Effects of Kava Alkaloid, Pipermethystine, and Kavalactones on Oxidative Stress and Cytochrome P450 in F-344 Rats

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Kava-containing products remain popular in the United States and continue to be sold in health food stores and ethnic markets regardless of the fact that it was banned in Western countries such as Germany, France, Switzerland, Australia, and Canada, following reports of alleged hepatotoxicity. It is therefore critical to establish efficacy and verify adverse effects and/or herb-drug interactions for kava-kava (Piper methysticum). We have previously demonstrated that kava alkaloid, pipermethystine (PM), abundant in leaves and stem peelings, induces mitochondrial toxicity in human hepatoma cells, HepG2, as compared with the bioactive components, kavalactones (KL), abundant in the rhizome. The current study compared short-term toxic effects of PM in Fischer-344 (F-344) rats to acetone-water extracts of kava rhizome (KRE). Treatment of F-344 rats with PM (10 mg/kg) and KRE (100 mg/kg) for 2 weeks failed to elicit any significant changes in liver function tests or cause severe hepatic toxicity as measured by lipid peroxidation and apoptosis markers such as malondialdehyde, Bax, and Bcl-2. However, PM-treated rats demonstrated a significant increase in hepatic glutathione, cytosolic superoxide dismutase (Cu/ZnSOD), tumor necrosis factor α mRNA expression, and cytochrome P450 (CYP) 2E1 and 1A2, suggesting adaptation to oxidative stress and possible drug-drug interactions.

Key Words: kava; pipermethystine; kavalactones; oxidative stress; cytochrome P450.

Kava (‘awa in Hawaiian language) beverage prepared from the roots and rhizome of the kava plant, Piper methysticum, has been widely used for centuries throughout the South Pacific, from Papua New Guinea to Hawaii, without any apparent side effects except dermopathy (Lebot et al., 1997). In the late 1990s, commercial kava products gained immense popularity in Europe and North America as an effective treatment option for anxiety (O’Sullivan and Lum, 2004; Singh and Singh, 2002). By the end of 2001, alleged hepatotoxicity sparked a ban of kava-containing products in Western countries such as Germany, France, Switzerland, Australia, and Canada (Clouatre, 2004; Russmann et al., 2001; Schmidt et al., 2002). However, kava tinctures, capsules, teas, and dry kava powder continue to be sold in health food stores and ethnic markets within the United States regardless of a consumer advisory issued by the Food and Drug Administration concerning the potential hepatotoxicity of commercial kava products. It is therefore pertinent to identify the possible causative factors and mechanisms of kava-associated hepatotoxicity, if any.

Although kava drink is traditionally prepared from the underground roots and rhizome, commercial preparations in the late 1990s may have included stem peelings and above-ground parts due to easy availability and high demand (Dragull et al., 2003). Stem peelings and leaves contain high concentrations of kava alkaloid, pipermethystine (PM), as compared to the physiologically active kavalactones (KL) that are abundant in roots and rhizome (Dragull et al., 2003). Our earlier in vitro studies demonstrated that toxicity of PM in human hepatoma cells, HepG2, was associated with an insignificant increase in the production of reactive oxygen species (ROS), loss of mitochondrial membrane potential, reduced cellular ATP levels, and ultimately cell death (Nerurkar et al., 2004). However, the toxicities of PM, in vivo, remain unknown.

Kava root/rhizome extracts (KRE) and individual KL are known to be metabolized in vitro and in vivo, via modulation of various phase I drug-metabolizing enzymes, cytochrome P450s (CYP450) (Mathews et al., 2002, 2005; Zou et al., 2002, 2004, 2005). It has been speculated that adverse kava reactions may be in part due to modulation of CYP450, family of heme-thiolate enzymes, involved in the oxidative metabolism of a
variety of endogenous and exogenous lipophilic compounds. Poor coupling of the P450 catalytic cycle, specifically the ethanol-inducible CYP2E1 and CYP1A2, can result in continuous production of ROS (Caro and Cederbaum, 2004; Imaoka et al., 2004). ROS-associated oxidative stress and subsequent mitochondrial dysfunction are considered to be key elements of chemical-induced liver injury. Therefore, the objectives of this study were to investigate the in vivo toxicity of PM and the synergistic effects of PM and acetone-water KRE on mitochondrial function, oxidative stress, and CYP450 enzymes.

**MATERIALS AND METHODS**

**Preparation of KRE.** Kava rhizome (unpeeled, roots removed) of Hawaiian cv. Mahakea was cut into 4-cm cubes, dried at 60°C at normal pressure, and stored at −18°C until further processing. Before extraction, the chips were redried at 50°C and −50 kPa for 1 h and milled. The powder was first extracted with ethyl acetate, and the resulting supernatant was stored. The residue was further extracted with acetone/water (75:25, vol/vol) by shaking at 150 rpm for 8 h, and acetone was removed from the supernatant under reduced pressure. The aqueous phase was further partitioned with ethyl acetate 1:1 (vol/vol) three times, and the upper ethyl acetate phases were collected. All the ethyl acetate extracts were combined, dried over sodium sulfate, and filtered through silica gel. The resultant purified residue was further extracted with acetone/water (75:25, vol/vol) by shaking at 150 rpm for 8 h, and acetone was removed from the supernatant under reduced pressure. The aqueous phase was further partitioned with ethyl acetate 1:1 (vol/vol) three times, and the upper ethyl acetate phases were collected. All the ethyl acetate extracts were combined, dried over sodium sulfate, and filtered through silica gel (40-μm particle size and 60-Å pore size). KL concentration (sum of six major KL: kavain, dihydrokavain, methysticin, dihydromethysticin, yango- nin, and desmethoxyyangonin) in the solvent-free KRE was 62.67 ± 7.2% (Table 1, n = 5), as determined by gas chromatography–flame ionization detector (GC-FID) using authentic KL standards for calibration.

**Preparation of PM.** Kava leaves (cv. Mahakea) were dried at 60°C, crumbled, and stored at −18°C until extraction. Before extraction, the leaves were redried, milled to pass a 2-mm sieve, and extracted with acetone/water (75:25, vol/vol). Acetone was removed under vacuum, and the green extract was re-extracted with ethyl acetate. The green pigments were removed by activated charcoal, and the extract was filtered through silica gel. The resultant purified extract was applied onto a silica gel column packed with n-hexane/ethyl acetate (80:20, vol/vol), eluted with the same solvent mixture, and monitored by GC-FID. The column chromatography step was repeated to remove remaining yellow pigmentation and minor impurities that elute before PM.

**Animal treatment.** Male Fischer-344 (F-344) rats (200–220 g) were obtained from Charles River Laboratory (Wilmington, MA), housed individually, and maintained in an environment of 12 h dark/12 h light cycles at 68°C–72°F. Food and water were provided ad libitum. Experiments were conducted in accordance to the National Institutes of Health (NIH) guidelines and were approved by the University of Hawaii’s Institutional Animal Care and Use Committee. The rats were randomly split into four treatment groups containing five animals each: (1) control (received corn oil, 3.33 ml/kg/day), (2) PM (10 mg/kg/day), (3) KRE (100 mg/kg/day equivalent to 63 mg total KL/kg/day), and (4) PM + KRE. Both PM and KRE were mixed in corn oil and administered by intragastric gavage for 2 weeks to investigate the short-term, acute effects of PM and KL. Animals were fasted overnight before being sacrificed in a carbon dioxide chamber. Blood was collected by cardiac puncture and allowed to clot at room temperature for 30 min, and serum was collected by centrifugation at 1500 × g for 15 min at 4°C. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed by Diagnostic Laboratory Services (Honolulu, HI). Livers were excised, weighed, and examined for any gross changes. Fresh liver was used to measure hepatic reduced glutathione (GSH) content. The remaining liver was snap frozen in liquid nitrogen and stored in aliquots at −80°C until further analysis.

**Hepatic aconitase activity.** A 1% (wt/vol) liver homogenate was prepared in ice-cold sodium citrate buffer (0.2mM sodium citrate, 50mM Tris-HCl, pH 7.4) and centrifuged at 800 × g for 10 min to pellet tissue debris. The supernatant was assayed fresh for aconitase activity using a commercial assay kit (Oxis Research, Portland, OR).

**Hepatic ATP.** Frozen liver samples were homogenized in 10 volumes of ice-cold 0.25M sucrose, and homogenates were deproteinized by boiling for 5 min. The supernatant was collected after centrifugation at 10,000 × g for 10 min and stored at −80°C until analysis. Total ATP levels were analyzed using the Perkin Elmer (Boston, MA) ATPlite kit according to the manufacturer’s protocol and compared to ATP standard curves analyzed with each assay. ATP was expressed as μM/mg protein.

**Hepatic GSH activity.** Total hepatic GSH was measured according to published protocol with slight modifications (Hissin and Hilf, 1976). In brief, fresh liver was homogenized in phosphate buffer (100mM phosphate, pH 8.3, 5mM EDTA, 20% wt/vol) and centrifuged at 10,000 × g for 15 min. Supernatants were deproteinized with an equal volume of 10% trichloroacetic acid and frozen at −80°C until analysis. The final reaction was performed in a 96-well plate by adding deproteinized liver homogenates, phosphate buffer, and α-phthalaldehyde solution in a total volume of 200 μl. Fluorescence was read at 350/420 in the Victor2 Wallace multimetal plate reader (Perkin Elmer). Each fluorescence value was compared to GSH standards prepared freshly with each batch of samples and adjusted to μM GSH/g liver.

**Hepatic superoxide dismutase activity.** Liver homogenates were prepared in 10 volumes of ice-cold HEPES buffer (20mM HEPES, 1mM EGTA, 210mM mannitol, 70mM sucrose, pH 7.2) and centrifuged at 1500 × g for 5 min. The supernatant was further centrifuged at 10,000 × g for 15 min. The supernatant contained the cytosolic superoxide dismutase (SOD), and the pellet contained the mitochondrial SOD. The mitochondrial pellet was resuspended in 600 μl of HEPES buffer. Both fractions were frozen at −80°C for up to 1 month, and cytosolic (Cu/ZnSOD) and mitochondrial SOD (MnSOD) activities were analyzed using commercial Superoxide Dismutase Assay kit (Cayman Chemicals, Ann Arbor, MI).

**Analysis of hepatic lipid peroxidation markers.** Lipid peroxidation (LPO) was determined by measuring the amounts of malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) using the Bioxytech LPO-586 kit (Oxis Research). In brief, liver homogenates were prepared with 2.5 volumes of ice-cold PBS (20mM, pH 7.4) containing fresh butylated hydroxytoluene (5mM final concentration), centrifuged at 5000 × g for 10 min, and supernatants were analyzed according to the manufacturer’s protocol.

**Preparation of hepatic microsomes and mitochondrial extracts.** Hepatic microsomes and mitochondrial extracts were prepared from frozen livers as described previously (Nelson et al., 2001; Pon and Schon, 2001). For microsomal preparation, frozen livers were homogenized in four volumes of ice-cold homogenization buffer (0.1M potassium phosphate, 0.125M KCl, 0.25m sucrose, 1mM EDTA, pH 7.4) and centrifuged at 12,000 × g for 20 min to pellet microsomes and cell debris. Resulting supernatant was further

<table>
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<tr>
<th>KLs</th>
<th>Abbreviation</th>
<th>MW</th>
<th>% (wt/wt)</th>
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<tbody>
<tr>
<td>Dihydrokavain</td>
<td>DHK</td>
<td>258.3</td>
<td>13.05</td>
</tr>
<tr>
<td>Kavain</td>
<td>K</td>
<td>247.5</td>
<td>11.46</td>
</tr>
<tr>
<td>Desmethoxyyangonin</td>
<td>DMY</td>
<td>222.7</td>
<td>8.46</td>
</tr>
<tr>
<td>Dihydromethysticin</td>
<td>DHM</td>
<td>245.4</td>
<td>11.36</td>
</tr>
<tr>
<td>Yangonin</td>
<td>Y</td>
<td>204.6</td>
<td>8.34</td>
</tr>
<tr>
<td>Methysticin</td>
<td>M</td>
<td>247.6</td>
<td>10.00</td>
</tr>
<tr>
<td>Total</td>
<td></td>
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<td>62.67</td>
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centrifuged at 138,000 × g for 60 min to pellet the microsomal fraction. The pellet was resuspended in microsomal buffer (0.1M Tris-Base, 0.125M KCl, pH 7.4) and centrifuged again at 138,000 × g for 60 min. The supernatant was discarded, and the pellet was suspended in microsomal buffer and stored at −80°C until use.

For mitochondrial preparations, frozen liver was homogenized in 10 volumes of mitochondrial isolation buffer (20mM HEPES, 210mM mannitol, 1mM EGTA, 70mM sucrose, 2mM Tris-Cl, pH 7.2, containing 1% protease inhibitor cocktail [Roche, Indianapolis, IN]) with six strokes in a Dounce homogenizer. The homogenate was centrifuged at 750 × g for 10 min, and the supernatant was subjected to further centrifugation at 10,000 × g for 15 min to pellet mitochondrial fraction. The supernatant was collected as the cytosolic fraction while the pellet was resuspended as the mitochondrial fraction in mitochondrial isolation buffer. Mitochondrial and cytosolic fractions were stored at −80°C until use. Protein concentrations were determined using Bradford protein assay reagent according to the manufacturer’s instructions (Bio-Rad Laboratories, Hercules, CA).

**Western blot analysis of CYP450, uncoupling protein 2, cytochrome C, and heat shock protein 70.** Microsomal CYP450 proteins and mitochondrial uncoupling protein 2 (UCP-2), cytochrome C (Cyt C), and heat shock protein 70 (HSP70) were analyzed by Western blotting. Proteins (0.1, 1.25, 2.5, 11, and 25 μg/lane for CYP2D6, CYP2E1, CYP3A4, CYP1A2, HSP70, respectively) were separated on 10% or 12% SDS-PAGE and transferred to a nitrocellulose membrane, blocked in 1% bovine serum albumin or 5% nonfat dry milk, and incubated overnight with specific primary antibodies against CYP1A2 and CYP2E1 (Research Diagnostics, Flanders, NJ), CYP2D6 (BD Biosciences, Bedford, MA), CYP3A4 (Affinity Bioreagents, Golden CO), and Cyt C, UCP-2, and HSP70 (Santa Cruz Biotechnology, Santa Cruz, CA). After washing, blots were probed with appropriate secondary antibodies for 2 h at room temperature (Santa Cruz Biotechnology). Proteins were detected using electrochemiluminescence kit (Amersham Biosciences, Piscataway, NJ).

**Semiquantitation of Bax, Bcl-2, and tumor necrosis factor α gene expression.** Bax, Bcl-2, and tumor necrosis factor α (TNF-α) mRNA gene expression were determined by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was extracted using RNA-Bee (Tel-Test, Friendswood, TX), and 2 μg RNA was reverse transcribed into complementary DNA. Bcl-2 expression levels were quantitated using commercial primers (Sigma, Saint Louis, MO, catalog# APO-PCR), while Bax and TNF-α expression levels were quantitated using published primers and cycling conditions (Table 2) (Kono et al., 2005; Wang et al., 2004). PCR reactions were performed in a GeneAmp PCR System 9700 (Applied Biosciences), and amplicons were size fractionated on a 2% agarose gel, visualized with ethidium bromide staining, and semiquantitated with Kodak 1D image analysis software. The intensity of the amplicons was expressed as ratio of the gene of interest against a housekeeping gene, GAPDH or β-actin.

**Statistical analysis.** All data are presented as mean ± SD. Biochemical and molecular analyses were performed in duplicate or triplicate, with five animals in each group. A one-way analysis of variance model was used to compare mean effects among groups. Normality and homogeneity of variance were checked using multivariate log-normal plots. When appropriate, the data were transformed using a logarithm or square root function. Post hoc comparisons were done via the two-stage Ryan-Einot-Gabriel-Welsch multiple range test. p Values ≤ 0.05 were considered to be significant.

**RESULTS**

Both PM and KRE had no effect on daily food intake and liver weight as compared to control rats (Table 3). Although rats in all experimental groups lost body weight, the KRE group rats demonstrated a significant trend for increased weight loss of 42 g as compared to control, PM, and PM + KRE groups (Table 3). PM alone had no significant effect on either serum AST or ALT levels, as compared with control rats (Table 3). Interestingly, while KRE and PM + KRE groups had no

| **TABLE 2** | Primer Sequences and Cycling Conditions for Semiquantitation of Target Gene Expression |
|---|---|---|---|
| Primer | Sequence | Amplicon size (bp) | Cycling conditions |
| Bax (F) | 5’ GAC ACC TGA GCT GAC CTT GG 3’ | 310 | 94°C for 45 s, 56°C for 30 s, 72°C for 1 min; 27 cycles |
| Bax (R) | 5’ GAG GAA GTC CAG TGT CCA GC 3’ | 278 | 94°C for 45 s, 51°C for 1 min 15 s, 74°C for 1 min; 40 cycles |
| TNF-α (F) | 5’ ATG AGC ACA GAA AGC ATG ATG 3’ | 660 | Changed according to different target genes |
| TNF-α (R) | 5’ TAC AGG CTT GTC ACT CGA ATT 3’ | 254 | 94°C for 45 s, 56°C for 30 s, 72°C for 1 min; 27 cycles |
| β-actin (F) | 5’ CCA ACC GTG AAA AGA TGA CC 3’ | 216 | 94°C for 45 s, 56°C for 30 s, 72°C for 1 min; 27 cycles |
| β-actin (R) | 5’ CAG GAG GAA TGA TCT TG 3’ | 254 | 94°C for 45 s, 56°C for 30 s, 72°C for 1 min; 27 cycles |

| **TABLE 3** | Body Weights, Food Intake, Liver Weights, and Serum AST and ALT levels |
|---|---|---|---|---|
| | Control | PM | KRE | PM + KRE |
| Initial body weight (g) | 346.6 ± 17.0 | 348.6 ± 16.9 | 361.4 ± 9.0 | 347.8 ± 11.6 |
| Final body weight (g) | 322.8 ± 19.8 | 321.2 ± 24.6 | 319.2 ± 31.7a | 324.4 ± 20.5 |
| Food intake (g/day) | 11.4 ± 3.5 | 10.84 ± 2.1 | 10.2 ± 3.1 | 11.6 ± 1.4 |
| Liver weight (mg/g body weight) | 36.0 ± 5.8 | 35.1 ± 2.6 | 36.8 ± 3.0 | 37.9 ± 4.7 |
| AST (U/L) | 100.6 ± 10.5 | 96.4 ± 15.9 | 102.8 ± 27.2 | 108.2 ± 36.9 |
| ALT (U/L) | 78.2 ± 21.5 | 72.8 ± 4.2 | 62.6 ± 5.6 | 58.8 ± 8.8 |

*Note. Values are expressed as means ± SD, n = 5.

a*p < 0.05 compared to initial body weight within KRE group.
Studies from our laboratory indicated that PM significantly increased ROS production, reduced cellular ATP, and induced loss of mitochondrial ΔΨm in HepG2 (Nerurkar et al., 2004), as well as in rat hepatoma cell line, H4IIEC3 (data not shown). In the current study, mitochondrial aconitase activity was analyzed as an indirect marker of ROS. PM alone significantly increased aconitase activity by 66%, while KRE and KRE + PM–treated rats demonstrated a nonsignificant increase (Fig. 1A). In contrast to our in vitro studies, total ATP levels were unaffected by both PM and KRE (Fig. 1B). However, UCP-2 which is a negative regulator of ROS production was significantly decreased by 30–40% in the KRE and PM + KRE treatment groups as compared with control but was unchanged in PM-treated animals (Fig. 1C, *p < 0.05).

PM-treated rats showed a significant increase (88%) in hepatic TNF-α mRNA expression as compared to control rats, while the KRE and PM + KRE groups showed a nonsignificant 30–40% increase (Fig. 2). Since our in vitro studies indicated that PM, rather than individual KL, induced apoptosis in HepG2 cells, we measured hepatic mRNA expression of antiapoptotic protein Bcl-2, the proapoptotic protein Bax, and the release of Cyt C from mitochondria, as indicators of apoptosis in vivo. Neither PM nor KRE had any effect on either Bax, Bcl-2 gene expression as measured by RT-PCR, or the release of Cyt C as measured by Western blotting (data not shown).

Hepatic LPO products, MDA and HAE, were analyzed using commercial kits. Both PM and KRE had no effect on either MDA or HAE levels in vivo (Fig. 3A). Hepatic SOD and GSH were enzymatically measured as markers of antioxidant defense mechanisms. Cu/ZnSOD activity was significantly increased by 18% in the PM group and by 24% in PM + KRE–treated rats as compared with controls (*p < 0.05, Fig. 3B). MnSOD was significantly increased by 32% only in the combination group (*p < 0.05, Fig. 3C). Similarly, PM and PM + KRE–treated rats demonstrated a significant 35–54% increase in hepatic GSH activity (*p < 0.05, Fig. 3D). KRE alone had no effect on Cu/ZnSOD, MnSOD, or GSH activity (Figs. 3B–D, respectively).

Figure 4 depicts the changes in hepatic microsomal CYP450 protein levels in F-344 rats treated with PM and KRE. While PM alone demonstrated a nonsignificant increase in CYP1A2, KRE and PM + KRE significantly increased hepatic CYP1A2 protein levels by 98% (*p < 0.05, Fig. 4A). CYP2E1 was also increased by 40 to 50% in the KRE and PM + KRE groups (Fig. 4B), while CYP2D6 protein levels were increased in only PM + KRE group by 50% (Fig. 4C). CYP3A4 protein levels remained unchanged in all groups (Fig. 4D).

**DISCUSSION**

Although small doses of kava induce muscle relaxation and/or drowsiness, long-term and excessive use of kava can lead to malnutrition, weight loss, and apathy. In our study, KRE and PM had no effect on food intake. However, rats in the KRE group alone lost significant weight compared to initial body weights within the group. KRE-associated weight loss may be...
similar to the lower body mass index observed among the “very heavy” kava drinkers of Aboriginal community (Clough et al., 2003b, 2004). Nonsignificant weight loss was also observed in other groups which probably reflects the effects of intragastric feeding and large SD within each group of animals.

To date, there is no documentation of PM exposure in humans. The dosage was chosen based on our initial studies with mice which demonstrated a lack of PM toxicity with 1 mg/kg/day administered for 2 months (unpublished observations). Rats were treated for 2 weeks to mimic a short-term interaction between heavy KL dosage and incidental consumption of PM in humans. KL dosage (63 mg/kg/day) used in our study was 10 times higher than the daily recommended dosage for human consumption (6–7 mg/kg/day) and may be comparable to mimicking the “heavy kava drinkers.”

While heavy kava use has been linked to liver function abnormalities such as increased Gamma glutamyltranspeptidase (GGT) (Clough et al., 2003a,b), serum AST, ALT, alkaline phosphatase, and lactate dehydrogenase were unaffected in rats receiving aqueous kava extracts up to 500 mg KL/kg/day (Singh and Devkota, 2003; Sorrentino et al., 2006). Similarly, we also failed to observe any effect on serum AST and ALT levels in rats treated with PM or KRE. Nevertheless, serum ALT levels in KRE groups were lower than in control rats which may also result from malnutrition observed in Aboriginal community (Clough et al., 2003b, 2004). Overall, increases of ALT and AST were never confirmed in kava drinkers, with the exception of two New Caledonian women.
consuming high concentrations of kava beverage over a period of 4–5 weeks (Russmann et al., 2003), while GGT was moderately and reversibly elevated in kava-consuming populations (Clough et al., 2003a; Russmann et al., 2003). Thus, if kava did not increase AST and ALT levels in animal models and kava drinkers of aboriginal communities, is kava alone the culprit in the Swiss and German cases of hepatotoxicity (Schmidt, 2003)?

Mitochondria are a major source of ROS production and the earliest target of drug-induced liver injury. Excess ROS is capable of reacting with iron-sulfur (4Fe-4S) centers in NADH dehydrogenase, aconitase, and other enzymes that may account for inhibition of ATP production. Similar to our earlier in vitro studies, PM and KRE significantly increased ROS production in vivo, as measured indirectly by mitochondrial aconitase activity, but had no effect on mitochondrial function as measured by cellular ATP levels. Loss of ΔΨm is a common event following toxicant exposures leading to cellular necrosis or apoptosis. Alternatively, permeability changes to the outer mitochondrial membrane and collapse of ΔΨm are known to be associated with the release of Cyt C from mitochondria to cytosol, ultimately initiating the mitochondrial death pathway (Regula et al., 2003). Although PM was able to lower ΔΨm, initiate caspase activation, and induce apoptosis in HepG2 cells (Nerurkar et al., 2004), it had no effect on Bax and Bcl-2 mRNA expression, or Cyt C release from mitochondria to cytosol in rats treated either singly or in combination with KRE, suggesting lack of apoptosis. Although PM increases hepatic ROS, it does not induce apoptosis in vivo, which could probably reflect differences in metabolizing capacity of the two systems employed.

Mitochondria are also the site of oxidative phosphorylation during which substrate oxidation by the electron transport chain builds a proton gradient (ΔΨm) and fuels ATP synthesis. Reentry of protons into mitochondrial matrix without ATP synthesis may lower ΔΨm and ROS formation due to “mild uncoupling.” Although ATP levels were unaffected, reduction in UCP-2 levels in PM-treated rats may be indicative of ROS, release of proinflammatory cytokines, or persistent activation of nuclear factor kappa B (NF-κB). It is highly possible that PM may induce inflammatory responses as indicated by increased TNF-α expression.

Increased expression and/or activity of antioxidant enzymes are indicative of adaptive mechanism to ROS-induced oxidative stress (Haddad, 2004; Koch et al., 2004). Concomitant induction of both Cu/ZnSOD and MnSOD in the PM + KRE group is
quite interesting as these genes can be both regulated by NF-κB, a known target of TNF-α (Rojo et al., 2004). Kava and its derivatives were recently demonstrated to inhibit TNF-α–induced activation of NF-κB and its translocation to the nucleus (Folmer et al., 2006). In our studies, while PM significantly increased TNF-α mRNA expression, KRE probably reduced TNF-α activation and normalized the PM-associated increase, thereby corroborating the studies published by Folmer et al. (2006). However, normalization of PM-induced TNF-α expression by KRE did not reduce the PM-associated increase in Cu/ZnSOD activity. It was recently demonstrated that protection against oxidative stress by PI3K/Akt pathway involves up regulation of Cu/ZnSOD via NF-κB activation (Rojo et al., 2004). Based on these observations, it is tempting to speculate that Cu/ZnSOD may be regulated by TNF-α.

SOD enzymes are an important antioxidant defense system that converts superoxide anion to hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) that is further converted to water and molecular oxygen by glutathione peroxidase using GSH as a cofactor. Studies suggest that GSH is important in phase II metabolism and detoxification of KL and that excess KL may rapidly deplete intracellular GSH resulting in hepatic damage due to oxidative stress (Denham et al., 2002). Elevation of SOD enzymes by PM + KRE treatment is suggestive of oxidative stress while the increased hepatic GSH levels indicate responses of the antioxidant defense mechanism to stress. Adaptation to mitochondrial oxidative stress can cause an increase in protective chaperone proteins such as HSP70. Our study indicated that PM and KRE demonstrated increased ROS production and induced adaptive responses to stress as indicated by increased aconitase, SOD, and GSH levels with concomitant reduction in UCP-2 proteins and increase in TNF-α expression. However, lack of severe PM-associated toxicity in vivo, as opposed to our earlier in vitro study (Nerurkar et al., 2004), may be due to the differences in the drug-metabolizing capacity of HepG2 cells and F344 rats. Additionally, the differences in kava variety used (Isa vs. Mahakea) may have partially contributed to the differences in toxicity observed in vitro and in vivo.

It has been hypothesized that kava-drug interactions are highly likely to occur due to inhibition of CYP450 enzymes, which are responsible for metabolizing a majority of the therapeutic drugs (Anke and Ramzan, 2004). Although in vitro models have demonstrated KL as strong inhibitors of CYP450 enzymes (Mathews et al., 2002; Unger et al., 2002; Zou et al., 2002), increased P450 observed in our studies is probably due to differences in model (in vivo rat model vs. cell culture) and use of KL mixture rather than individual KL.

Mathews et al. (2005) demonstrated that seven daily doses of 391 mg/kg KL administered to male F-344 rats increased the overall hepatic P450 content by 35% and markedly increased CYP1A2, CYP2B1, CYP2C6, and CYP3A1/2 activity. Similarly, CYP1A2, 2B1, and 3A1 were significantly increased in F-344 rats fed 1.0 and 2.0 g/kg kava extract for 90 days by gavage (Clayton et al., 2006). In our study, lower dose of KL (63 mg/kg), as compared to the above-mentioned studies, was also able to elicit increases in hepatic expression of CYP1A2, CYP2E1, and CYP2D6. The possibility that kava-consuming individuals may be concomitantly consuming additional medications and/or alcohol highly increases the risk for kava-drug interactions. Induction of CYP2E1 in rat liver microsomes has been shown to cause an increased production of superoxide and hydrogen peroxide molecules (Wu and Cederbaum, 2005). Thus, induction of CYP2E1 by PM and KRE may be indicative of ROS generation.

Traditionally, kava has been safely consumed for centuries without much documented adverse hepatotoxicity. Both in vitro and in vivo studies thus far have failed to link both traditional and solvent-based kava extracts to liver toxicity, even at extremely high doses (Nerurkar et al., 2004; Singh and Devkota, 2003; Sorrentino et al., 2006). Overall, our study indicated that PM may induce ROS production and generate early stress responses but fails to elicit a severe toxicity response to induce apoptosis. Although KL have been demonstrated to be bioavailable in animals and humans (Keledjian et al., 1988; Rasmussen et al., 1979; Zou et al., 2005), to date there is no evidence of actual exposure, bioavailability, and/or the pharmacokinetics of PM in humans. In summary, the lack of severe PM toxicity in rats may reflect possible differences in absorption, metabolism, and/or the variety of kava used. Studies are further warranted to determine the effects of excess KL consumption on oxidative stress in heavy kava drinkers.

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