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Effects of Kava (Kava-kava, 'Awa, Yaqona, *Piper methysticum*) on c-DNA-expressed cytochrome P450 enzymes and human cryopreserved hepatocytes

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Summary

The effects of the herbal product kava (*Kava kava*, 'Awa, Yaqona, *Piper methysticum*) on human P450 isoforms were studied *in vitro* using both cDNA-expressed human enzymes and cryopreserved human hepatocytes. Increasing concentrations of an ethanolic extract of dried kava root and three purified kava lactones (methysticin, desmethoxyyangonin, and yangonin) were tested for their ability to inhibit the catalytic activity of a panel of P450 isoforms (1A2, 2A6, 2C9, C2C19, 2D6, 2E1, and 3A4) present as c-DNA expressed-enzymes and in previously cryopreserved human hepatocytes. In addition, the test compounds' effect on hepatocyte viability was evaluated by measuring cellular ATP content. In both models, the kava extract and the three kava lactones were found to be potent inhibitors of CYPs 1A2, 2C9, 2C19, 2E1, and 3A4 with IC_{50} values of approximately 10 μM. The test compounds were also moderately cytotoxic to human hepatocytes (EC_{50} values of approximately 50 μM). Methysticin was the most potent enzyme inhibitor as well as the most cytotoxic, followed by (in order of potency:) the kava root extract, desmethoxyyangonin, and yangonin. Our results suggest that the drug interaction and hepatotoxic potential of kava should be further investigated.

Key words: Kava, P450 inhibition, cytotoxicity, *Piper methysticum*, hepatocytes, hepatotoxicity

Introduction

Kava is the term applied to both the plant (*Piper methysticum* Forster f.) and to the mildly psychoactive beverage from which it is prepared. Kava is cultivated and used traditionally throughout the South Pacific for its relaxing effects and has been described as a "social lubricant" that facilitates the smooth running of daily life (Lebot et al. 1997). Kava extracts and resins have been used in European phytomedicines as sedatives, tranquilizers, and muscle relaxants, and for the treatment of menopausal symptoms, urinary tract and bladder disorders (Blumenthal et al. 2000; Lindenberg and Pitule-Schodel, 1990; Warnecke, 1991). In the US, kava is sold as a dietary supplement under the provisions of the Dietary Supplemental Health and Education Act of 1994, and as of 2000, it was the ninth most popular herbal product in terms of global sales (Blumenthal, 2001). The kava lactones are considered to be the major psychoactive components of kava. Approximately 17 have been characterized and six – kavain, methysticin, dihydrokavain, dihydromethysticin, yangonin, and desmethoxyyangonin, (chemical structures shown in Fig. 1) – are considered to be the most important pharmacologically.

Earlier studies in one of our laboratories (GH) revealed that five kava lactones – kavain, methysticin, dihydrokavain, dihydromethysticin, and desmethoxyyangonin – inhibited at least one recombinant human CYP450 enzymes at a concentration less than 10 μM

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(Zou et al. 2002a). We conducted these current studies because the increased use of herbal products in the United States has raised concerns about their possible interactions with standard medications (Almeida and Grimsley, 1996; Cantor, 1997; Janetzky and Morreale, 1997; Miller, 1998; Cupp, 1999; Gold et al. 2001; Izzo and Ernst, 2001; Williamson, 2001) and because other dietary components such as grapefruit juice have been shown to alter the pharmacokinetics of co-administered drugs significantly (D'Arcy, 1995; Lewis et al. 1995; Schmiedlin-Ren et al. 1997; Fuhr, 1998). We reasoned that some herbal products might produce similar interactions and tested this hypothesis by evaluating 25 constituents of some commonly used herbal compounds *in vitro* for their ability to inhibit a panel of human recombinant CYP isoforms: CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 (Zou et al. 2002a). The compounds evaluated in this study were "marker" compounds (components of herbs thought to be associated with their biological activity or used for purposes of quality control) and the positive controls were drugs known to produce clinically significant drug-drug interactions. Increasing concentrations of the test compounds were incubated with specific surrogate substrates and the IC_{50} values (the concentration required to inhibit the metabolism of the surrogate substrates by 50%) were estimated then compared with those obtained for positive control inhibitory drugs (furafylline, sulfaphenazole, tranylcypromine, quinidine, and ketoconazole). This study revealed that components of garlic, St. John's Wort, *Ginkgo biloba*, and Kava were potent inhibitors ($IC_{50} \leq 10\ \mu M$) of recombinant human P450 enzymes. Kava was chosen for further evaluation as a potential inhibitor of drug metabolizing enzymes because five of the kava test compounds exhibited potent inhibition of P450 enzymes and because kava extracts have been associated recently with fatal hepatotoxicity (Strahl et al. 1998; Escher et al. 2001; Kraft et al. 2001; Russmann et al. 2001a; Russmann et al. 2001b), possibly through the formation of reactive metabolites (Russmann et al. 2001a).

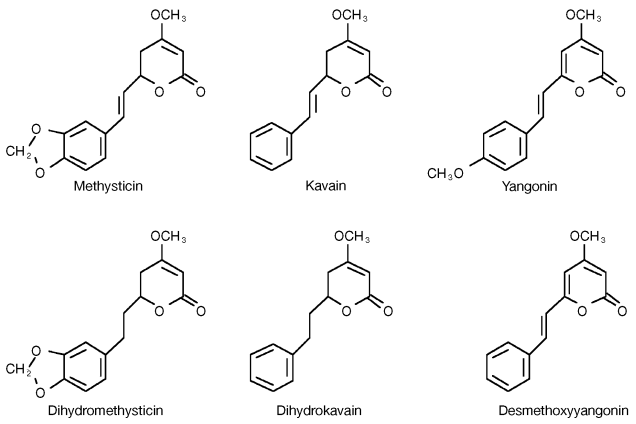

Fig. 1. Chemical formulae of the six most abundant lactones found in the ethanolic extract of dried kava root.

In the study reported herein, we tested an ethanolic extract of kava and three purified kava lactones (methysticin, desmethoxyyangonin, and yangonin) for their effects on P450 enzymes expressed in a baculovirus/insect cell system (the recombinant CYPs described above) and in cryopreserved human hepatocytes. Methysticin and desmethoxyyangonin were chosen because they were found to be two of the most potent enzyme inhibitors in our previous study. Yangonin was evaluated in cryopreserved hepatocytes only because its strong native fluorescence and quenching prevented its analysis in recombinant P450 assays which use fluorogenic substrates (Zou et al. 2002b). We expanded our evaluation of kava's effects by including additional CYP isoforms not tested previously (i.e., 2A1 and 2E1) and by using intact cryopreserved human hepatocytes, which may be a physiologically relevant model of the liver. In this report we show that an ethanolic extract of kava root and three kava lactones – methysticin, desmethoxyyangonin, and yangonin – are potent inhibitors of human P450 catalytic activity and are moderately cytotoxic to human hepatocytes.

Materials and Methods

Test Compounds

The kava lactones desmethoxyyangonin, dihydrokavain, dihydromethysticin, kavain, methysticin, and yangonin were obtained from PhytoCal, Inc. (Addipharma, Hamburg, Germany). The purity of these compounds was certified by the supplier to be >97% and was verified in our laboratory by HPLC (see method below). A dried, ethanolic extract of kava root was kindly provided by Paulo Altaffer of Nat-Trop, a division of New World Enterprises, Inc., San Leandro, CA. Concentrations of the respective kava lactones in this extract were measured by HPLC (see method below and results in Table 3). cDNA-derived CYP450 isoforms, substrates, and fluorescent products were obtained from Gentest Corporation, Woburn, MA, or from the suppliers shown in Table 1. Cryopreserved human hepatocytes were prepared by InVitro Technologies, Baltimore, MD and the suppliers of substrates, metabolites, and positive controls used in this assay are shown in Table 2.

HPLC Analysis of the Kava Root Extract

Concentrations of the kava lactones (desmethoxyyangonin, dihydrokavain, dihydromethysticin, kavain,

methysticin, and yangonin) in the kava root extract were determined by HPLC using a modification of Method 101.007 of the Institute for Nutraceutical Advancement (INA) [http://www.nsfina.org/methods/kavaset.html]. All solvents were HPLC grade and were obtained from Fisher Scientific (Pittsburgh, PA). Analyses were performed using a Shimadzu HPLC Model LC-M8A (Columbia, MD) equipped with a system controller (Model SCL 10A), a photodiode array detector (Model SPD M10A), a controlled-temperature chamber (Model CTO-10AS) set at 40 °C, and a Perkin-Elmer Model ISS-100 autosampler (Norwalk, CT). The analytical column was a Beta Basic-8, 3 μm, 4.6 × 150 mm i.d. (Keystone Scientific, Bellefonte, PA) with flow rate 1 ml/min and a linear gradient mobile phase of 20 to 40% acetonitrile:isopropanol, 1:1 (B) in 0.1% phosphoric acid (A) from 0 to 20 min, 90% B from 20–25 min, and equilibration with 20% B from 25 to 30 min. The UV absorption was monitored at 190 to 400 nm with quantitation at 220 nm. Concentrations of the six kava lactones were determined by peak area using external standard analysis. A 5-point calibration curve (5, 10, 25, 50, 100 μg/ml) was prepared daily and quality control samples (5 and 100 μg/ml) were analyzed with each batch of samples to monitor precision and reproducibility of the method. Coefficients of vari-

Table 1. Experimental conditions for the cDNA-expressed P450 assays.

CYP	1A2	2A6	2C9	2C19	2D6	2E1	3A4
Amount Enzyme/ well (pmol)	0.5	1.0	1–2	0.5–1	1.5	2.0	2.0 (BFC) or 6.0 (BzRes)
Krebs-Heinslet Buffer	100 mM	None	25 mM	50 mM	100 mM	100 mM	200 mM
Pluronic F68	None	None	None	None	None	None	0.01% (only with BzRes)
Substrate	CEC	Coumarin	MFC	CEC	AMMC	MFC	BFC, BzRes
Concentration	5 mM	3 μM	75 mM	25 mM	1.5 mM	100 μM	50 mM
Source	Ultrafine Chemicals	Sigma-Aldrich	Sigma-Aldrich	Ultrafine Chemicals	Gentest	Sigma-Aldrich	Gentest
Metabolite	CHC	7-HC	HFC	CHC	AHMC	HFC	HFC (BFC) Resorufin (BzRes)
Source	Molecular Probes	Sigma-Aldrich	Gentest	Molecular Probes	Gentest	Gentest	Sigma-Aldrich (Resorufin) Gentest (HFC)
Inhibitor positive control	Furafylline	Tranyl-cypromine	Sulfa-phenazole	Tranyl-cypromine	Quinidine	DDTC	Ketoconazole
Concentration	100 μM	100 μM	10 μM	500 μM	0.5 μM	100 μM	5 μM
Source	Ultrafine Chemicals	Sigma-Aldrich	Ultrafine Chemicals	Sigma-Aldrich	Sigma-Aldrich	Sigma-Aldrich	Ultrafine Chemicals
Incubation time	30 min	15 min	45 min	45 min	45 min	45 min	30 min (BFC) 45 min (BzRes)
Excitation wavelength	410	390	410	410	390	409	410 (BFC) 544 (BzRes)
Emission wavelength	460	460	538	460	460	530	538 (BFC) 590 (BzRes)

Abbreviations: CEC, 7-Ethoxy–3-cyanocoumarin; CHC, 7-Hydroxy–3-cyanocoumarin; MFC, 7-Methoxy-4-trifluoromethylcoumarin; 7-HFC, 7-Hydroxy-4-trifluoromethylcoumarin; BFC, 7-Benzyloxy-4-trifluoromethylcoumarin; AMMC, 3-[2-(N,N-diethyl-N-methylamino)ethyl]-7-methoxy-4-methylcoumarin; AHMC, 3-[2-(N,N-diethylamino)ethyl]-7-hydroxy-4-methylcoumarin hydrochloride; BzRes, Resorufin Benzyl Ether; DDTC, Diethyldithiocarbamic acid; 7-HC, 7-hydroxycoumarin.

ation (% CV) for the six kava lactones were 4.8 to 11.6 at 5 μg/ml and 4.8 to 7.4 at 100 μg/ml.

cDNA-Expressed Enzyme Assays:
Test compounds were evaluated for their ability to inhibit the catalytic activity of seven cDNA-expressed human cytochrome P450 isoforms (CYP1A2, CYP2A6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4) by means of fluorometric inhibition assays conducted in 96-well (200 μl volume) microtiter plates (Corning Costar, Cambridge, MA). A 50% inhibitory concentration (IC_{50} value) was estimated for each test substance and each enzyme, according to the method of Crespi et al. (Crespi and Penman, 1997; Crespi, 1999). Assay conditions are described on the Gentest Corporation website (www.gentest.com), in our previous studies (Henderson et al. 1999; Zou et al. 2002a), and in Table 1. This method is described in detail on the Gentest Corporation website (www.gentest.com) and summarized in Table 1. Reaction conditions were optimized so that the generation of the products of each assay was linear over the range of concentrations used in these assays and the fluorescent signal of the products was 3 to 5 times background fluorescence.

Table 2. Experimental conditions for the cryopreserved hepatocyte assays.

CYP	1A2	2A6	2C9	2C19	2D6	2E1	3A4
Cells/well	0.5×10^6	0.5×10^6	0.5×10^6	0.5×10^6	0.5×10^6	0.5×10^6	0.5×10^6
Substrate	Phenacetin	Coumarin	Tolbutamide	s-Mephenytoin	Dextro-methorphan	Chlorzoxa-zone	Testosterone
Concentration	15 μM	8 μM	150 μM	20 μM	8 μM	100 μM	50 μM
Source	Sigma	Sigma	Sigma	BioMol Research	Sigma	ICN Biomedicals	Sigma
Metabolite	Acet-aminophen	7-OH Coumarin	4-OH Tolbutamide	4-OH s-Mephenytoin	Dextror-phan	6-OH Chlorzoxazone	6-OH Testosterone
Source	Sigma	Sigma	Sigma	BioMol Research	Sigma	ICN Biomedicals	Sigma
Positive Control	Furafylline	Tranyl-cypromine	Sulfa-phenazole	Omeperazole	Quinidine	4-Methyl pyrazole	Ketoconazole
Concentration	1 μM	1 μM	10 μM	25 μM	2.5 μM	250 μM	1 μM
Source	Gentest	Sigma	Sigma	Sigma	Sigma	Sigma	Research Diagnostics
Incubation time	1 hr	1 hr	1 hr	1 hr	1 hr	1 hr	1 hr
Incubation temp	37°	37°	37°	37°	37°	37°	37°
CO_2 conc	5%	5%	5%	5%	5%	5%	5%

Table 3. Kava lactone concentrations in the ethanolic extract of dried kava root.

Analyte	MW	Concentration (μg/mg extract)	Concentration (% extract)	Concentration (% total lactones)	Concentration (μmol/mg extract)
Desmethoxyyangonin	228.26	12.58	1.26	4.5	0.06
Dihydrokavain	232.26	69.72	6.97	25.0	0.30
Dihydromethysticin	276.26	38.23	3.82	13.7	0.14
Kavain	230.26	54.53	5.45	19.5	0.24
Methysticin	274.26	69.18	6.92	24.8	0.25
Yangonin	258.26	34.93	3.49	12.5	0.14
Total Kava Lactones		279.17	27.92	100.00	1.13

Briefly, test compounds were incubated with each cDNA-expressed P450 isoform, an NADPH regenerating system, and a fluorogenic probe. For all enzymes except CYP2D6, this buffer contained 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate and 0.4 U/ml glucose-6-phosphate dehydrogenase. For CYP2D6, the cofactor concentrations were 0.0082 mM $NADP^+$, 0.41 mM glucose-6-phosphate, and 0.4 U/ml glucose-6-phosphate dehydrogenase. Because of the broad specificity of the 3A4 isoform, two substrates were used for its evaluation: resorufin benzyl ether (BzRes) and (BFC) 7-benzyloxy-4-trifluoromethylcoumarin. Because yangonin exhibited such high intrinsic fluorescence at the excitation and emission wavelengths used in the fluorometric assays for CYPs 2C9, 2C19, and 3A4 (BzRes substrate) (Zou et al. 2002b) it could not be tested by this method and was evaluated only in the human hepatocyte model which does not use fluorogenic probes.

Test compounds (6 μl of a 10 mM acetonitrile stock solution) or positive control dissolved in acetonitrile were added to the first well in each row, mixed thoroughly, then diluted serially. Test compound concentrations ranged from 0.05–200 μM. Plates were pre-incubated at 37 °C for 10 min and the reaction initiated by the addition of 100 μl of pre-warmed enzyme/substrate (E/S) mixture. The E/S mixture contained buffer, cDNA-expressed P450, substrate, and Pluronic F68 (with BzRes only) as described in Table 1 and the amount was adjusted to give the final concentration shown in a reaction volume of 200 μl. Reactions were terminated at the various times shown in Table 1 by the addition of 75 μl of a 4:1, acetonitrile:0.5 M Tris base solution. Fluorescence per well was measured a Packard Fluorocount Microplate Fluorometer (Packard Instrument Company, Meriden, CT) using the excitation and emission wavelengths shown in Table 1. Generation of the products of each assay was linear over the range used for these assays. Data were exported and analyzed using an Excel spreadsheet. The IC_{50} values were calculated by linear interpolation.

Hepatocyte Cytotoxicity Assay

Test compounds were evaluated for non-specific cytotoxic effects on the cryopreserved hepatocytes by measuring their ability to interfere with ATP bioluminescence (Petty et al. 1995). Hepatocytes and the test compound (at concentrations of 1.0, 3.3, 10.0, 33.3, and 100 μM) were added to uncoated 24-well tissue culture plates (0.5×10^6 cells in 0.5 ml culture media per well) and incubated in a Forma incubator (Thermo Forma, Marietta, Ohio) at 37 °C/5% CO_2 for 4 h. The ATP substrate was added and amount of ATP measured using a Wallace Victor 1420 Multilabel Counter (Promega Corp., Madison, WI).

Hepatocyte Enzyme Inhibition Assays

Experimental conditions for the cryopreserved hepatocyte enzyme inhibition assays are summarized in Table 2. Cells were isolated, cryopreserved, and used according to previously published procedures (Li, 1997; Li et al. 1999; Easterbrook et al. 2001). Briefly, test compounds (at concentrations of 1.0, 3.3, 10.0, 33.3, and 100 μM), cryopreserved hepatocytes (mixed pool of 5 male and 5 female donors with ages ranging from 23–84 years old), substrates, and positive controls were incubated in Krebs-Heinslet Buffer using 24-well plates (500 μl of incubation medium per well) in a Forma incubator (Thermo Forma, Marietta, Ohio) at 37 °C/5% CO_2 for 1 h. The reaction was stopped with 500 μl of cold methanol or 100 μl of perchloric acid for S-mephenytoin or 150 μl acetonitrile for phenacetin. Samples were then centrifuged at 13,000 rpm for 10 min, the supernatant removed, and the amount of metabolites generated quantitated by HPLC (see methods below).

HPLC Analysis of Hepatocyte Incubate

Supernatants from hepatocyte incubations were analyzed without further treatment using a HP1100 Series high-performance liquid chromatography system consisting of a vacuum degasser, binary pumps, and both a variable wavelength and a fluorescence detector (Agilent Technologies, Menlo Park, CA). Luna C18 and phenylhexyl columns (2.00 × 150 mm. 4.6 × 250 mm, 4.6 × 150 mm; Phenomenex, Torrance, CA) were used for separation and the mobile phases consisted of combinations of acetonitrile, methanol, H_2O, trifluoroacetic, and/or ammonium acetate.

Results

Concentrations of Kava Lactones in the Kava Root Extract

Concentrations of the six kava lactones in the kava root extract are shown in Table 3. Total kava lactone concentration in the root extract was 28%, which is slightly higher than the 3–20% reported to be in the dried root (Lebot and Levesque, 1996), but considerably lower than some phytomedicine preparations standardized to contain 70% kava lactones (Malsch and Kieser, 2001). Ratios of individual lactones in the kava root extract are generally consistent with those reported for the dried plant (Kubatova et al. 2001); however, kava lactone concentrations vary considerably among various cultivars (Lebot and Levesque, 1996). The chemotype of the kava root extract used in the present study is similar to the chemotype of kava reported to be used in phytomedicines (i.e., dihydrokavain > methysticin > kavain > dihydromethysticin > yangonin > desmethoxy-

yangonin) except that dihydromethysticin was present in slightly higher concentrations than kavain.

Effects of Kava on the Catalytic Activity of cDNA-Expressed P450s

Data in Table 4 and Fig. 2 show that rCYP isoforms 1A2, 2C9, 2C19, 2E1 and 3A4 were inhibited by at least one of the kava test compounds, whereas isoforms 2A6 and 2D6 were not inhibited at 200 μM, the highest concentration tested. Kava root extract inhibited isoforms 1A2, 2C9, 2C19, and 3A4 at concentrations less than 10 ug/ml, a concentration equivalent to less than 2 μM for each of the individual kava lactones. Conversion of μg/mg kava root extract to μM was accomplished using values in Table 3. Methysticin was a potent inhibitor of 2C19 and 3A4 and a moderate inhibitor of 1A2, 2C9, and 2E1. Desmethoxyyangonin was a potent inhibitor of 1A2 and 2C19 and a moderate inhibitor of 2C9 and 3A4. Desmethoxyyangonin inhibited the catalysis of one 3A4 substrate (BFC) but not the other (BZREZ). Because of yangonin's high intrinsic fluorescence it was difficult to evaluate using this test system; however, yangonin clearly inhibited the 1A2 and 2C19 isoforms, but had no effect on 2A6, 2D6, or 2E1.

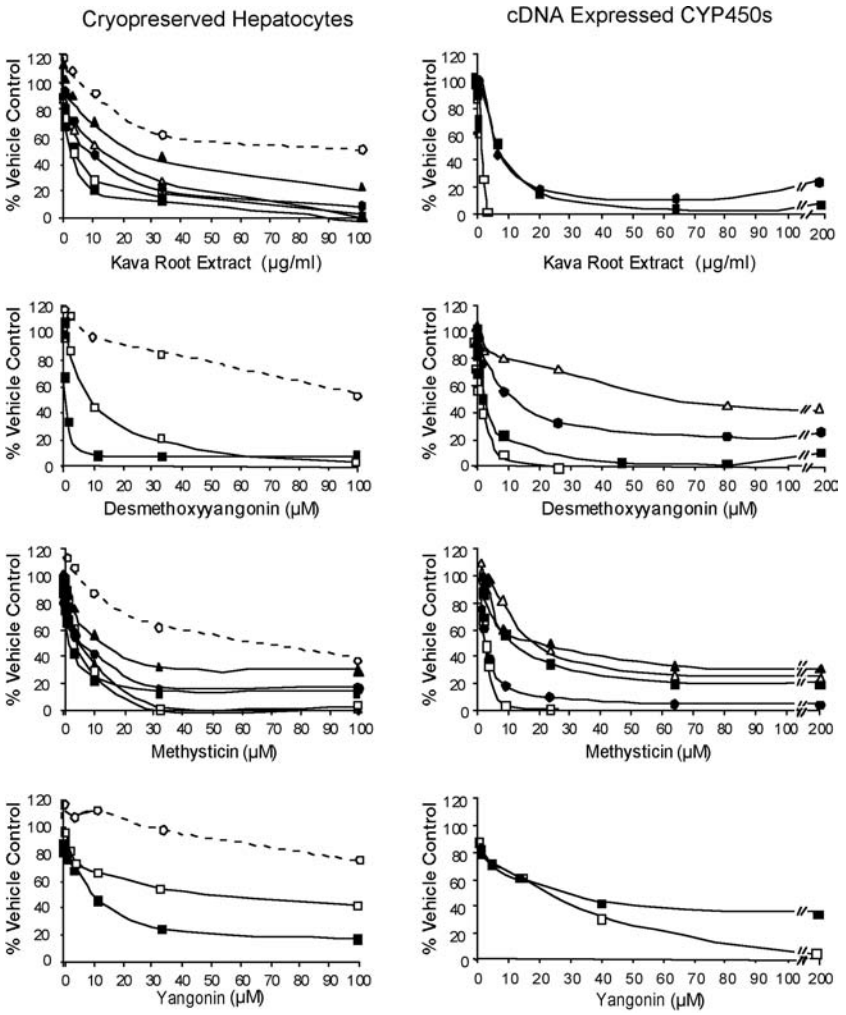

Fig. 2. Inhibitory effects of kava root extract, desmethoxyyangonin, methysticin, and yangonin on the catalytic activity of cDNA-expressed P450 isoforms and cryopreserved human hepatocytes. Test compounds and enzymes or hepatocytes were incubated as described under Materials and Methods and in Tables 1 and 2. Isoforms evaluated were 1A2 (■), 2C9 (△), 2C19 (□), 2E1 (▲), and 3A4 (●). ATP bioluminescence (○) was measured as an index of cell viability. Data shown are expressed as percent change from vehicle control and represent the mean of four determinations (duplicate analysis on two different occasions) for cDNA-expressed isoforms, three determinations for hepatocytes, and four determinations of ATP content. Standard deviations for data points were typically smaller than the size of the symbol.

Table 4. IC_{50} values (μM) for kava compounds obtained using c-DNA-expressed human P450 enzymes.

Test Compound	CYP1A2	CYP2A6	CYP2C9	CYP2C19	CYP2D6	CYP2E1	CYP3A4 (BzRes)	CYP3A4 (BFC)
Methysticin	12.5	NI	16.4	0.9	NI	19.6	10.2	1.5
Desmethoxyyangonin	1.7	NI	50.1	0.5	NI	NI	NI	20.0
Yangonin	19.9	NI	NI	22.6	NI	NI	NI	NI
Kava Root Extract[a]	5.4	NI	7.6	1.8	NI	NI	5.7	4.6
Positive Control	Furafylline	Tranylcypromine	Sulfaphenazole	Tranylcypromine	Quinidine	DDTC	Ketoconazole	Ketoconazole
	1.65	0.9	0.29	5.46	0.012	1.9	0.123	0.017

Values shown represent the mean of four determinations (duplicate analyses on two different occasions). NI indicates no inhibition at the highest concentration tested

[a] Micromolar concentrations for the kava root extract are estimated from the amounts of the six kava lactones present in the extract as shown in Table 3.

Table 5. IC_{50} values (μM) for kava compounds obtained using cryopreserved human hepatocytes.

Test Compound	CYP1A2	CYP2A6	CYP2C9	CYP2C19	CYP2D6	CYP2E1	CYP3A4
Methysticin	2.4	NI	5.5	4.8	NI	7.2	7.1
Desmethoxyyangonin	1.4	NI	NI	9.4	NI	NI	NI
Yangonin	12.1	NI	NI	58.9	NI	NI	NI
Kava Extract[a]	4.4	NI	18.4	3.8	NI	18.0	15.1
Positive Control	Furafylline	Tranylcypromine	Sulfaphenazole	Omeperazole	Quinidine	4-Methylpyrazole	Ketoconazole
% Inhibition	87	12	88	22	57	77	92
Concentration	2 μM	2 μM	20 μM	50 μM	5 μM	500 μM	2 μM

Values shown represent the mean of three determinations. NI indicates no inhibition at the highest concentration tested

[a] Micromolar concentrations for the kava root extract are estimated from the amounts of the six kava lactones present in the extract as shown in Table 3.

Table 6. Comparison of IC_{50} Values (μM) obtained using c-DNA-expressed human P450 isoforms with those obtained using cryopreserved human hepatocytes.

Test Compound	1A2		2A6		2C9		2C19		2D6		2E1		3A4	
	rCYP	Hepat.	rCYP	Hepat.	rCYP	Hepat	rCYP	Hepat.	rCYP	Hepat.	rCYP	Hepat.	rCYP	Hepat
Methysticin	12.5	2.4	NI	NI	16.4	5.5	0.9	4.8	NI	NI	19.6	7.2	10.2 /1.5	7.1
Desmethoxyyangonin	1.7	1.4	NI	NI	50.1	NI	0.5	9.4	NI	NI	NI	NI	NI	NI
Yangonin	19.9	12.1	NI	NI	NI	NI	22.6	58.9	NI	NI	NI	NI	NI	NI
Kava Root Extract[a]	5.4	4.4	NI	NI	7.6	18.4	1.8	3.8	NI	NI	NI	18.0	5.5/4.6	15.1

Values shown represent the mean of four determinations using rCYPs and three determinations with hepatocytes. NI indicates no inhibition at the highest concentration tested

[a] Micromolar concentrations for the kava root extract are estimated from the amounts of the six kava lactones present in the extract as shown in Table 3.

Effects of Kava on the Catalytic Activity of Cryopreserved Human Hepatocytes

As shown in Table 5 and Fig. 3, the effects of the kava test compounds on cryopreserved hepatocytes were similar to those observed in recombinant enzymes. Hepatocyte isoforms 2A6 and 2D6 were not inhibited by any of the kava components at the highest concentration tested in this assay – 100 μM. Isoforms 1A2, 2C9, 2C19, 2E1 and 3A4 were inhibited at concentrations ranging from 1.4–58.9 μM. Methysticin and the kava root extract inhibited five of the seven isoforms evaluated in hepatocytes, desmethoxyyangonin, and yangonin inhibited two isoforms.

Comparison of Inhibition Data Obtained in the Different Systems

Considering the source of enzymes and the differences in substrates used, the IC_{50} values obtained in the two test systems (shown in Table 6) agreed generally. Both assays were consistent in their findings that all the kava test compounds were potent inhibitors of 1A2 and 2C19, but had no effect on isoforms 2A6 and 2D6. For the other five isoforms, results did not agree in two instances: desmethoxyyangonin's effects on 2C9 and kava root extracts effects on 2E1. Desmethoxyyangonin demonstrated no inhibitory activity of 2C9 in the hepatocyte assay, but showed moderate inhibition of this isoform in the rCYP assay (IC_{50} value of 50 μM).

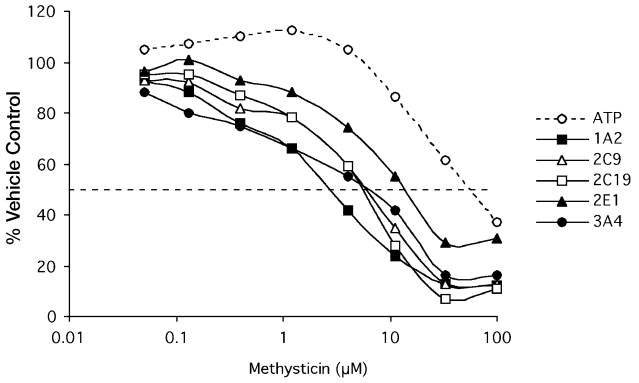

Fig. 3. Comparison of the inhibitory effects of methysticin on hepatocyte viability with its effects on the catalytic activity of the individual P450 isoforms. Isoforms evaluated were 1A2 (■), 2C9 (△), 2C19 (□), 2E1 (▲), and 3A4 (●). ATP bioluminescence (○) was measured as an index of cell viability. Data shown are expressed as percent change from vehicle control and represent the mean of four determinations (duplicate analysis on two different occasions) for cDNA-expressed isoforms, three determinations for hepatocytes, and four determinations for ATP content. Standard deviations for data points were typically smaller than the size of the symbol.

The kava root extract did not inhibit 2E1 in the rCYP assay, but inhibited this isoform in the hepatocyte assay (IC_{50} value of 18.0 μM). In all other cases the results of the assays differed quantitatively, but not qualitatively. The greatest quantitative difference observed was desmethoxyyangonin's effect on 2C19. Although both assays showed desmethoxyyangonin to be a potent inhibitor of this isoform, the IC_{50} value was 9.4 μM in hepatocytes and 0.5 μM in rCYPs.

Effects of Kava on Hepatocyte Viability

Compared with the vehicle control, all test compounds produced an initial increase and then a decrease in ATP bioluminescence (Fig. 2). At 100 μM concentrations, kava root extract, methysticin, and desmethoxyyangonin decreased ATP approximately 50%. The compounds that produced the greatest effects on rCYP activity – kava root extract, methysticin, and desmethoxyyangonin – also produced the greatest effects on hepatocyte viability. Fig. 3, which compares methysticin's inhibitory effects on hepatocyte viability with its inhibitory effects on P450s, shows that although all curves were sigmoidal and demonstrate dose-dependent inhibition, approximately 10–20 times higher concentrations are required to produce cytotoxicity than are required to produce P450 inhibition. Further, because methysticin produces significant CYP inhibition at concentrations that do not decrease ATP, it is likely that these two effects are mediated by different mechanisms.

Discussion

Results obtained from these studies showed that an ethanolic extract of dried kava root extract and two individual kava lactones, desmethoxyyangonin and methysticin, are potent inhibitors of human cytochrome P450s. Isoforms 1A2, 2C9, 2C19, 2E1, and 3A4 were inhibited by one or more of these compounds in at least one of the test systems with IC_{50} values <10 μM. Yangonin was found to be a moderate inhibitor of 1A2 and 2C19 with IC_{50} values ranging from 12.1 to 58.9 μM. In these assays, compounds with IC_{50} values below 10 μM are considered "potent" inhibitors; whereas, compounds with IC_{50} values below 50 μM are considered "moderate" inhibitors (Dierks, 2001) .

These compounds are also weak inhibitors of human hepatocyte viability (EC_{50} values approximately 100 μM); however, the concentrations required to decrease cell viability were well above those required to inhibit P450s. Results obtained from both assay methods were generally consistent; however, there were differences in the IC_{50} values obtained in the c-DNA-ex-

pressed cells and the cryopreserved hepatocytes. These differences are most likely due to the different substrates used in the respective assays. In addition, the recombinant CYP assays do not address conjugating enzyme metabolism or metabolic pathway switching. Considering the differences in the test systems and the probe substrates used, the results are surprisingly consistent and agree generally with the recent report of Mathiews et. al. who found that kava components inhibit P450 activities in human liver microsomes (Mathiews et. al. 2002).

Methysticin was the most potent inhibitor of CYP activity and of hepatocyte viability followed by (in order of potency) the kava root extract, desmethoxyyangonin, and yangonin. These findings suggest that kava could potentially decrease the metabolic clearance of a number of co-administered drugs. Also, because kava inhibits CYP isoforms that are polymorphic (CYP2C9, CYP2C19) its effects could vary among genetically different individuals. In addition, the individual kava lactones as well as the whole root extract were found to be moderate inhibitors of hepatocyte viability.

It is difficult to predict the *in vivo* effects of kava on drug metabolism based on these *in vitro* data. Both studies clearly show that components of kava inhibit drug metabolizing enzymes. These compounds could, however, inhibit CYP enzymes after a single dose, but induce after repeated doses; thus, the resultant effects of kava on co-administered drugs could be quite complex. A recent *in vitro* study using a reporter gene assay for the human pregnane X receptor, hPXR, demonstrated that 100 μM of a kava extract increased CYP3A4 mRNA significantly in primary hepatocyte cultures (Raucy, 2003). This suggests that kava may induce CYP3A4; however, the concentration used was high, not pharmacological, and, in our experience, likely to be cytotoxic. Finally, there is little known about kava pharmacokinetics in humans; therefore, it is difficult to predict whether concentrations of the lactones in hepatocytes could reach the low micromolar concentrations necessary to inhibit P450 enzymes or the higher concentrations required for cytotoxicity. Relatively large amounts of these very lipid soluble compounds are, however, ingested by regular users of kava. For example, the daily intake of kava lactones by Pacific Islanders using kava as a traditional beverage has been estimated to range from 100–1,000 mg (Kilham, 1996; Cass and McNally, 1998). This compares favorably with the 60–240 mg total kava lactones consumed daily by patients taking kava for its anxiolytic effects (Pittler and Ernst, 2000).

In summary, our results suggest that the drug interaction and hepatotoxic potential of kava in humans should be investigated further.

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