PHARMACOKINETICS AND DISPOSITION OF THE KAVALACTONE KAWAIN: INTERACTION WITH KAVA EXTRACT AND KAVALACTONES IN VIVO AND IN VITRO

JAMES M. MATHEWS, AMY S. ETHERIDGE, JOHN L. VALENTINE, SHERRY R. BLACK, DONNA P. COLEMAN, PURVI PATEL, JAMES SO, AND LEO T. BURKA

Department of Drug Metabolism and Pharmacokinetics, RTI International, Research Triangle Park, NC 27709 (J.M.M., A.S.E., J.L.V., S.R.B., D.P.C., P.P., J.S.), National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, NC 27709 (L.T.B).
Running title:

Modulation of metabolism by kava extract and kavalactones

James M. Mathews
RTI International
P.O. Box 12194
3040 Cornwallis Road
RTP, NC 27709
(919) 541-7461, FAX (919) 541-6499, e-mail mathews@rti.org

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ABBREVIATIONS: P450, cytochrome P450; Y, yangonin; K, kawain; LSS, liquid scintillation spectrometry; DMY, desmethoxyyangonin; DHM, dihydromethysticin; DHK, dihydrokawain; M, methysticin; Pgp, P-glycoprotein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Abstract:

Reported adverse drug interactions with the popular herb kava have spurred investigation of the mechanisms by which kava could mediate these effects. In vivo and in vitro experiments were conducted to examine the effects of kava extract and individual kavalactones on cytochrome P450 and P-glycoprotein activity. The oral pharmacokinetics of the kavalactone, kawain (100 mg/kg), were determined in rats with and without coadministration of kava extract (256 mg/kg) to study the effect of the extract on drug disposition. Kawain was well absorbed, with >90% of the dose eliminated within 72 h, chiefly in urine. Compared to kawain alone, coadministration with kava extract caused a tripling of kawain AUC_{0-8 h} and a doubling of C_{max}. However, a 7-day pretreatment with kava extract (256 mg/kg/day) had no effect on the pharmacokinetics of kawain administered on day 8. The 7-day pretreatment with kava extract only modestly induced hepatic P450 activities. The human hepatic microsomal P450s most strongly inhibited by kava extract (CYP2C9, CYP2C19, CYP2D6, CYP3A4) were inhibited to the same degree by a "composite" kava formulation composed of the six major kavalactones contained in the extract. K_i values for the inhibition of CYP2C9 and CYP2C19 activities by methysticin, dihydromethysticin, and desmethoxyyangonin ranged from 5–10 μM. Kava extract and kavalactones (≤ 9 μM) modestly stimulated P-glycoprotein ATPase activities. Taken together, the data indicate that kava can cause adverse drug reactions via inhibition of drug metabolism.
Kava kava, or kava, is an intoxicating beverage used by South Pacific Islanders and is traditionally prepared as an aqueous extract of the roots of the kava plant (*Piper methysticum*). Kava extracts have been used in Europe and North America as a mild anxiolytic and for their intoxicating properties. The physiological activity of kava resides in pyrone- or dihydropyrone-containing components called kavalactones. Six kavalactones (Fig. 1) account for approximately 96% of the total kavalactones (Lebot and Lévesque, 1989). Commercially available kava formulations have been primarily ethanol or acetone extracts, standardized to a specified kavalactone content. In 2002, sales of kava extracts were either severely restricted or prohibited in Europe due to several cases of serious hepatotoxicity attributed to kava consumption. These new reports of hepatotoxicity after centuries of apparently safe use in the South Pacific may be attributed to differences in the manner in which the commercial extract is prepared using organic solvents. The traditional aqueous extract is made from the rhizome and contains glutathione, which offers protection against hepatotoxicity (Whitton et al., 2003). Common commercial kavas are made from leaves and stems as well, which contain additional alkaloids with structural features that have been associated with toxic responses (Dragull et al., 2003, Nerurkar et al., 2004). Additionally, adverse drug interactions with kava have been attributed to inhibition of P450 enzymes by components of the extract resulting in increased toxicity of other drugs taken concurrently (Mathews et al., 2002).

The National Toxicology Program (NTP) has conducted 2-week and 13-week toxicology studies in rodents with kava extract (NTP, 2005). The 13-week study included oral doses from 0.125 to 2.0 g/kg/d administered over 5 days. Some mortality was observed in the 1.0 and 2.0 g/kg/d dose groups, generally during the first week. At these doses the animals were described as ataxic and lethargic, apparently due to the pharmacological action of the extract, but only after the first dose. After 13 weeks, centrilobular hypertrophy in mice and hepatocyte hypertrophy in rats was observed in the 1.0 and 2.0 g/kg groups. The effect was generally graded as minimal to mild. None of the animals exhibited hepatic necrosis. The low level of hepatotoxicity in rodents suggested that the reported toxicities in humans may have resulted from drug-drug-like interactions or some other indirect mechanism. Individual kavalactones do not exhibit the same biological activity as the whole extract, suggesting that the extract can modulate the metabolism and/or transport of one or more kavalactones. For example, yangonin (Y) possesses weak CNS activity following oral or intraperitoneal administration but has significant activity when given intravenously or in
combination with the other kavalactones (Meyer, 1967). Higher brain concentrations of kawain (K) or yangonin were observed in mice following administration of kava resin than from equivalent amounts of the individual kavalactones. We previously reported that some of the methylenedioxyphenyl-containing components of the kava extract effectively inhibit several cytochrome P450 enzymes and form carbene-P450 complexes in human liver microsomes (Mathews et al., 2002). Using probe-based assays indicative of CYP1A2, 2A6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A, and 4A9/11 catalytic activities, kava extract had the greatest inhibitory effect toward CYPs 2C9, 2C19, 2D6, and 3A. The kavalactones dihydromethysticin (DHM), methysticin (M), and desmethoxyyangonin (DMY) were especially potent, whereas kawain and dihydrokawain (DHK) had no effect at concentrations up to 10 µM.

In the present work, we have extended these earlier in vitro studies by examining the effects of the extract on the pharmacokinetics of kawain in rats. Kawain essentially acted as an example of a drug whose pharmacological/toxicological effect is altered by kava extract. Daily doses of kava commonly used in clinical trials for the treatment of anxiety (210 mg kavalactones) (Jellin et al., 2002) are equivalent to about 3 mg/kg/day, but consumption of 1–4 g kavalactones per day (ca. 60 mg/kg) has been reported for regular consumers of kava (Russmann et al., 2005). Accordingly, allowing for a reasonable allometric scaling factor between rat and human, a dose of 100 mg/kg was selected for the present studies in rat.
Materials and Methods

Chemicals. DL-Kawain was obtained from Indofine Chemical Co., Inc. (Belle Mead, NJ), and (+)-kawain was supplied by ChromaDex, Inc. (Laguna Hills, CA). Identity was verified by $^1$H and $^{13}$C NMR, and by GC/MS. (+)-Kawain was resolved from (-)-kawain present in the racemic mixture by HPLC, using a Chiralpak AD column (4.6 x 250 mm) and an isocratic mobile phase of 95:5 (v:v) hexane:isopropanol at a flow rate of 1 ml/min. Kawain enantiomers were detected by measuring absorbance at 245 nm using an Applied Biosystems 759A absorbance detector. Using this system (HPLC System A), the isomers eluted at 38 and 44 min and were present in approximately equal proportion. The fraction at 38 min coeluted with the (+)-kawain standard, as well as with a single peak, accounting for ca. 70% of the integrated area, present in kava extract. This peak was isolated and analyzed by GC/MS and $^1$H-NMR. Both spectra verified the identification of this peak as kawain. $[^{14}C]$DL-Kawain, randomly labeled with carbon-14 in the phenyl ring, was supplied by Wizard Laboratories (West Sacramento, CA). The specific activity of $[^{14}C]$kawain was 61.9 mCi/mmol. Radiochemical purity was determined using HPLC System A described above. The column effluent was monitored with an IN/US Systems, Inc. β-RAM radioactivity detector (Tampa, FL) equipped with a 600-μl glass solid scintillate cell. Following injection of $[^{14}C]$kawain, the column effluent was collected in fractions, and the radioactivity in each fraction was quantitated by liquid scintillation spectrometry (LSS). It was found to be 96.6% radiochemically pure, with $[^{14}C]$(+)-kawain accounting for 48% of the radioactivity. $[^{14}C]$(+)-Kawain was isolated using HPLC System A as described above for nonradiolabeled kawain. Isolated $[^{14}C]$(+)-kawain was reconstituted in methanol, and the radiochemical purity was determined to be ca. 95% by HPLC. Individual natural kavalactones isolated from kava, DMY, DHM, K, DHK, M, and Y were supplied by ChromaDex, Inc., and their identities were verified by GC/MS and $^1$H-NMR. Kava extract (Kavapure Kava PE 40%) was supplied by PureWorld Botanicals (South Hackensack, NJ). Kava extract was dissolved in methanol and analyzed by HPLC System A. This material was also analyzed to determine the content of the six major kavalactones using HPLC System A and the above standards. Alkamuls EL-620/L, a vegetable oil-based vehicle, was purchased from Rhodia (Cranbury, NJ).

NMR and Mass Spectrometry. $^{13}$C NMR spectra were obtained using a Brucker Avance 300 MHz NMR spectrometer; 500 MHz $^1$H-NMR spectra were obtained using a Bruker AMX-500 (500 MHz) NMR.
spectrometer. All NMR samples were dissolved in deuterochloroform prior to analysis. GC/MS data were obtained on a Hewlett Packard instrument consisting of an HP6890 Gas Chromatograph with a DB5-MS column (J & W Scientific, 30 m, 0.25 mm id, 0.25 µm film thickness) and a HP5973 MSD (mass selective detector). The injection port temperature was 250°C, and the oven temperature was 100°C during the first 2.5 min of the run and then ramped to 280°C at 15°C/min, and held at that temperature for 10 min. Ionization was achieved by electron impact (70 keV).

**HPLC Methods.** Kawain extracted from plasma was analyzed using HPLC System B consisting of a Phenomenex Luna C8(2) column (150 mm x 4.6 mm), with a mobile phase of acetonitrile and water at a flow rate of 1 ml/min. The initial mobile phase condition of 20% acetonitrile was held for 1 min and then increased to 80% acetonitrile linearly over 7 min. These conditions were held for 10 min and then returned to initial conditions linearly over 1 min. A Waters 2695 Alliance System was used to analyze the samples from pharmacokinetic studies. Detection and quantitation of kawain was made using a Waters 2487 absorbance detector monitored at 245 nm. To insure HPLC System B was sufficient to resolve kawain from other kavalactones, samples of kawain, kava plus kawain, and kawain plus kava extracted from plasma were submitted for LC-MS analysis. The HPLC column and gradient conditions described above were used for the analysis. Results indicated that the mass spectrum of the fraction containing kawain was identical to that of kawain standards, and was free of the analytes present in the adjoining fractions. Therefore, HPLC System B was used to quantitate kawain extracted from plasma obtained from rats that had received a co-administration of kawain and kava extract.

**Microsomal Incubations.** Incubations were prepared by adding either kava extract (dissolved in methanol) or an individual kavalactone (dissolved in acetone) to each tube for a final assay concentration of 1, 10, or 100 µM kavalactones. The solvent was allowed to evaporate. Control incubation tubes contained vehicle in place of kava extract or kavalactone. Similarly, a "composite" kava formulation comprised of kavalactones present in the same concentration as that of the extract was prepared such that a kavalactone composite at a final incubation concentration of 1 mM kavalactones contained 123 µM DHM, 217 µM K, 218 µM DHK, 245 µM Y, 71.8 µM DMY, and 128 µM M. Microsomes and assay buffer were added to control and treated tubes as described below, and the tubes were preincubated at 37°C for 10 min. NADPH was then added, and the incubations were maintained at 37°C for an additional 15 min.
Following initiation of the reactions by addition of probe substrate, assays were performed as described below.

**Cytochrome P450 Activity Assays.**

**Human Liver Microsomes:** Pooled samples of human liver microsomes were obtained from Tissue Transformation Technologies (TTT, Edison, NJ). The pools were a good representation of the average enzyme activities found for the inventories of TTT human liver microsomes. Incubations to determine the effect of kava extract or the individual kavalactones on acetalanilide hydroxylase (CYP1A2), coumarin 7-hydroxylase (CYP2A6), paclitaxel 6α-hydroxylase (CYP2C8), tolbutamide hydroxylase (CYP2C9), S-mephenytoin 4-hydroxylase (CYP2C19), dextromethorphan O-demethylase (CYP2D6), and p-nitrophenol hydroxylase (CYP2E1) activities were measured as previously described (Mathews et al., 2002). Midazolam 1'-hydroxylase (CYP3A) activity was measured using the method of Patki et al. (2003). The reaction mixture contained 6 µM midazolam, 50 mM phosphate buffer (pH 7.4), 0.06 mg microsomal protein, and 1 mM NADPH in a final volume of 0.25 ml. Reactions were initiated by the addition of midazolam and were allowed to proceed for 5 min at 37°C before termination by the addition of 100 µl ice cold acetonitrile. Following centrifugation, samples were analyzed for 1'-hydroxymidazolam by chromatography on a Zorbax Rx-C18 column with an isocratic mobile phase of 20:35:45 acetonitrile:methanol:10 mM potassium phosphate buffer, pH 7.4 and a flow rate of 1.4 ml/min. 1'-Hydroxymidazolam was detected and quantitated by measuring absorbance at 220 nm. Standards were prepared containing known amounts of 1'-hydroxymidazolam, and a standard curve was calculated by using linear regression analysis. Production of 1'-hydroxymidazolam was quantitated by linear regression of the area under the UV absorbance curve for each incubation sample.

Kinetic analysis of the inhibition of CYP2C9 by DMY, DHM, and M and of CYP2C19 by DHM in human liver microsomes was accomplished by modifying the conditions in the initial inhibition studies. For CYP2C9 kinetics, DMY, DHM, and M concentrations were 0–60, 0–30, and 0–25 µM respectively. Tolbutamide concentrations were 60–600 µM. For CYP2C19 kinetics, DHM concentrations were 0–15 µM and S-mephenytoin concentrations were 25–300 µM. Control and treated tubes containing microsomes and buffer were preincubated for 10 min at 37°C. Substrate was added and the tubes were preincubated for an additional 15 min prior to the initiation of the reaction by the addition of NADPH.
Each data set of measured enzyme activities as a function of substrate concentration at each inhibitor concentration was fitted individually to the Michaelis-Menten equation by non-linear regression using PRISM (GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, CA)). Estimates of $K_i$ (intercept of the $K_m/V_{max}$ axis) were obtained from linear regression of a plot of $K_m/V_{max}$ vs. inhibitor concentration.

**Rat liver microsomes:** Microsomes were prepared from male F344 rats treated with 1 g/kg or 256 mg/kg kava extract or vehicle for 7 days, and hepatic cytochrome P450 content and activities were determined as described above or as previously described (Mathews et al., 1998) for acetanilide hydroxylase (CYP1A2), dextromethorphan O-demethylase (CYP2D1), p-nitrophenol hydroxylase (CYP2E1), and midazolam 1'-hydroxylase (CYP3A1/2). 2α-, 6β-, and 16β-Testosterone hydroxylase activities, markers of CYP2C11, 3A1/2, and 2B1 activities, respectively, were determined by the method of Wood et al. (1983). Progesterone 21-hydroxylase activity, a marker for the activity of CYP2C6, was determined by the method of Swinney et al. (1987).

**P-glycoprotein ATPase Assay.** Human P-glycoprotein (Pgp), expressed in a baculovirus expression system obtained from Gentest Corp. (Woburn, MA), was used for determination of verapamil-stimulated, vanadate-sensitive ATPase activity. Reaction mixtures contained TRIS-MES buffer (50 mM TRIS-MES, 2 mM EGTA, 50 mM KCl, 2 mM dithiothreitol and 5 mM sodium azide), 40 μg Pgp membranes and 0, 0.087, 0.87, or 8.7 (+)-kawain or dihydrokawain (8.6 μM), desmethoxyyangonin (8.8 μM), yangonin (7.8 μM), or kava extract (2 μg/ml) in a final volume of 60 μl. Control ATPase reaction mixtures were prepared as described above with the addition of 100 μM sodium orthovanadate, an inhibitor of Pgp, to allow discrimination of non-Pgp-mediated ATPase activity. Reactions were initiated by the addition of 0.24 μmol MgATP and incubated for 20 min at 37°C prior to termination by the addition of 30 μl 10% SDS and Antifoam A. Following addition of 200 μl detection reagent (1:4 35 mM ammonium molybdate in 15 mM zinc acetate:10% ascorbic acid), reactions were incubated at 37°C for an additional 20 min. The liberation of inorganic phosphate was quantitated by measuring visible absorbance at 630 nm and comparing with a phosphate standard curve.
Animals. RTI International is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Animal procedures detailed in this report are in accordance with the "Guide for the Care and Use of Laboratory Animals" (National Research Council, 1996). Male F-344 rats (180–250 g) purchased from Charles River (Raleigh, NC) were provided Purina Rodent Chow and tap water ad libitum.

Pharmacokinetic Studies. Rats were anesthetized with ketamine/xylazine (7:1, 60 mg/kg) and an indwelling cannula was placed in the right external jugular vein one day prior to initial dosing for serial blood collection. Cannulae were Dow Corning silastic tubing, 0.025-inch inside diameter; 0.047-inch outside diameter. For the single i.v. dose pharmacokinetic study, rats (n = 4) received 7 mg/kg (+)-kawain, formulated in plasma:Alkamuls (80:20, v:v), by injection into the lateral tail vein. The total injection volume was 1 ml/kg. For oral dose pharmacokinetic studies, rats were divided into three treatment groups to investigate 1) the pharmacokinetics of a single dose administration of (+)-kawain (n = 4), 2) the effects of kava coadministration on (+)-kawain pharmacokinetics (n = 4), and 3) the effects of repeated dosing of kavalactones on (+)-kawain pharmacokinetics (n = 5). For the single oral dose administration study, rats received 100 mg/kg (+)-kawain only. In the coadministration study, (+)-kawain was formulated with kava such that the total dose of (+)-kawain delivered to rats was 100 mg/kg. The total kavalactone dose that each rat received was 256 mg/kg. In the repeated oral dose administration study, rats were dosed with kava by gavage for seven days. The kavalactone dose was 100 mg/kg on each day of dosing. On the day following the last kava dose (Day 8), rats received a single oral dose of 100 mg/kg (+)-kawain only. All oral doses contained (+)-kawain and/or kava formulated in Alkamuls:water (4:1, v:v) and were administered by intragastric gavage. The dose volume was 5 ml/kg. For the single oral and oral coadministration studies, blood was collected at predose and at 0.033, 0.083, 0.167, 0.25, 0.5, 1, 1.5, 2, 4, 6, and 8 h following kawain administration. For the repeated dose oral administration study, blood samples were drawn at predose, 0.083, 0.25, 0.5, 1, 3, 5, 10, 15, and 24 h. The volume of blood removed at each time point (ca. 300 µl) was replaced with an equal volume of heparinized plasma obtained from naïve rats. Blood samples were maintained on wet ice until processed for plasma by centrifugation at 9500g for 10 min. Plasma samples were maintained at -20°C until analyzed.
Analysis of Plasma Samples for Kawain: Plasma kawain content was determined by HPLC following extraction with methanol. Aliquots of ca. 0.1 ml plasma were extracted with 900 µl methanol, thoroughly mixed, then centrifuged at 9500g for 10 min. The methanolic supernatant was removed and stored in the dark at -20°C until further sample preparation. Just prior to HPLC analysis, supernatants were evaporated to dryness by vacuum centrifugation, and resuspended in 200 µl of 50:50 (v:v) acetonitrile:water. Samples were then placed into Ultra-free MC Centrifugal filtrate tubes (0.45 µm; Millipore, Bedford, MA and centrifuged for 10 min at 9500g. Filtrates (100 µL) were then analyzed by using HPLC System B. Plasma kawain concentrations were compared against a calibration curve generated by spiking naïve rat plasma with known concentrations of kawain. The kawain concentration ranges of the standard curves were 25–16000 ng/ml plasma in oral studies and 25–20,000 ng/ml plasma in i.v. studies. Linear regression of standard curves were performed after weighting the concentration-response curves by 1/X.

Pharmacokinetic Analysis: Plasma kawain concentration-time data from each rat were analyzed by noncompartmental pharmacokinetic methods using WinNonlin® (Pharsight Corporation, Cary, NC). Plasma kawain concentrations were converted from units of µg/g tissue, and are reported in units of µg/ml, assuming a plasma density of 1 g/ml (Brown et al., 1997). The following pharmacokinetic parameters were derived; \( C_{\text{max}} \), maximum achieved plasma concentration; \( T_{\text{max}} \), time to maximum observed concentration; \( \lambda_z \), terminal elimination rate constant, \( T_{1/2,\lambda_z} \), terminal elimination half-life; \( AUC_{0}^{8} \), area under the concentration-time curve from time zero to 8 h; \( AUC_{0}^{\infty} \), area under the concentration-time curve from time zero to infinity; \( V_{ss}/F \), apparent volume of distribution at steady state and \( Cl/F \), apparent oral clearance. Oral bioavailability \((F)\) following single dose oral administration was calculated as the ratio of the \( AUC_{0}^{\infty} \) following oral administration to that following i.v. administration and correcting for dose.

Disposition of \([^{14}C]\)Kawain in Rats. Intravenous and oral doses of (+)-kawain were formulated in plasma: Alkamuls (80:20, v:v) or Alkamuls, respectively, and contained 15–19 µCi radiolabel and an appropriate amount of nonradiolabeled (+)-kawain in a final dose volume of 1 ml/kg for i.v. doses and 5 ml/kg for oral doses. Single oral administration kava doses, co-administered with tracer levels of
[\textsuperscript{14}C\textsuperscript{(+)}]kawain, contained 17–19 µCi radiolabel and an appropriate amount of kava extract in a final dose volume of 5 ml/kg. Repeat oral administration kava doses were formulated in Alkamuls:water (4:1, v:v) and contained an appropriate amount of kava extract in a final dose volume of 5 ml/kg. Intravenous doses were drawn into a syringe equipped with a Teflon®-tipped plunger and a 27-gauge hypodermic needle and administered via the lateral tail vein. Oral doses were administered by gavage via a syringe equipped with a Teflon®-tipped plunger and a ball-tipped 16-gauge gavage needle.

Urine and feces were collected separately into round-bottom flasks cooled with dry ice. Collection intervals ended at 8 (urine only), 24, 48, and 72 h. Samples were stored in the dark at –20°C until analyzed. At the end of each experiment, rats were anesthetized with an intramuscular injection of ketamine:xylazine (7:1, 60 mg/kg). Blood was then withdrawn into a heparinized syringe by cardiac puncture. Animals were sacrificed by section of the diaphragm. Adipose tissues (3 samples), muscle (3 samples), skin (3 samples), as well as entire kidneys, liver, heart, lungs, and brain were removed and assayed for radioactivity content.

**Effect of Repeat Dosing of Kava Extract on P450 Levels in Rat.** Doses were formulated in Alkamuls:water (4:1, v:v) and administered in a dose volume of 5 ml/kg. Control values were from Alkamuls treated rats. Following repeat oral administration of 1 g/kg or 256 mg/kg kava extract (391 mg/kg and 100 mg/kg in kavalactones, respectively), or dose vehicle each day for seven days, animals were sacrificed and their livers excised and placed into individual beakers containing ice-cold 2.5 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer (pH 7.4) with 0.15 M KCl. The liver was homogenized in three volumes of ice-cold buffer using a Potter-Elvehjem homogenizer and then centrifuged at 9,000g for 20 min. Supernatants were centrifuged at 100,000g for 50 min. The resulting microsomal pellets were resuspended in fresh buffer and resedimented at 100,000g for 30 min. The washed microsomal pellet was resuspended in buffer (protein ca. 20 mg/ml), flash frozen in liquid N\textsubscript{2} and stored at –70°C.

Statistical Analyses: Pharmacokinetic values are reported as the mean ± SEM, obtained from the WinNonlin\textsuperscript{®} descriptive statistics module. Enzyme activities are reported as mean ± SD. Pharmacokinetic values and enzyme activities measured after repeat dose studies were compared by ANOVA followed by Dunnett’s test. Comparison of enzyme activity from in vitro inhibition studies using
whole kava extract and the reconstituted kava preparation were made using ANOVA followed by least-squares means comparisons. Statistically significant differences were determined at the $p < 0.05$ level.
Results

Inhibition of P450 Activity in Vitro in Human Liver Microsomes. We previously demonstrated that, of the nine human P450s examined, kava extract had the greatest inhibitory effect toward CYPs 2C9, C19, 2D6, and 3A (Mathews et al., 2002). As an extension of this work, the inhibition of CYP2C9, CYP2C19, CYP2D6, and CYP3A4 by a “composite” kava formulation comprised of kavalactones present in the same concentration as that of the extract was investigated, as were the kinetics of the inhibition of two of the forms most markedly effected, CYP2C9 and CYP2C19, in their interaction with three of their most potent kavalactone inhibitors, M, DHM, and DMY.

The kinetics of the inhibition of the CYP2C9-mediated 4-hydroxylation of tolbutamide by M and DMY, and of the CYP2C19-mediated 4-hydroxylation of S-mephenytoin are shown in Fig. 2. The \( K_i \) for the inhibition of this CYP2C9 activity by M and DMY was 5 and 9 \( \mu \)M, respectively, and that for the inhibition of CYP2C19 by DHM was 7 \( \mu \)M.

The inhibition of CYP2C9, CYP2C19, CYP2D6, and CYP3A by kava extract (39.1% kavalactones by weight) and by a composite mixture made by combining the six major kavalactones to concentrations equivalent to those in the kava extract was investigated. There were no statistically significant differences between extract and composite in the degree of inhibition of the activities of the four enzymes across the range of concentrations tested (1, 10, and 100 \( \mu \)M) (Fig. 3). DHK and Y were found to have little effect on those four enzymes, and the only statistically significant inhibition was a 30% inhibition of 2C9 activity by Y at 10 \( \mu \)M (data not shown).

Interaction of Kava Extract and Kavalactones with P-glycoprotein (Pgp). Membrane vesicles expressing human Pgp were incubated with kava extract and individual kavalactones. Following incubation with (+)-kawain at concentrations from 0–9 \( \mu \)M, Pgp ATPase activity increased in a concentration-dependent manner, but the activity was less than that of the positive control verapamil (20 \( \mu \)M) (Table 1). Further experiments with these vesicles were subsequently conducted, incubating with 8–9 \( \mu \)M dihydrokawain, desmethoxyyangonin, yangonin, and kava extract (3 \( \mu \)M in kavalactones). Pgp ATPase activities did not vary from control levels when vesicles were incubated with dihydrokawain, yangonin or kava extract. Incubation with desmethoxyyangonin resulted in a modest 2-fold increase in Pgp ATPase activity.
**Effect of Repeat Daily Dosing of Kava on Rat Cytochrome P450 Enzymes.** Following repeat oral administration of 256 mg/kg kava extract (100 mg/kg in kavalactones), to rats for seven days, total P450 content and the activities of hepatic CYP1A2, 2B1, 2C6, 2C11, and 2E1, were not significantly different from those of vehicle-treated control animals (Fig. 4). However, the activity of CYP2D1 was modestly increased (22%), and CYP3A1/2 activity, as measured by midazolam 1'-hydroxylase activity, was increased 72%. As measured by testosterone 6β-hydroxylase activity, CYP3A1/2 was not significantly increased. Following daily administration of 1 g/kg kava (391 mg/kg kavalactones) to male rats for seven days, total hepatic P450 content and the activities of all P450 enzymes measured were significantly altered. Hepatic P450 content was increased 35% in animals administered kava at this dose level, while the activities of CYP1A2, 2B1, and 2C6 were each increased >200% when compared to vehicle-treated control animals. There was also a 51% increase in the activity of CYP3A1/2, as measured by testosterone 6β-hydroxylase, while midazolam 1'-hydroxylase activity was increased 80%. In contrast to the marked induction of these enzymes, the activities of CYP2D1 and CYP2C11 were decreased 25 and 77%, respectively.

**Pharmacokinetics of Kawain in Rats.** Experiments were conducted to determine the pharmacokinetics of kawain following a single oral or i.v. dose, and after 7 days of repeated oral dose administration of kava. In addition, the pharmacokinetics of kawain coadministered with kava was investigated. Mean plasma kawain concentration-time curves following single i.v. and oral dosing, coadministration with kava, and after repeated dosing of kava are depicted in Fig. 5. Mean pharmacokinetic values are listed in Table 2.

Following i.v. administration, kawain was rapidly eliminated from the systemic circulation, decreasing from an initial plasma concentration ($C_0$) of 7.2 ± 0.6 µg/ml with a mean terminal half-life ($T_{1/2\lambda}$) of 0.63 h. By the last sampling time point, approximately 100% of kawain was eliminated from plasma. Systemic clearance and volume of distribution were 89 ± 6 ml/min/kg and 2.7 ± 0.9 l/kg, respectively, indicating a significant amount of kawain rapidly distributed out of the plasma into tissues and was rapidly cleared from the body.

Following single oral dose administration of 100 mg/kg (+)-kawain demonstrated that kawain was rapidly absorbed into the systemic circulation. Plasma $C_{max}$ (2.6 ± 0.2 µg/ml) was achieved by 24 h post
administration. Thereafter, plasma concentrations declined with a \( T_{1/2} \) of 1.3 h, which was approximately two times longer than the \( T_{1/2} \) following i.v. administration. The difference in the \( T_{1/2} \) estimates between i.v. and oral administration indicated that the rate of absorption across the gut was much slower than the rate of elimination of kawain from plasma. Mean bioavailability of kawain following a single oral dose (100 mg/kg) was appreciable at 50 ± 7%, relative to the 7 mg/kg i.v. bolus dose.

**Pharmacokinetics of Kawain After Coadministration with Kava.** Coadministration of kava extract with (+)-kawain in an oral dose formulation had a marked effect on the pharmacokinetics of kawain. With coadministration, the mean kawain \( C_{\text{max}} \) doubled to 5.4 ± 0.7 µg/ml, compared with oral administration of kawain alone, and the mean time to reach maximum plasma concentration (\( T_{\text{max}} \)) tripled to 2.5 ± 0.9 h. After \( C_{\text{max}} \) was attained plasma kawain concentrations were sustained to the last time point (i.e., 8 h). The terminal phase was not sufficiently quantitated to allow for accurate assessment of \( \lambda_z \) and subsequently, \( AUC_{0}^{\infty} \). Therefore, kawain \( AUC_{0}^{8} \) (AUC truncated to 0–8 h, Table 2) was estimated following all oral dose treatments for comparison of systemic exposure across oral kawain studies. Plasma kawain concentrations were generally higher at all time points compared with single dose administration of kawain alone. In addition, the kawain \( AUC_{0}^{8} \) values were approximately three times higher following coadministration of (+)-kawain with kava compared to (+)-kawain alone. This demonstrated a substantial increase in systemic exposure of kawain when coadministered with kava. However, as a result of the slow elimination from plasma, only approximately 78% of kawain (approximately 2 half-lives) was eliminated between the time of \( T_{\text{max}} \) and the 8 h time point, and additional pharmacokinetic measures could not be accurately estimated. However, the increase in \( AUC_{0}^{8} \) and an apparent prolonged half-life in animals coadministered kava and kawain compared with those animals that received kawain alone are indicative of drug-drug interactions that likely resulted in a decrease in systemic clearance.

**Pharmacokinetics of Kawain After Repeated Oral Dosing of Kava for 7 Days.** The average daily dose of kavalactones in kava administered for the 7-day period prior to the kawain administration was 102 ± 1 mg/kg. Of this amount, an average daily dose of 21.6 ± 0.2 mg/kg kawain was administered.
Kawain pharmacokinetics obtained following oral administration of (+)-kawain after seven days of repeated daily dosing with kava were not significantly different (student’s t-test; assuming equal variances) from those obtained following a single oral administration of (+)-kawain (Table 2). By 24 h it appeared that at least one kavalactone (i.e., kawain) present in kava was sufficiently cleared from the bloodstream. The rapid $T_{1/2z}$ following a single oral (+)-kawain dose (Table 2) predicted little accumulation of kawain with repeated daily dosing.

**Disposition of Oral Doses of $[^{14}C](+)$-kawain.** The disposition of 100 mg/kg oral doses of $[^{14}C](+)$-kawain is shown in Table 3. (+)-Kawain was primarily excreted in the urine following oral administration, with 77 ± 1% of the dose recovered from the urine in the 72 h following dose administration, and fecal excretion accounted for 14 ± 3% of the dose. About 0.4% of the dose was retained in the tissues sampled 72 h after administration of (+)-kawain, and tissue/blood ratios indicate that kawain does not have a propensity towards accumulation in any particular tissue. Relative excretion of radioactivity into urine and feces was virtually unchanged by 7 days of pretreatment with kava extract, but coadministration of kava extract shifted the profile to 58 ± 3% excreted in urine and 26 ± 8% in feces (data not shown). After an i.v. dose of kawain (7 mg/kg), 64 ± 5% and 19 ± 3% of the radioactivity were recovered in urine and feces, respectively.
Discussion

Although the metabolism of kawain has been studied by Rasmussen et al. (1979) and others using the racemic kavalactone, the present work is the first to report of the disposition of the naturally-occurring (+)-kawain in the absence of confounding effects of the (-)-isomer. The present studies extended previous in vitro work from this laboratory (Mathews et al., 2002) to determine 1) the inhibitory effects of DHK and Y toward human microsomal P450 activities; 2) the contribution, by the six kavalactones (comprising 39% by weight of the kava extract used), to inhibition observed with whole extract; 3) the enzyme kinetic constants for interactions with major P450s; 4) the interaction potential of all six major kavalactones with Pgp; and 5) the disposition of kawain in vivo in rats with and without coadministration or daily pretreatment with kava extract.

DHK and Y completed the initial investigations reported in Mathews et al. (2002). The only significant effect was a 30% inhibition of CYP2C9 by Y at a concentration of 10 µM. There were no statistically significant differences between the degree of inhibition of the major enzymes inhibited by kava (CYP2C9, CYP2C19, CYP2D6, and CYP3A4) and that by a composite mixture made to the same concentration of the six major kavalactones present in that extract. The six major kavalactones have been shown to be bioavailable in rat and mouse (Rasmussen et al., 1979; Keledjian et al., 1988). Evidence to date indicates that kavalactones are responsible for the inhibitory properties of kava, rather than other constituents such as the ubiquitous flavonoids, which are known CYP inhibitors, but are poorly bioavailable (Walle, 2004). The K_i for the inhibition of CYP2C9 activity by M and DMY, and that for the inhibition of CYP2C19 by DHM were all in the low micromolar range, suggesting that these naturally occurring compounds could be inhibitors in vivo. Unfortunately, human clinical trials designed to measure the circulating levels of kavalactones have been abandoned due to concerns about hepatotoxicity, making it difficult to predict whether the in vitro K_i values correspond to physiologically relevant concentrations. The kavalactones were found to be relatively poor Pgp substrates at concentrations as high as 2 µM, consistent with the findings of Hay et al. (2002).

In vivo experiments in rats demonstrated that coadministration of kava extract with kawain caused a tripling of kawain plasma AUC_{0-8 h} over that for kawain alone, and a doubling of C_{max}. A 7-day pretreatment with kava extract (256 mg/kg) caused no statistically significant changes in kawain
pharmacokinetics, consistent with the minimal changes in hepatic P450 levels found in a parallel group of animals administered the same repeat dose regimen. Induction studies conducted in rats administered 256 mg/kg daily doses of kava for seven days confirmed that only CYP3A activity was markedly induced, consistent with the induction pattern reported by Ma et al. (2004) with rat hepatocytes. At a higher daily dose of 1000 mg/kg, kava markedly induced CYP1A2, CYP2B1, and CYP2C6 activity, yet suppressed CYP2C11 activity. Excretion of radioactivity after either oral or i.v. administration of [14C]kawain ranged from ca. 80–90%; comparison of the extent of this presumably absorbed radioactivity with the calculated oral bioavailability of kawain (ca. 50%) indicates a substantial oral first pass extraction of this kavalactone.

Mechanisms by which kava extract altered the pharmacokinetics of kawain could include inhibition of cytochrome P450 and/or Pgp.. The current work indicates that inhibition of P450-mediated metabolism may well be the most important factor in the decreased clearance of kawain. An average single dose of extract containing 70 mg of kavalactones (Jellin et al., 2002) administered to a 70 kg person (1 mg/kg) and dissolced in 500 ml of gastrointestinal fluid (Yu et al., 2002) would achieve a concentration of 140 µg/ml (ca. 500–600 µM). At these concentrations, inhibition of intestinal Pgp and possibly P450 may increase absorption and explain the speculation of Keledjian et al. (1988) that kavalactones work synergistically to increase absorption. These authors also noted that levels of kavalactones rise synergistically in mouse brain, but the rat data and Pgp data reported in the present work suggest that circulating kavalactone concentrations may be too low to affect Pgp at the blood brain barrier. Additionally, these authors did not measure plasma concentrations of kavalactones, and the increased concentrations in brain may have been driven by the higher plasma concentrations found when a single kavalactone is coadministered with kava extract. Currently, there is a tendency to standardize herbal products to a single, presumably active, constituent. This work demonstrates that the presence of other constituents has great impact on the ultimate pharmacokinetics of the whole herb, and that a more accurate assessment of bioequivalence of preparations requires attention to those constituents as well.

In summary, kawain is absorbed and excreted at a moderate rate, mostly in urine, and does not accumulate in tissues. Kava extract increases the oral bioavailability of kawain in rat, and presumably that of drugs taken by patients using that extract concomitantly with pharmaceutical agents. Kavalactones, rather than other components of the extract, are responsible for the observed inhibition of
P450 enzymes, and this phenomenon is likely the most important factor in causing kava-mediated changes in pharmacokinetic profiles.
Acknowledgments. The authors are grateful to Kathy Ancheta for her assistance in preparation of the manuscript.
References


Footnotes

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**Fig. 1.** Chemical structures of the major kavalactones

**Fig. 2.** Kinetic analysis of P450 enzyme inhibition by kavalactones

**Fig. 3.** Activities of hepatic microsomal cytochrome P450 enzymes following incubation of human hepatic microsomes with whole kava extract or a composite kavalactone mixture

Human liver microsomes were incubated with kava extract or a composite kavalactone mixture and NADPH (for 15 min) prior to determination of individual P450 enzyme activities. *Expressed as tolbutamide hydroxylase. 100% value (control) = 0.06 +/- 0.00 nmol/mg protein/min. **Expressed as mephenytoin 4-hydroxylase. 100% value (control) = 5.43 +/- 0.69 pmol/mg protein/min. ***Expressed as dextromethorphan O-demethylase. 100% value (control) = 4.93 +/- 0.95 nmol/mg protein/h. ****Expressed as testosterone 6ß-hydroxylase. 100% value (control) = 2.26 +/- 0.49 nmol/mg protein/min. Values are means +/- standard deviations.

**Fig. 4.** P450 content and activities of hepatic microsomal cytochrome P450 enzymes following repeat oral administration of kava extract.

Rats were administered daily oral doses of (A) 256 mg kava extract/kg or (B) 1 g kava extract/kg for 7 days. Rats were sacrificed on day 8 and hepatic microsomes prepared prior to determination of individual P450 enzyme activities. Enzymes and their activities are as follows: *CYP1A2, acetanilide hydroxylase; **CYP2B1, testosterone 16ß-hydroxylase; ***CYP2C6, progesterone 21-hydroxylase; ****CYP2C11, testosterone 2α-hydroxylase; *****CYP2D1, dextromethorphan O-demethylase; ******CYP2E1, p-nitrophenol hydroxylase; *******CYP3A1/2, testosterone 6ß-hydroxylase, and ********midazolam 1'-hydroxylase. Values are means +/- standard deviations. *Significantly different from control (p<0.05).

**Fig. 5.** Mean plasma kawain concentration-time curves in rats following 7 mg/kg i.v. dose administration (panel A, ■) and 100 mg/kg oral doses of kawain (panel B). Single oral kawain dose administration (■), kawain coadministered with kava (×), repeated kava dose administration for 7 days followed by a single dose of kawain on day 8 (♦) are depicted in panel B. Error bars are standard deviation for mean concentrations at each time point.
Table 1.

Effect of (+)-Kawain and Individual Kavalactones on P-glycoprotein ATPase Activity

**A) (+)-Kawain**

<table>
<thead>
<tr>
<th>(+)-Kawain</th>
<th>ATPase Activity (nmol Pi/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (MeOH)</td>
<td>0.20 ± 0.12</td>
</tr>
<tr>
<td>87 nM</td>
<td>3.35 ± 0.00</td>
</tr>
<tr>
<td>0.87 µM</td>
<td>4.09 ± 1.04</td>
</tr>
<tr>
<td>8.7 µM</td>
<td>6.14 ± 0.08</td>
</tr>
<tr>
<td>Verapamil (positive control)(^a)</td>
<td>29.4 ± 4.1</td>
</tr>
</tbody>
</table>

**B) Kavalactones**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ATPase Activity (nmol Pi/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (MeOH)</td>
<td>1.41</td>
</tr>
<tr>
<td>Dihyrodiokawain (8.6 µM)</td>
<td>ND(^b)</td>
</tr>
<tr>
<td>Desmethoxyyangonin (8.8 µM)</td>
<td>2.97 ± 4.19</td>
</tr>
<tr>
<td>Yangonin (7.8 µM)</td>
<td>1.17 ± 4.65</td>
</tr>
<tr>
<td>Kava(^c)</td>
<td>1.73 ± 1.00</td>
</tr>
<tr>
<td>Verapamil (positive control)(^a) (20 µM)</td>
<td>29.6 ± 2.0</td>
</tr>
</tbody>
</table>

\(^a\) Verapamil was assayed at a concentration of 9 µg/ml.

\(^b\) Not detectable.

\(^c\) 2 µg extract/ml, 3.3 µM in kavalactones.
### Table 2.

Mean Plasma Kawain Pharmacokinetics in Male Rats

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Route</th>
<th>Kawain Dose*</th>
<th>Body Weight</th>
<th>$C_{\text{max}}$</th>
<th>$T_{\text{max}}$</th>
<th>$\lambda_z$</th>
<th>$T_{1/2z}^b$</th>
<th>$\text{AUC}_0^c$</th>
<th>$\text{AUC}_\infty^c$</th>
<th>$V_{ss}/F$</th>
<th>$\text{Cl/F}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single Dose</td>
<td>IV Mean</td>
<td>7.19</td>
<td>0.185</td>
<td>7.2$^d$</td>
<td>0</td>
<td>1.5</td>
<td>0.63</td>
<td>ND$^g$</td>
<td>82</td>
<td>2.7$^e$</td>
<td>89$^e$</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>0.058</td>
<td>0.0019</td>
<td>0.6$^d$</td>
<td>0.4</td>
<td>0.23</td>
<td>0.36</td>
<td>0.9$^d$</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single Dose</td>
<td>Oral Mean</td>
<td>96.7</td>
<td>0.208</td>
<td>2.6$^d$</td>
<td>0.88</td>
<td>0.58</td>
<td>1.3</td>
<td>530</td>
<td>550</td>
<td>32</td>
<td>190</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>0.659</td>
<td>0.00250</td>
<td>0.2$^d$</td>
<td>0.24</td>
<td>0.082</td>
<td>0.2</td>
<td>75</td>
<td>75</td>
<td>3</td>
<td>23</td>
</tr>
<tr>
<td>Co-administration</td>
<td>Oral Mean</td>
<td>99.3</td>
<td>0.203</td>
<td>5.4$^d$</td>
<td>2.5</td>
<td>ID$^f$</td>
<td>ID</td>
<td>1500$^g$</td>
<td>ID</td>
<td>ID</td>
<td>ID</td>
</tr>
<tr>
<td>of kava extract</td>
<td>SEM</td>
<td>0.897</td>
<td>0.00108</td>
<td>0.7$^d$</td>
<td>0.9</td>
<td></td>
<td></td>
<td>110</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repeated Dose</td>
<td>Oral Mean</td>
<td>102</td>
<td>0.188</td>
<td>4.7$^d$</td>
<td>0.4</td>
<td>0.57</td>
<td>1.5</td>
<td>420</td>
<td>450</td>
<td>28</td>
<td>260</td>
</tr>
<tr>
<td></td>
<td>SEM</td>
<td>1.53</td>
<td>0.00222</td>
<td>1.2$^d$</td>
<td>0.2</td>
<td>0.15</td>
<td>0.4</td>
<td>61</td>
<td>75</td>
<td>5</td>
<td>44</td>
</tr>
</tbody>
</table>

* Dose at single or last treatment administration.

$^b$ Calculated as harmonic mean.

$^c$ $C_0$ obtained by extrapolation of concentration-time data to time zero.

$^d$ ND – Not determined.

$^e$ Calculated using 100% bioavailability.

$^f$ ID – Insufficient data available for calculation.

$^g$ Statistically different from single dose oral administration treatment group (ANOVA, P<.05). IV-treated animals were not compared against other groups for statistical evaluation.
Table 3.
Disposition of Radioactivity 72 h After an Oral Dose of [\(^{14}\)C]Kawain (100 mg/kg) to Male F-344 Rats\(^a\)

**Cumulative Excretion of Radioactivity**

<table>
<thead>
<tr>
<th>End of Collection Period (h)</th>
<th>Percent of Dose Recovered in:</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urine</td>
<td>Feces</td>
</tr>
<tr>
<td>8 h</td>
<td>31.1 ± 5.3</td>
<td>31.1 ± 5.3</td>
</tr>
<tr>
<td>24 h</td>
<td>62.2 ± 4.7</td>
<td>6.9 ± 3.9</td>
</tr>
<tr>
<td>48 h</td>
<td>71.1 ± 2.9</td>
<td>13.1 ± 2.7</td>
</tr>
<tr>
<td>72 h</td>
<td>76.8 ± 1.2</td>
<td>13.8 ± 2.7</td>
</tr>
</tbody>
</table>

**Distribution in Tissues**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>ng-eq Kawain per g Tissue</th>
<th>Tissue/Blood Ratio</th>
<th>% Dose in Total Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipose</td>
<td>439 ± 363</td>
<td>0.390 ± 0.379</td>
<td>0.0417 ± 0.0358</td>
</tr>
<tr>
<td>Blood</td>
<td>1240 ± 243</td>
<td>UNITY</td>
<td>0.0810 ± 0.0136</td>
</tr>
<tr>
<td>Brain</td>
<td>38.8 ± 4.7</td>
<td>0.0317 ± 0.0039</td>
<td>0.0003 ± 0.0000</td>
</tr>
<tr>
<td>Heart</td>
<td>309 ± 30.5</td>
<td>0.253 ± 0.038</td>
<td>0.0009 ± 0.0001</td>
</tr>
<tr>
<td>Kidney</td>
<td>2370 ± 153</td>
<td>1.95 ± 0.35</td>
<td>0.0175 ± 0.0008</td>
</tr>
<tr>
<td>Liver</td>
<td>3150 ± 266</td>
<td>2.58 ± 0.42</td>
<td>0.129 ± 0.006</td>
</tr>
<tr>
<td>Lung</td>
<td>497 ± 34</td>
<td>0.408 ± 0.061</td>
<td>0.0018 ± 0.0001</td>
</tr>
<tr>
<td>Muscle</td>
<td>121 ± 15</td>
<td>0.101 ± 0.028</td>
<td>0.0432 ± 0.0065</td>
</tr>
<tr>
<td>Skin</td>
<td>622 ± 155</td>
<td>0.505 ± 0.132</td>
<td>0.104 ± 0.026</td>
</tr>
</tbody>
</table>

Total in tissues 0.420 ± 0.0590

\(^a\) n = 4.
Figure 1.

- Yangonin (Y)
- 5,6-Dehydrokawain (Desmethoxyyangonin, DMY)
- Methysticin (M)
- 7,8-Dihydromethysticin (DHM)
- Kawain (K)
- 7,8-Dihydrokawain (DHK)
Figure 2.

A. CYP2C9 inhibition by DMY

\[ K_{i} = 9.2 \, \mu M \]

B. CYP2C9 inhibition by M

\[ K_{i} = 5.0 \, \mu M \]

C. CYP2C19 inhibition by DHM

\[ K_{i} = 6.5 \, \mu M \]
Figure 3.
Figure 4.

A. 256 mg/kg/day kava extract

B. 1.0 g/kg/day kava extract
Figure 5.

A

B

Plasma Kawain (μg/g)

Plasma Kawain (ng/g)

Time (h)

Time (h)