Title
Metabolism of Liquiritigenin

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Abbreviations:

ER, estrogen receptor; CYP, cytchrome P450; LC-MS, high-performance liquid chromatography- mass spectrometry; MS, mass spectrometry; MSD, mass selective detector; MS/MS, tandem mass spectrometry; SERM, selective estrogen receptor modulators.

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Abstract

Liquiritigenin (2,3-dihydro-7-hydroxy-2-(4-hydroxyphenyl)-(S)-4H-1-benzopyran-4-one) is one of the major active compounds of MF101, a drug currently in clinical trials for the treatment of hot flashes and night sweats in postmenopausal women. MF101 is a selective estrogen receptor β agonist, but does not activate the estrogen receptor α. Incubation with pooled human liver microsomes yielded a single metabolite. Its structure was elucidated using MS/MS in combination with analysis of the fragmentation patterns. The metabolite resulted from the loss of two hydrogens and rearrangement to the stable 7,4'-dihydroxyflavone. The structure was also confirmed by comparison to authentic standard material. Maximum apparent reaction velocity (V_{max}) and Michaelis-Menten constant (K_m) for the formation of 7,4'-dihydroxyflavone were 32.5 nmol/g protein/min and 128 µmol/L, respectively. After correction for protein binding (free fraction F_u= 0.84), the apparent CL_{int} for 7,4'-dihydroxyflavone formation was 0.3 mL/g/min. Liquiritigenin was almost exclusively metabolized by CYP3A enzymes. Comparison of liquiritigenin metabolism in human liver microsomes isolated from 16 individuals showed 9.5-fold variability in metabolite formation (3.4 to 32.2 nmol/g protein/min). An estrogen receptor luciferase assay indicated that the metabolite was a 3-fold more potent activator of the estrogen receptor β than the parent compound and did not activate the estrogen receptor α.

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Introduction

Flavonoids have been recognized for their anticarcinogenic, antioxidant and anti-inflammatory properties. Over 4,000 flavonoids have been isolated and identified from many types of fruits, vegetables and herbs. Chemically, they can be categorized into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones. Liquiritigenin is the 7,4'-dihydroxyflavanone and is found most notably in licorice. Licorice is one of the oldest and most commonly used Chinese herbal medicines and is used in many different medications. Usually, it is found concomitantly with liquiritin, which is the 4'-glycoside of liquiritigenin (Wang ZY and Nixon DW, 2001; Moon YJ et al, 2006).

Liquiritigenin is an active ingredient of MF101, a drug currently in clinical trials for the relief of post-menopausal symptoms in women. The drug is an ethanol/aqueous extract of 22 herbal species used in traditional Chinese medicine (Cvoro et al., 2007). Due to its effects on the estrogen receptor, MF101 belongs to a class of compounds referred to as selective estrogen receptor modulators (SERMS). Most clinically used estrogen receptor agonists or antagonists similarly affect both estrogen receptor α and estrogen receptor β. SERMS discriminate between estrogen receptor α and β (Riggs and Hartmann, 2003). MF101 is a potent agonist of estrogen receptor β, but does not activate estrogen receptor α (Cvoro et al., 2007). Most importantly, it is not implicated in tumor formation as a result of estrogen receptor α activation (Cvoro et al., 2007). MF101 is an oral drug designed for the treatment of hot flashes and night sweats in peri-menopausal and menopausal women. In animal studies, the compound did not adversely alter reproductive hormones or promote tumor formation in the breast or uterus, suggesting that MF101 will not increase the risk of either breast or uterine cancer (Cvoro et al., 2007; Hillerns et al., 2005).

As of today, liquiritigenin metabolism has mainly been studied in rats. In the rat, liquiritigenin is metabolized to five glucuronide and sulfate conjugated metabolites (Shimamura et al., 1990; 1993) that are actively excreted into bile, but can also be found in urine (Shimamura et

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al., 1993; 1994). Nikolic and Van Breemen (2004) studied the oxidative metabolism of liquiritigenin using rat liver microsomes and found six metabolites: 7,3',4'-trihydroxyflavone, a hydroxyl quinone metabolite, two A-ring dihydroxy metabolites, 7,4'-dihydroxyflavone and 7-hydroxychromone.

However, the human metabolism of liquiritigenin is still largely unknown. As a first step, it was the goal of our study to assess the oxidative metabolism of liquiritigenin by human liver microsomes, to elucidate the structures of the metabolites formed, to identify the cytochrome P450 enzymes involved in human oxidative metabolism of liquiritigenin, to assess inter-individual variability of liquiritigenin metabolite formation in human liver microsomes, to evaluate potential inter-species differences and to test the metabolites' activity as estrogen β-receptor agonists.

Materials and Methods

Materials

Liquiritigenin and 7,4'-dihydroxyflavone were purchased from Extrasynthese (Lyon, France) or Indofine Chemicals (Hillsborough, NJ). Human liver microsomes (pooled from 50 donors or from 16 individual donors), animal liver microsomes and recombinant CYP450s (expressed in *E.Coli.*) were purchased from Xenotech (Lenexa, KS). The NADPH regenerating system containing glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP and $MgCl_2$ was purchased premixed from BD Biosciences (San Jose, CA). All solvents were of HPLC grade and obtained from Fischer or J.T. Baker. 2',4'-dihydroxychalcone was used as an internal standard for quantification by LC-MS and was purchased from Indofine (Hillsborough, NJ). Liquiritigenin stock solutions were prepared with 100% methanol (10 mg/mL) and were diluted with methanol as necessary. Liquirtigenin,

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7,4'-dihydroxyflavone and 2',4'-dihydroxychalcone stock solutions (*vide infra*) were stored at -80°C. U2OS cells were obtained from the American Type Culture Collection (Rockville, MD).

General procedure for microsomal incubations

Microsomal incubations contained liquiritigenin (0.04 mmol/L), 200 mg/L microsomal protein and NADPH regenerating system in phosphate buffer pH7.4 (0.1 mol/L). Final volume was either 500 or 1000 µL. Samples were incubated at 37ºC for 5 min before initiating the reaction by addition of microsomal proteins. Thereafter, samples were incubated at 37°C for 60 min. Reactions were terminated and proteins precipitated by adding a protein precipitation solution (0.2 mol/L $ZnSO_4$/methanol, 30:70 v/v) that also contained the internal standard 2',4'-dihydroxychalcone at a concentration of 1 µg/mL. Samples were vortexed on a DS-500 orbital shaker (VWR International, West Chester, PA) at 500 rpm for 30 minutes and were centrifuged at 24,400g at 4ºC for 5 min. Supernatants were transferred into HPLC vials for LC-MS analysis.

Quantification of liquiritigenin and its major metabolite using LC-MS

An Agilent series 1100 HPLC system in combination with a mass selective detector was used for quantitative analysis (all Agilent Technologies, Santa Clara, CA). One hundred µL of the extracted sample was injected onto an extraction column (4.6 x 12.5 mm Eclipse XDB-C8, 5 µm particle size, Agilent Technologies) with a mobile phase of 20% methanol in 0.1% formic acid at a flow rate of 5 mL/min. A switching valve was activated after one minute and the analytes backflushed from the extraction column onto two sequentially linked analytical columns (4.6 x 250 mm Eclipse XDB-C8, 5 µm particle size, Agilent Technologies). The following gradient was used: methanol/ 0.1% formic acid 20/80 v/v for 1 min, to 75/25 v/v in 17 min, to 98/2 v/v within 3 minutes and 98/2 v/v for 4 min, then the columns were re-equilibrated to starting conditions. The flow rate was set to 1 mL/min and the column temperature was kept at 65ºC.

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The mass spectrometer was run in negative scan mode detecting a mass range of *m/z* = 150-500. The drying gas flow was set to 12 L/min, the nebulizer pressure to 50 psi, the drying gas temperature to 350ºC, the capillary voltage to 3000 V, and the fragmentor to 100 V. Analytes were quantified in extracted ion mode using the following ions: liquiritigenin (*m/z* = 255), the metabolite of liquiritigenin (7,4'-dihydroxyflavone, m/z = 253), and 2',4'-dihydroxychalcone (internal standard, *m/z* = 239, all $[M-H]^-$).

For quantification, areas-under-the-peak were corrected using the internal standard and were compared with a 7,4'-dihydroxyflavone calibration curve. Since extraction recovery depends on the amount of protein in the incubation mixture, two sets of calibration curves were prepared based on two different microsomal protein concentrations (0.2 and 0.5 mg/ml human liver microsomes solution). The metabolite was added at the following final concentrations in calibration samples: 0, 5, 10, 25, 50, 100, 250, 500, 1000, and 1500 nmol/L. Samples were extracted immediately by adding protein precipitation solution containing the internal standard (2',4'-dihydroxychalcone, 0.2 mol/L $ZnSO_4$/methanol, 30/70, v/v) as described above.

The assay for 7,4'-dihydroxyflavone had the following key performance parameters. The lower limit of quantitation (LLOQ) was determined as the lowest quantity consistently achieving accuracy ≤ ±20% of the nominal concentration and a precision ≤ 20%. Lower limits of detection: 10 nmol/L; LLOQs: 25 nmol/L; ranges of linear response: 25- 1500 nmol/L (r^2 > 0.9992, n=6). There were no carry over, matrix interferences or relevant ion suppression as tested using the procedure proposed by Müller et al. (2002). The analytes (liquiritigenin, 7,4'-dihydroxyflavone, and internal standard were stable in the extract for at least 48 hours at +4°C (temperature in the autosampler). Intra-day and inter-day accuracies and precisions were <10% (except at the lower limit of quantitation: <20%).

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Identification of liquiritigenin metabolite structures.

For structural identification, metabolites were separated using HPLC and MS spectra (m/z= 100 to 1600) and MS/MS spectra (m/z= 50 to 300) were recorded on an Agilent QTOF 6510 instrument (Agilent Technologies, Santa Clara, CA). Metabolites were identified based on their accurate mass determination and fragmentation patterns in positive and negative mode. The hypothetical structures of the metabolites were verified by comparison with the authentic, commercially available materials (HPLC retention, exact mass and MS/MS fragments).

Evaluation of the time-dependency of liquiritigenin metabolite formation.

A solution of liquiritigenin (0.04 mmol/L final concentration) and NADPH regenerating system in phosphate buffer was aliquoted into 1.5 mL conical polypropylene tubes with snap-on lids (n=6 for each time point). Following pre-incubation for 5 min, the reaction was initiated by adding human liver microsomes to each sample for a final protein concentration of 200 mg/L. Samples were incubated for 0 (stopped immediately), 5, 10, 15, 20, 30, 45, 60, 90, or 120 min. An additional set of samples was incubated, but no NADPH had been added (controls). Reactions were terminated by addition of the protein precipitation/internal standard solution and further processed as described above.

Evaluation of the dependency of liquiritigenin metabolite formation on the microsomal protein concentration.

A solution of liquiritigenin (0.04 mmol/L final concentration) and NADPH regenerating system in phosphate buffer was aliquoted into 1.5 mL conical polypropylene tubes with snap-on lids (n=6 for each protein concentration). Following pre-incubation for 5 min, the reaction was initiated by adding microsomes to a final protein concentration of 0, 10, 50, 100, 250, 500, 1000, and 2500 mg/L. Samples were incubated for 60 minutes and samples extracted as described above.

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Estimation of the apparent Michaelis-Menten constant (K_m) and the maximum metabolite formation velocity (V_{max})

Microsomal incubations were conducted with final liquiritgenin concentrations of 0, 20, 40, 60, 80, 100, 150, 200, 250, 300, 400 and 500 µmol/L (n=6 per concentration). Samples were pre-incubated for 5 min and the reaction was started by adding human liver microsomes to a final microsomal protein concentration of 500 mg/L. Samples were incubated for 60 min at 37ºC. Reactions were stopped by adding 250 µL protein precipitation/internal standard solution and were extracted as previously described. Incubation mixtures without NADPH incubated for 60 min were used as controls. Apparent K_m and V_{max} were determined after data fitting using the Enzyme Kinetics Module (Version 1.3) of the Sigma Plot software (version 9.0, SPSS Inc., Chicago, IL).

Determination of the non-specific binding of liquiritigenin (F_u) to microsomal protein

Solutions of human liver microsomes (500 mg/L microsomal protein) and 80 µmol/L liquiritigenin were prepared (both in 0.1 M phosphate buffer pH7.4). Using a 96-well equilibrium dialyzer plate (molecular weight cut-off 5 kDa, Harvard Apparatus, Holliston, MA) the liquiritigenin solution was dialyzed against the microsomal solution or phosphate buffer only (200 µL per well, n=6 per experiment) at 37°C in a Big Shot II rotator oven (Boekel Scientific, Feasterville, PA) for 24 hours. The volume of liquid in each well was checked to ensure that no volume increase or decrease occurred due to osmosis. Seventy-five µL of ice-cold zinc sulfate (0.2 mol/L)/ methanol (3/7 *v/v*) containing 1 µg/L of the internal standard 2',4'-dihydroxychalcone and 300 µL of acetonitrile were added to 150 µL of the solution from each well. The samples were vortexed after each addition, shaken for 10 min, then centrifuged at 24,400g for 5 min. The supernatant was analyzed for liquiritigenin by LC/MS as described above. The fraction of unbound substrate (F_u) was determined by comparison of substrate concentration in wells originally containing the substrate. To confirm that

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equilibrium was achieved, control wells that contained no microsomal proteins were compared and equal concentrations of liquiritigenin were found.

Determination of the human cytochrome P450 enzymes responsible for the oxidative metabolism of liquiritigenin and interindividual variability.

The following E. coli expressed human cytochrome P450 enzymes were tested at 25 nmol/L: 1A1, 1A2, 2B6, 2C8, 2C9, 2 C9*2, 2C19, 2D6, 2 D6*2, 2 D6*10, 2 D6*39, 2E1, 3A4, and *E. coli* control membrane protein extracts. Solutions of liquiritigenin (0.04 mmol/L) and NADPH regenerating system in 0.1 M phosphate buffer were pre-incubated for 5 min, then P450 enzymes were added to start the reaction. Samples were incubated for 120 minutes and extracted as described earlier.

As a second approach, liquiritigenin was incubated with human liver microsomes isolated from 16 individuals using the procedure described above. The relative activities of individual cytochrome P450 enzymes among the individual microsomal preparations had been determined by the manufacturer using specific cytochrome P450 substrates (see Table 1). We correlated the activities of the individual cytochrome P450 enzymes with the formation of liquiritigenin metabolites. This experiment was also used to assess inter-individual variability of human oxidative liver metabolism of liquiritigenin.

Species-dependent differences in liquiritigenin oxidative liver metabolism.

Microsomes from the following species were tested: Rhesus monkey, Cynomolgus monkey, beagle, mini pig, guinea pig, Sprague Dawley rat, Fischer rat, CD1 mouse, and B6C3F1 mouse. A stock solution of NADPH regenerating system and liquiritigenin (0.04 mmol/L) in phosphate buffer (0.1 mol/L) was aliquoted into 1.5 mL conical polypropylene tubes with snap-on lids (n=6/ species). After a 5 min pre-incubation period, microsomes (0.4 mg/mL protein) were added to each sample, then incubated for 60 minutes at 37ºC prior to being extracted as previously described.

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Assessment of the β-estrogen receptor agonist activity of the major liquiritigenin metabolite.

After the major liquiritigenin metabolite generated by human liver microsomes was identified as 7,4'-dihydroxyflavone, its binding to and activation of estrogen receptors was examined and compared to the one of liquiritigenin.

Estrogen receptor binding assay. The relative binding affinity of 7,4'-dihydroxyflavone and liquiritigenin to pure, full-length receptors was determined using the estrogen receptor α and β competitor assay kits (Invitrogen Life Technologies, Carlsbad, CA). Fluorescence polarization of fluorophore-tagged estrogen bound to the α or β receptor, while in the presence of increasing amounts of competitor ligand or extract, was determined (10 readings per well; 0.02 millisecond integration time; G factor = 1.1087) using a GENios Pro microplate reader (Tecan Systems, San José, CA) with fluorescein excitation (485 nM) and emission (530 nM) filters. The vehicle ethanol was used as the negative control. Each 7,4'-dihydroxyflavone and liquiritigenin concentration was tested in triplicate.

Estrogen receptor transfection and luciferase assay. U2OS cells were grown to 85% confluency, trypsinized from 150 mm plates, centrifuged in 50 mL conical tubes, and resuspended in phosphate buffered saline containing 0.1% glucose. Cells were aliquoted (500 µL) into 0.4 cm cuvettes with 3 µg reporter plasmid and 1 µg of estrogen receptor α or β expression vectors. Cells were electroporated using a Genepulser II (Bio-Rad, Hercules, CA) and were resuspended in phenol red free DMEM/F12 media with 4% charcoal-dextran stripped fetal bovine serum. Cells were plated at 1 mL per well in 12-well plates and treated with varying dilutions of 7,4'-dihydroxyflavone or liquiritigenin overnight for 18 hours. Cells were lyzed with one freeze thaw cycle and 200 µL of 1x Reporter Lysis Buffer (Promega, Madison, WI). Activity was determined using the Luciferase Assay System (Promega) in a Veritas luminometer (Turner BioSystems, Sunnyvale, CA).

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Results

Human liver microsomes (pooled from 50 donors) metabolized liquiritigenin almost exclusively to 7,4'-dihydroxyflavone (Figure 1). The *m/z* of the metabolite $[M-H]^-$ of 253.0506 indicated a loss of two hydrogens. Comparison of the metabolite fragments (Figure 2B) with those of liquiritigenin (Figure 2A) showed that the hydrogen loss occurred at the C2 and C3-positions. The metabolite structure was confirmed using commercially available authentic 7,4'-dihydroxyflavone (Figure 2C). HPLC retention times, fragmentation patterns and exact masses of the parent and fragments were identical. In order to ensure that this was an enzymatic reaction and to exclude the involvement of electrophilic reactive intermediates, the incubation was performed in the presence of 10 mg/L and 1.5 g/L reduced glutathione (data not shown). In both cases, no glutathione adducts were found and the amount of metabolite formed was almost identical to the incubation without glutathione suggesting that no reactive electrophilic intermediates were involved in the liquiritigenin metabolism reaction observed after incubation with human liver microsomes. Also, we were unable to find any traces of hydroxylated metabolites which would be expected if the mechanism involves hydroxylation. No other metabolites were found using pooled human liver microsomes, not even when single ion mode (SIM) detection was used to specifically look for metabolites with m/z values compatible with the metabolites described by Nikolic and Van Breemen (2004) after incubation of liquiritigenin with rat liver microsomes. Incubation of the 7,4'-dihydroxyflavone itself yielded minimal amounts of two metabolites with a molecular weight gain of +16 (not identified).

Time and protein dependency of 7,4'-dihydroxyflavone formation by pooled human liver microsomes was established. Liquiritigenin was incubated with microsomes from 0-120 min. The reaction was linear up to 60 min. Protein dependency was tested over a microsomal protein concentration range from 0-2500 mg/L. The reaction was found linear up to 1000 mg/L protein. Based on these results, incubation times of 60 min and protein concentrations of 500 mg/L were chosen to assess the formation kinetics of 7,4'-dihydroxyflavone by pooled

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human liver microsomes. The formation of 7,4'-dihydroxyflavone followed Michaelis-Menten kinetics (Figure 3). The estimated apparent K_m was 128 µmol/L and the apparent V_{max} was 32.5 nmol/g protein/min. The unbound intrinsic clearance was calculated: $CL_{int} = V_{max}/ K_m \cdot F_u$, where F_u is the unbound fraction of substrate in solution during microsomal incubation. An average F_u of 0.84 was calculated from equilibrium dialysis experiments. Based on this data, the apparent CL_{int} for 7,4'-dihydroxyflavone formation was 0.3 mL/g/min.

In specific cytochrome P450 enzyme assays, cytochrome P450 enzymes 2C8, 2C9, 2E1 and 3A5 generated small amounts of 7,4'-dihydroxyflavone, while cytochrome CYP3A4 clearly produced the most significant amounts (Figure 4). Inter-individual variation was studied using hepatic microsomes from 16 human donors. The formation rates of 7,4'-dihydroxyflavone ranged from 3.4 to 32.2 nmol/g protein/min (Figure 5) indicating up to 9.5-fold inter-individual differences. No correlation between 7,4'-dihydroxyflavone formation rates and ethnic background, gender or age was determined, however due to the limited number of microsomal preparations in this study (16 individuals) no conclusive information was obtained. Testosterone hydroxylation is known to be catalyzed by cytochromes P450 3A4 and 3A5 (Chang et al., 1963). When 7,4'-dihydroxyflavone formation was plotted against the testosterone hydroxylation rates, as provided by the manufacturer of the individual human liver microsomes, there was a significant correlation (r^2= 0.896; Figure 6 and Table 1), further supporting this isozyme's primary role in the oxidative metabolism of liquiritigenin. Interestingly, microsomes of the individuals with the highest cytochrome P450 3A4/5 activity also generated traces of two hydroxylated metabolites. However, again, the amounts were too small for further assessment of their structures. Specific activities of none of the other major cytochrome P450 enzyme activities tested by the manufacturer showed a significant correlation with the individual 7,4'-dihydroxyflavone formation rates (Table 1). Besides cytochrome P450 3A4/5, the best correlation (r^2= 0.57, statistically not significant) was found for cytochrome P450 2B6, however, incubation of liquiritigenin with the isolated cytochrome P450 enzyme did not produce any detectable 7,4'-dihydroxyflavone. Based on those results

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it was concluded that cytochrome P450 3A4/5 enzymes are mainly responsible for the metabolism of liquiritigenin to 7,4'-dihydroxyflavone in the human liver. This was further confirmed by inhibition studies with the specific cytochrome P450 inhibitor ketoconazole and specific CYP3A antibodies based on pooled human liver microsomes. A mean half-maximal inhibition concentration (IC_{50}) of 0.9 nmol/L was estimated for ketoconazole after curve-fitting using the Sigma Plot enzyme kinetics module and the inhibition constant (K_i) was determined to be 0.09 nmol/L. The maximum inhibition of liquiritigenin metabolite formation that could be reached with ketoconazole was 76.4%. Specific CYP3A antibodies (BD Gentest, Woburn, MA) inhibited 7,4'-dihydroxyflavone formation by pooled human liver microsomes by >50%.

Human microsomes were compared with microsomes from different animal species (Figure 7). The formation rates of 7,4'-dihydroxyflavone ranged from 1.7 to 5.0 nmol/g protein/min, with the exception of the Guinea pig that exhibited the lowest formation rates at 0.8 nmol/g protein/min.

The metabolite 7,4'-dihydroxyflavone binds to both, the estrogen receptors α and β (Figure 8A). However, the IC_{50} in the competitive binding assay based on fluorophore-tagged estrogen was 10-fold lower for the β- than for the α-receptor; 0.59 (0.48-0.71) µmol/L versus 6.2 (1.4- 26.3) µmol/L, all median (95% confidence interval). Most importantly, 7,4'-dihydroxyflavone specifically activated the β-receptor (EC_{50} 0.23 (0.14- 0.35) µM, median 95% confidence interval), while activation of the estrogen receptor α was not different from the vehicle control (Figure 8B). In comparison, IC_{50} values of the parent compound liquiritigenin in the competitive estrogen receptor binding tests were 2.8 µmol/L (median, 95% confidence interval: 2.1- 3.5 µmol/L) for estrogen receptor α, and 0.41 µmol/L (median, 95% confidence interval: 0.32- 0.50 µmol/L) for estrogen receptor β. Liquiritigenin activated the β-receptor (EC_{50}: 0.69 µmol/L) while activation of the estrogen receptor α was not different from the vehicle control.

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Discussion

In the only other published study of the oxidative metabolism of liquiritigenin, Nikolic and van Breemen (2004) found that rat liver microsomes metabolized liquiritigenin to 7,4'-dihydroxyflavone and several hydroxylated products, including 3'- and 1'-hydroxylated metabolites and two metabolites hydroxylated at the 5, 6 or 8 carbon (the exact position was not identified). *In vitro* metabolism of liquiritigenin with pooled human liver microsomes, however, yielded almost exclusively the 7,4'-dihydroxyflavone. Traces of two hydroxylated metabolites that could not be fully characterized were detected only when liquiritigenin was incubated with isolated CYP3A4 or after incubation with liver microsomes from an individual with high CYP3A activity. Based on our results, we conclude that 7,4'-dihydroxyflavone is the major metabolite generated by human liver microsomes. Incubation of the metabolite 7,4'-dihydroxyflavone with pooled human liver microsomes under the same conditions yielded only traces of two hydroxylated metabolites, though both structures could not be elucidated due to the low quantities generated.

Although we made an extensive effort to detect potential hydroxylated intermediates in the reaction pathway from liquiritigenin to 7,4'-dihydroxyflavone, none could be detected. Therefore we hypothesize a quinone methide intermediate rather than a hydroxylation-dehydration mechanism. Due to the very low intrinsic formation clearance of 7,4'-dihydroxyflavone (see also below), we were unable to generate sufficient quantities of the metabolite to confirm its structure by NMR spectroscopy. However, the high-resolution mass spectra including fragments in MS/MS spectra as well as the HPLC retention time of the metabolite were identical to that of authentic 7,4'-dihydroxyflavone material.

Since microsomes are a mixture of different enzymes only apparent enzyme kinetic parameters could be determined. The apparent maximum formation velocity (V_{max}= 32.5 nmol/g protein/min) is low in comparison to most clinically relevant CYP3A substrates. Also, the affinity of the substrate to the microsomal enzymes is relatively low as indicated by an apparent K_m value of 128 µmol/L.

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The intrinsic clearance (CL_{int}= V_{max}/K_m) is a parameter commonly used for quantitative *in vitro-in vivo* allometric scaling (Ashford *et al.*, 1995; Iwatsubo *et al.*, 1996; Houston and Carlile, 1997). It has been recognized that the binding of the substrate to microsomes can hamper the prediction of the metabolic clearance *in vivo*. Thus, correction of the K_m with the unbound substrate fraction during incubation (f_u) gives a much better correlation between *in vitro* drug metabolism and in vivo pharmacokinetics (Houston, 1994; Obach, 1996; Iwatsubu et al., 1997; Austin et al., 2002). The apparent intrinsic formation clearance after correction for non-specific protein binding of 7,4'-dihydroxyflavone with 0.30 mL/g/min is similar to that of pravastatin with 0.20 mL/g/min (Jacobsen et al., 1999). Microsomal oxidative metabolism of pravastatin is not considered a clinically relevant elimination pathway (Christians et al., 1998). This can also be assumed to be the case for liquiritigenin. However, flavonoids in general are readily conjugated by phase II enzymes (Zhang et al., 2007; Zamek-Gliszczynski et al., 2006). Previous studies demonstrated that liquiritigenin undergoes significant metabolism by conjugation in the rat (Shimamura et al., 1990; 1993), but no other species have been examined.

The only cytochrome P450 enzyme that converted liquiritigenin to 7,4'-dihydroxyflavone in significant amounts was CYP3A4, as demonstrated by incubations with isolated cytochrome P450 enzymes as well as correlation to specific cytochrome P450 enzyme activities in human liver microsomes from 16 individuals. CYP3A is the most abundant cytochrome P450 enzyme in humans and it has been estimated that CYP3A4 is the major drug metabolizing enzyme for nearly 50% of all currently marketed drugs (Yan and Caldwell, 2001; Shimada et al.,1994). This raises the issue of potential drug-drug interactions. A significant cytochrome P450 drug interaction may occur when two or more drugs compete for the same enzyme and when the metabolic reactions catalyzed by this enzyme constitute the major elimination pathway (Rowland and Matin, 1973; Lin and Lu, 1998). Furthermore, a large interindividual variability of metabolite formation by CYP3A enzymes in the human liver is well established

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(Shimada et al., 1994; Thummel et al., 1994). Based on our results, it is unlikely that drug-drug interactions at cytochrome P450 enzymes will affect liquiritigenin pharmacokinetics.

Tsukamoto et al. (2005) isolated several CYP3A inhibitors from licorice, among them the liquiritigenin glycoside (liquiritin), but it remains unclear as to whether liquiritigenin contributes to the CYP3A inhibitory effect of licorice. Our data suggests that clinically relevant competitive drug-drug interactions at microsomal enzymes seem rather unlikely. Since there was no relevant metabolism of liquiritigenin by cytochrome P450 enzymes other than CYP3A4, it is unlikely that drugs that interact with other cytochrome P450 enzymes will modify liquiritigenin elimination or that liquiritigenin will competitively inhibit their metabolism.

7,4'-Dihydroxyflavone showed significant activity as a β-estrogen receptor agonist. Its binding affinity to the estrogen receptor β as estimated based on the IC_{50} in the competitive estrogen binding assay was similar to that of its parent liquiritigenin, however binding to the estrogen receptor α was lower indicating better selectivity. The estrogen receptor luciferase assay suggested that the metabolite was a 3-fold more potent activator of the estrogen receptor β than the parent compound and activation was highly estrogen receptor β-specific. The contribution of both 7,4'-dihydroxyflavone and liquiritigenin to the positive effect of the drug on the relief of postmenopausal symptoms in humans is currently unknown. It was not possible to evaluate the human pharmacokinetics of 7,4'-dihydroxyflavone after formation by cytochrome P4503A enzymes from its parent liquiritigenin since the MF101 preparation contains 7,4'-dihydroxyflavone extracted from plants that cannot be differentiated from the metabolite. The efficacy of MF101 is most likely the result of a combination of multiple ingredients in the herbal drug, including these two key ingredients.

In conclusion, the oxidative metabolism of liquiritigenin in the human liver by cytochrome P4503A enzymes results mostly in the active metabolite 7,4'-dihydroxyflavone. However, the intrinsic formation clearance of the metabolite was relatively low and based on our data, it is reasonable to assume that, although active, the metabolite does not significantly contribute to the overall biological activity of liquiritigenin. Recent *in vivo* studies in rats confirmed this

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conclusion. After oral gavage and intravenous bolus injection, the metabolite was not detectable in most plasma samples and only sporadically in urine and feces (own unpublished data). Phase II metabolism of liquiritigenin could have greater relevance to its excretion in humans. Glucuronidation, sulfation and glutathione adduction reactions have been shown to be a major elimination pathway for a variety of flavonoids (Zhang et al., 2007; Zamek-Gliszczynski et al., 2006). Ongoing studies in our group are currently focused on phase II metabolism of liquiritigenin as the potential major elimination pathway of liquiritigenin in humans.

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Legends to Figures

Figure 1

*Metabolism of liquiritigenin by human liver microsomes. Th*e metabolite 7,4'-dihydroxyflavone is the almost exclusive product. LC/MS chromatograms $[M-H]^-$ are shown after incubating liquiritigenin with human liver microsomes for 0 min (**A**) and 60 min (**B**).

Figure 2

*MS/MS spectra of liquiritigenin (**A**), the metabolite of liquiritigenin formed by human liver microsomes (**B**) and the commercial 7,4'-dihydroxyflavone reference material (**C**).* Mass spectra were obtained in negative ion mode. Fragment ions were detected by high resolution MS/MS-time-of-flight spectrometry.

Figure 3

*Michaelis-Menten **(A)** and Lineweaver-Burk **(B)** plots of 7,4'-dihydroxyflavone formation by pooled human liver microsomes.* Increasing concentrations of liquiritigenin were incubated with 500 mg/L pooled human liver microsomes for 60 min at 37°C. Data points represent means ± standard deviations (n=6). Data was analyzed using the Sigma Plot enzyme kinetics software module (version 1.3).

Figure 4

Formation of 7,4'-dihydroxyflavone by isolated cytochrome P450 enzymes. Bars represent means ± standard deviations (n=6).

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Figure 5

Formation of 7,4'-dihydroxyflavone by human liver microsomes isolated from 16 different individuals. Bars represent means ± standard deviations (n=6). The majority of microsomal preparations were from Caucasians between the ages of 18-65 with the following exceptions: ID number 140 were liver microsomes isolated from an 8-year old African-American male, 158 were from a 36-year old African-American female, 215 from a 6-year old Caucasian male, 227 from a 55-year old Hispanic female, 236 from a 17-year old Asian male, and 352 from a 7-year old Hispanic female.

Figure 6

Correlation between cytochrome P4503A activity as measured by testosterone hydroxylation and 7,4'-dihydroxyflavone formation by human liver microsomes isolated from 16 different individuals. The correlation analysis is based on the mean 7,4'-dihydroxyflavone formation rates shown in Figure 7. The correlation between 7,4'-dihydroxyflavone formation and the activity of other specific activities of cytochrome P450 enzymes is listed in Table 1.

Figure 7

Formation of 7,4'-dihydroxyflavone by liver microsomes from different species. Following species were tested: Rhesus and Cynomolgus monkeys, Beagle, Minipig, Guinea pig, Sprague-Dawley and Fisher rat, CD1 and B6C3F1 mouse and human. Data points are presented as means ± standard deviations (n=6). The animal microsomes were pools from 3-1000 animals depending on animal size and as provided by the manufacturer. The human liver microsomes were a pool of 50 individuals.

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Figure 8

*Binding to **(A)** and activation of **(B)** estrogen receptors by the liquiritigenin metabolite 7,4'-dihydroxyflavone.* Binding was measured using a competitive estrogen binding assay and activity using an estrogen receptor luciferase reporter assay. All data points are means ± standard errors of the mean (n=3). Abbreviation: ER: estrogen receptor.

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Tables

Table 1

Cytochrome P450 enzyme	Specific substrates	Correlation coefficient r^2
1A2	EROD	0.011
2A6	Coumarin	0.150
2B6	S-Mephenytoin demethylation	0.571
2C8	Paclitaxel	0.403
2C9	Diclofenac	0.313
2C19	S-Mephenytoin hydroxylation	0.180
2D6	Dextromethorphan	0.136
2E1	Chlorzoxazone	0.082
3A4/5	Testosterone	0.896
4A9/11	Lauric acid	0.002
Total CYP450		0.701

Correlation between 7,4'-dihydroxyflavone formation by human liver microsomes isolated from 16 different individuals and specific activities of individual cytochrome P450 enzymes. The results of activity assays using specific cytochrome P450 substrates were taken from the data sheet supplied by the microsomes' manufacturer Xenotech (Lenexa. KS). Abbreviation: EROD: Ethoxyresorufin-O-deethylase. Total cytochrome P450 concentrations were measured using CO difference spectrum at 450 nm.

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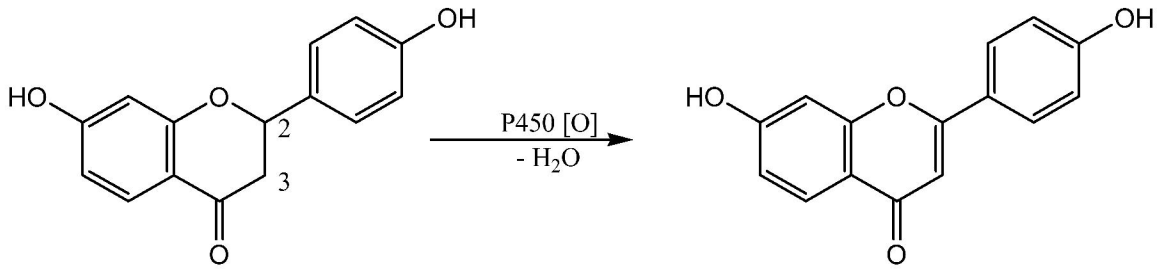
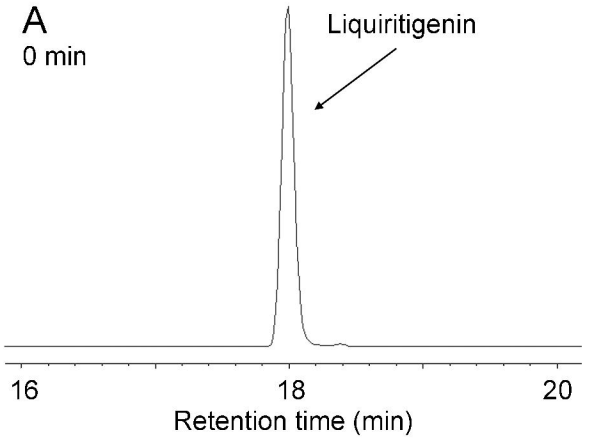
Figure 1



liquiritigenin

7,4'-dihydroxyflavone



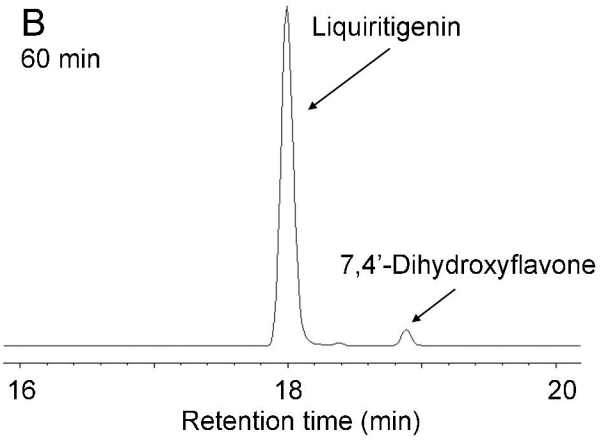
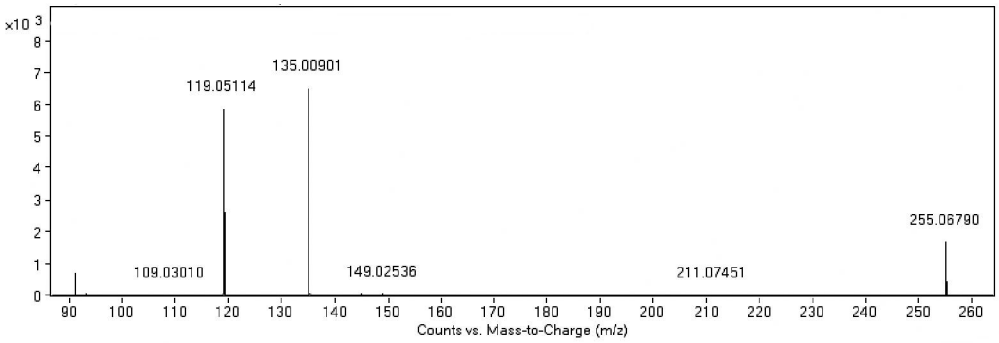
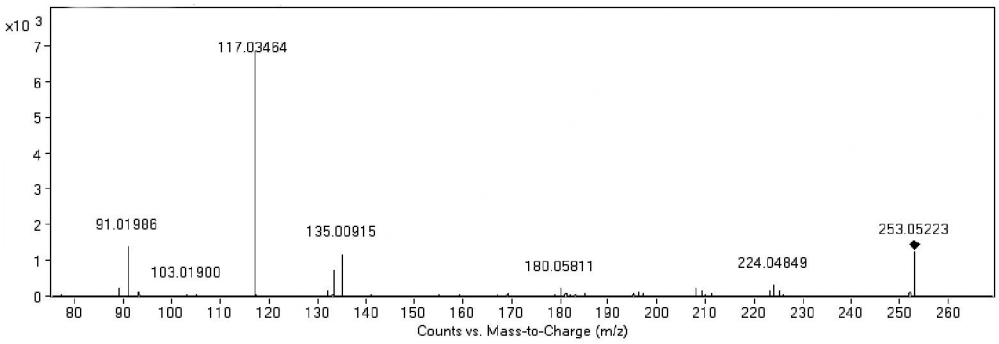


Figure 2

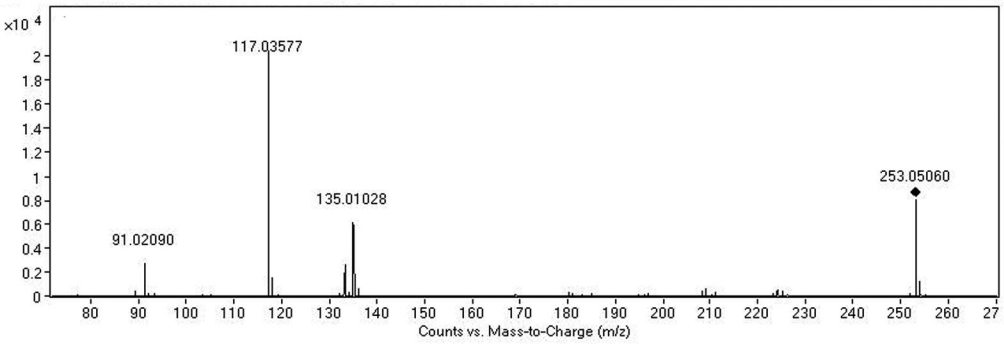
A


x10 3
135.00901
119.05114
255.06790
109.03010
149.02536
211.07451
Counts vs. Mass-to-Charge (m/z)

B


x10 3
117.03464
91.01986
135.00915
253.05223
103.01900
180.05811
224.04849
Counts vs. Mass-to-Charge (m/z)

C


x10 4
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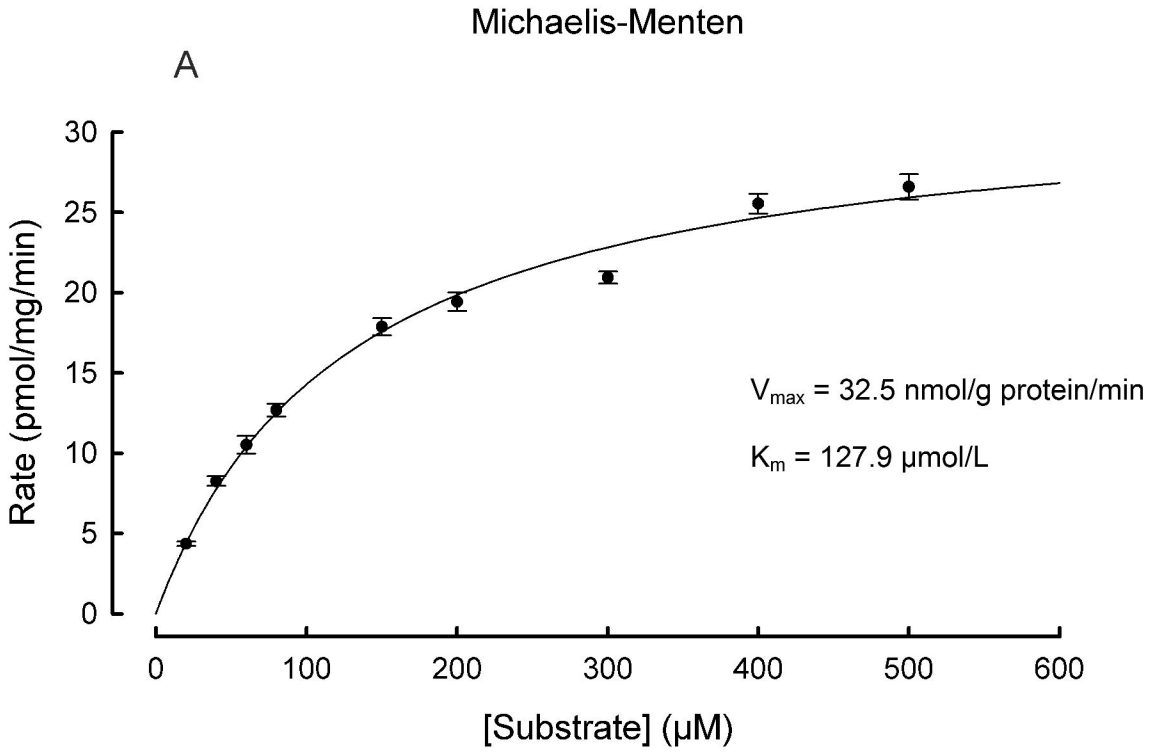
253.05060
135.01028
91.02090
Counts vs. Mass-to-Charge (m/z)

Figure 3


Michaelis-Menten
A
Rate (pmol/mg/min)
30
25
20
15
10
5
0
0
100
200
300
400
500
600
[Substrate] (μM)
V_{max} = 32.5 nmol/g protein/min
K_m = 127.9 μmol/L

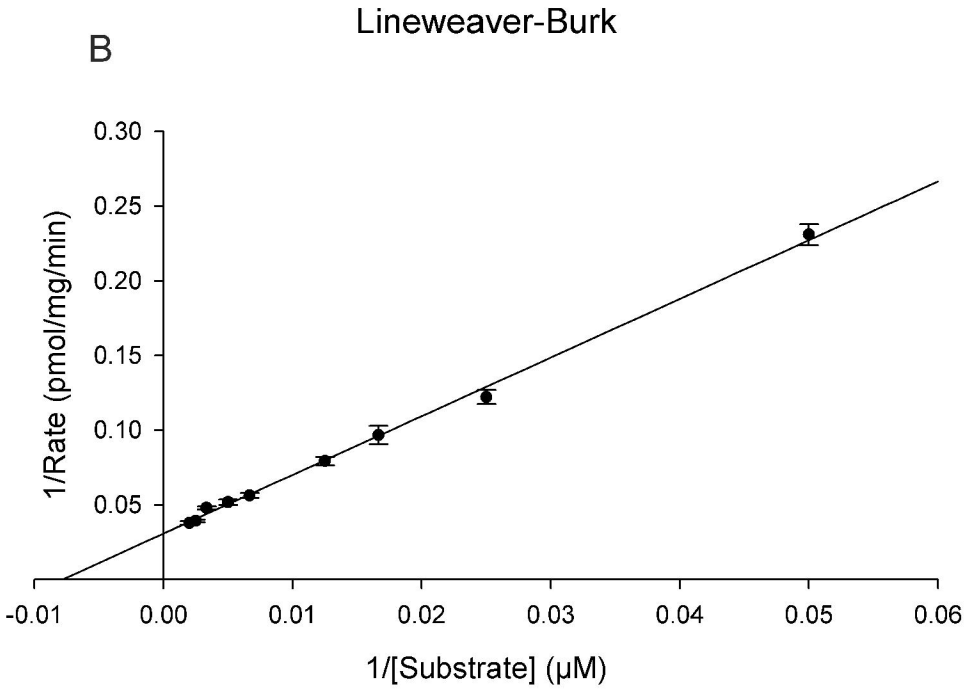

Lineweaver-Burk
B
1/Rate (pmol/mg/min)
0.30
0.25
0.20
0.15
0.10
0.05
-0.01
0.00
0.01
0.02
0.03
0.04
0.05
0.06
1/[Substrate] (μM)

Figure 4

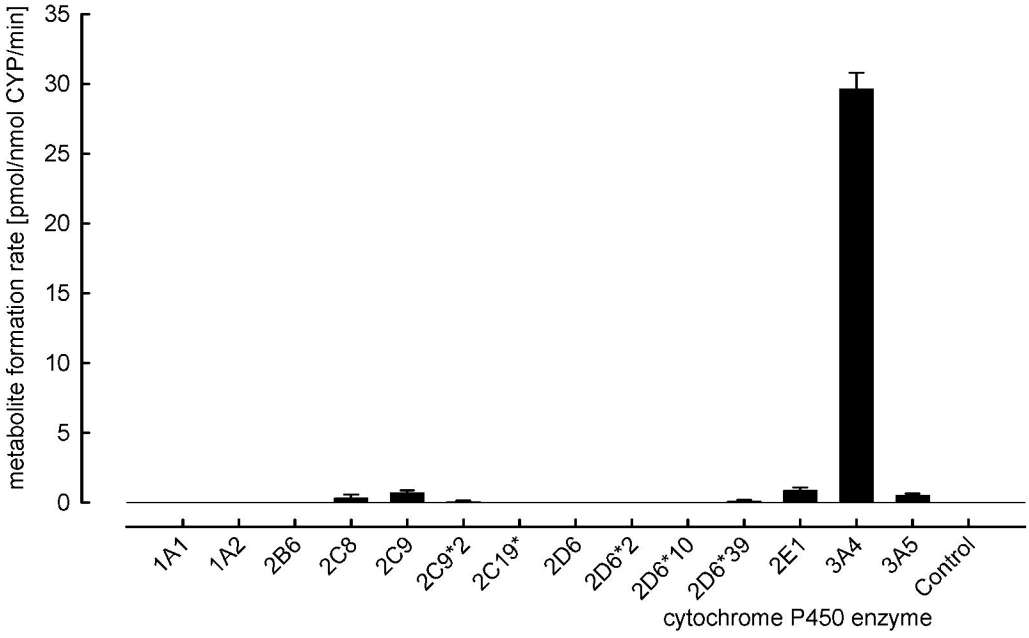

metabolite formation rate [pmol/nmol CYP/min]
35
30
25
20
15
10
5
0
1A1
1A2
2B6
2C8
2C9
2C9*2
2C19*
2D6
2D6*2
2D6*10
2D6*39
2E1
3A4
3A5
Control
cytochrome P450 enzyme

Figure 5

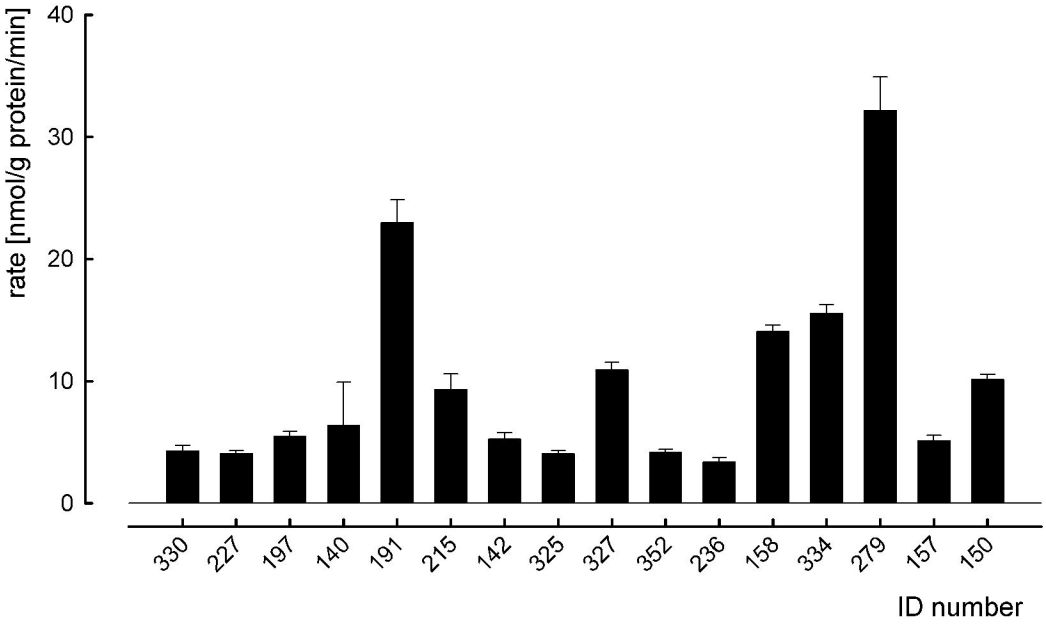

rate [nmol/g protein/min]
40
30
20
10
0
330
227
197
140
191
215
142
325
327
352
236
158
334
279
157
150
ID number

Figure 6

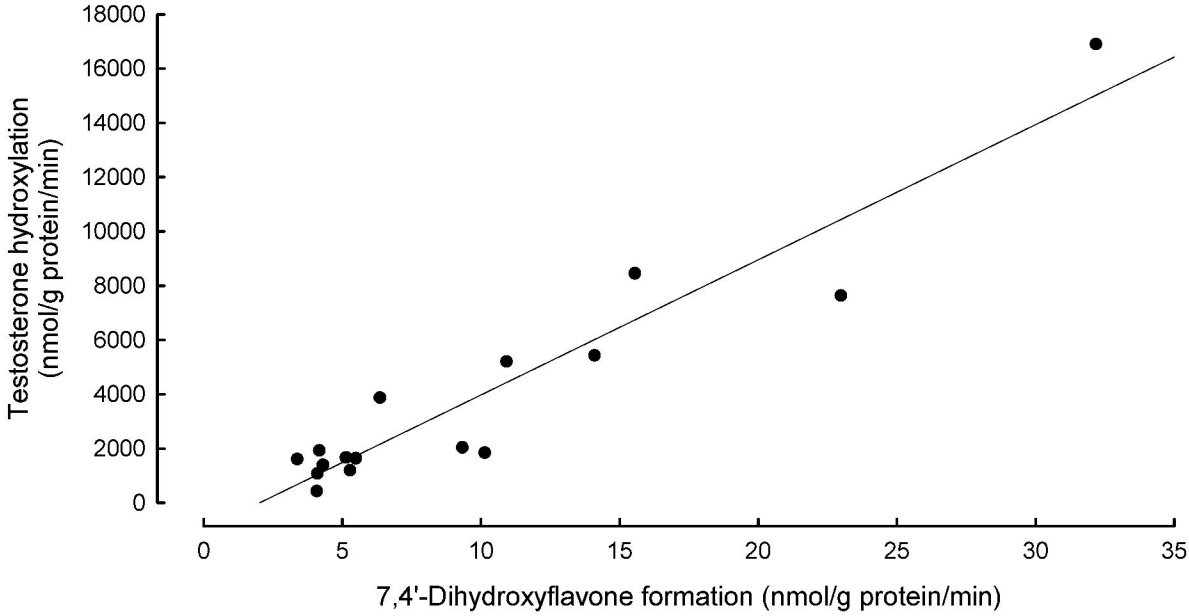

Testosterone hydroxylation
(nmol/g protein/min)
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
0
5
10
15
20
25
30
35
7,4'-Dihydroxyflavone formation (nmol/g protein/min)

Figure 7

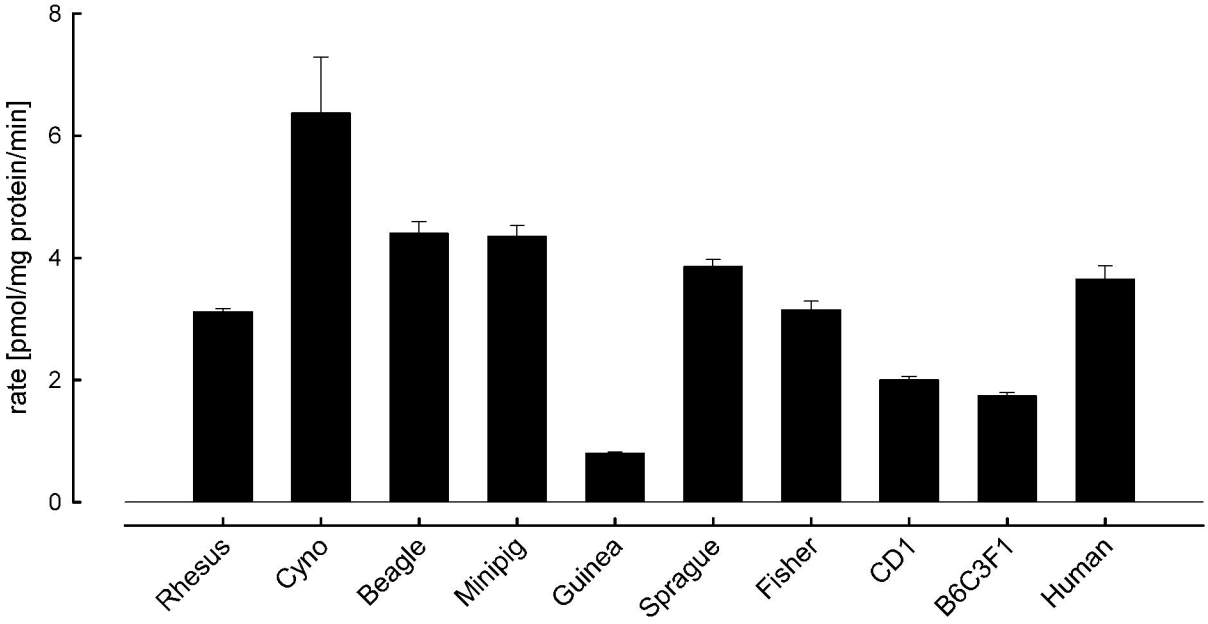
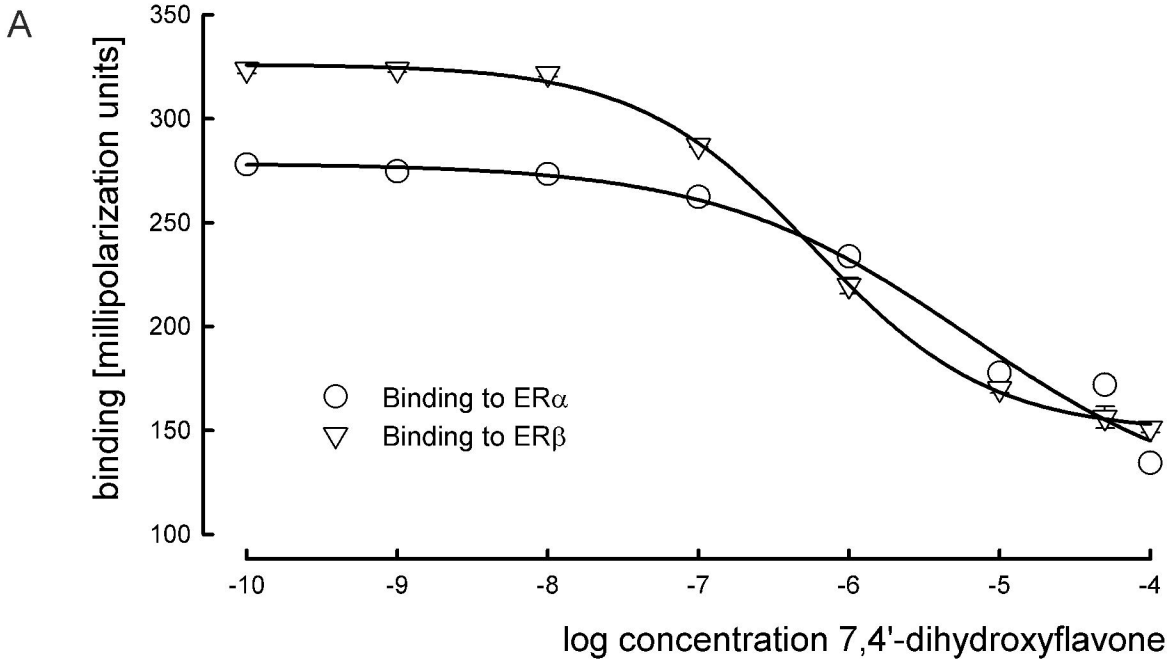
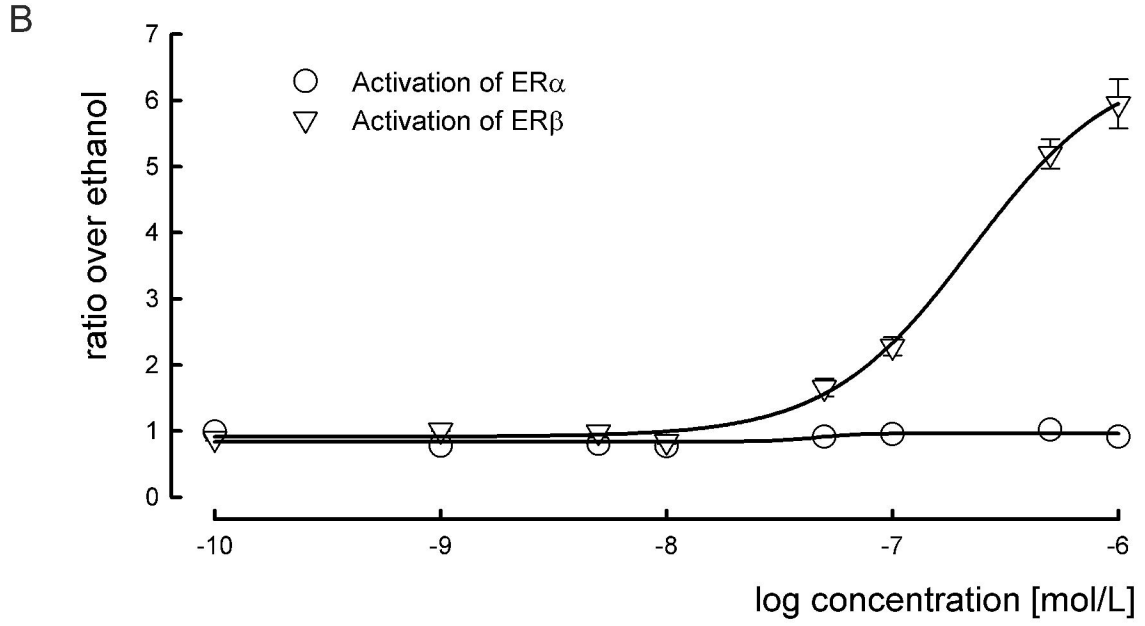

rate [pmol/mg protein/min]
8
6
4
2
0
Rhesus
Cyno
Beagle
Minipig
Guinea
Sprague
Fisher
CD1
B6C3F1
Human

Figure 8


A
binding [millipolarization units]
350
300
250
200
150
100
Binding to ERα
Binding to ERβ
-10
-9
-8
-7
-6
-5
-4
log concentration 7,4'-dihydroxyflavone


B
ratio over ethanol
7
6
5
4
3
2
1
0
Activation of ERα
Activation of ERβ
-10
-9
-8
-7
-6
log concentration [mol/L]