In Vitro Toxicity of Kava Alkaloid, Pipermethystine, in HepG2 Cells Compared to Kavalactones

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Kava herbal supplements have been recently associated with acute hepatotoxicity, leading to the ban of kava products in approximately a dozen countries around the world. It is suspected that some alkaloids from aerial kava may have contributed to the problem. Traditionally, Pacific Islanders use primarily the underground parts of the shrub to prepare the kava beverage. However, some kava herbal supplements may contain ingredients from aerial stem peellings. The aim of this study was to test the in vitro effects of a major kava alkaloid, pipermethystine (PM), found mostly in leaves and stem peelings, and kavalactones such as 7,8-dihydromethysticin (DHM) and desmethoxyyangonin (DMY), which are abundant in the roots. Exposure of human hepatoma cells, HepG2, to 100 μM PM caused 90% loss in cell viability within 24 h, while 50 μM caused 65% cell death. Similar concentrations of kavalactones did not affect cell viability for up to 8 days of treatment. Mechanistic studies indicate that, in contrast to kavalactones, PM significantly decreased cellular ATP levels, mitochondrial membrane potential, and induced apoptosis as measured by the release of caspase-3 after 24 h of treatment. These observations suggest that PM, rather than kavalactones, is capable of causing cell death, probably in part by disrupting mitochondrial function. Thus, PM may contribute to rare but severe hepatotoxic reactions to kava.

Key Words: piper methysticum; kava; kavalactones; 7,8-dihydromethysticin; desmethoxyyangonin; pipermethystine; apoptosis.

In the past 2 years, the potential hepatotoxicity of kava (Piper methysticum) has attracted much attention (Currie and Clough, 2003). The rhizome and roots of kava, a shrubby plant of the pepper family, have been used in the South Pacific as part of a recreational and ceremonial drink for 2000 years (Lebot et al., 1997). Currently, kava is consumed extensively in Vanuatu, Samoa, Tonga, and Fiji. Traditionally, the under-ground parts of the plant are mashed, mixed with water or coconut milk, and strained. The resulting aqueous suspension is also used as a medicine for urinary tract infections and other physical conditions (Bilia et al., 2002). Kava has been compared to benzodiazepines such as diazepam (valium) or alprazolam (xanax) and recognized as a "natural" alternative to these antianxiety medications (Malsch and Kieser, 2001; Singh and Singh, 2002). Kava was commonly prescribed or available as an over-the-counter herbal remedy in Europe and became increasingly popular in the United States as a mild anxiolytic and sedative agent.

Pathophysiologica effects on the liver have been associated with both prolonged use of very high doses of kava as a traditional drink (Clough et al., 2003) and short-term use of moderate doses of kava in the form of herbal supplement (Stickel et al., 2003). Kava-containing products have been banned in several countries (Bundesanstalt für Arzneimittel und Medizinprodukte, 2002; Food and Drug Administration 2002; Currie and Clough, 2003) or advisories have been issued (Food and Drug Administration 2002; Centers for Disease Control and Prevention, 2003) due to suspected hepatotoxicity, including cases requiring liver transplants and resulting in death (Centers for Disease Control and Prevention, 2003; Currie and Clough, 2003). Published case reports involving kava herbal supplements indicate fulminant hepatic failure, severe acute hepatitis, pan-acinar necrosis, collapse of hepatic lobules, and apoptosis, as well as increases in bilirubin, aspartate aminotransferase (AST), and alanine aminotransferases (ALT) (Gow et al., 2003; Humberston et al., 2003; Smith, 1983; Stickel et al., 2003; Stoller, 2000). In contrast, fulminant hepatic failure has not been documented with traditional kava use among Pacific Islanders (Moulds and Malani, 2003). However, abnormally high levels of serum γ-glutamyl transferase (GGT) and alkaline phosphatase (ALP), but normal ALT, have been noted among heavy kava drinkers of aboriginal communities in Australia (Clough et al., 2003; Currie and Clough, 2003; Mathews et al., 1988). Whereas hepatotoxicity cases involving kava herbal supplements demonstrated high levels of ALT, these effects of traditional kava use did not suggest acute inflammation. However, two cases of reversible liver toxicity...
from New Caledonia have recently been attributed to the traditional kava drink (Russmann et al., 2003). Alternatively, concomitant use of kava with conventional therapeutic depressants could cause drug-drug interactions and hepatotoxicity. In vitro studies suggest that inhibition of human P450 enzymes, which are responsible for metabolism of more than 90% of pharmaceuticals, by kavalactones could potentially lead to drug-drug interactions and probably liver toxicity (Mathews et al., 2002). Generally, the disparity in toxicity patterns could arise from genetic differences in human metabolism (Russmann et al., 2001), differences in extraction procedures (Currie and Clough, 2003; Whitton et al., 2003), and/or from differences in kava raw material use (Dragull et al., 2003).

Public records indicate that, in addition to rhizomes and roots, so-called peelings, scrapings, and shavings from the base of the plant have been major commodities in kava trade due to ample supply, low cost, and increased demand on the pharmaceutical and herbal supplement markets (Government of Samoa, 1998; Dragull et al., 2003). The underground parts of kava contain high quantities of kavalactones, the pharmacologically active ingredients of kava, while alkaloids are present only in trace amounts. Pipermethystine in particular was below a detectable limit of 0.02% in dry commercial root powders sold for drinking purposes, but on average exceeded 0.2% in freshly dried stem peelings (Dragull et al., 2003). This suggests that PM is not a major constituent of the kava drink and is avoided for ingestion by traditional practices. Alkaloids are concentrated in leaves and stem peelings (Dragull et al., 2003; Smith, 1979, 1983). A recent study in rats demonstrated that aqueous kava extracts (200–500 mg kavalactones/kg/day) did not adversely affect liver function tests for up to 4 weeks (Singh and Devkota, 2003). Based on these observations, we hypothesize that kava alkaloids, rather than kavalactones, could play a role in inducing cellular hepatotoxicity (Dragull et al., 2003).

In this study, we tested our hypothesis using human hepatoma cell line, HepG2, which provides a reproducible in vitro model system to identify potential liver toxins. To date, no data are available regarding kava alkaloid metabolism or toxicity. Initial toxicity studies in HepG2 cells indicated that desmethoxyyangonin (DMY) and dihydromethysticin (DHM) were more cytotoxic than the other kavalactones tested. The present study was conducted, using DHM and DMY as “representative” lactones, to test the potential of kavalactones and alkaloids to induce apoptosis as a potential mechanism of toxicity.

**MATERIALS AND METHODS**

**HepG2 cultures.** HepG2 cells, obtained from American Type Culture Collection (ATCC, Manassas, VA), were used before passage 12 and initially cultured in T75 flasks in minimum essential medium eagle (MEME) with 10% fetal bovine serum (FBS) and antibiotics. The cells were subsequently incubated at 37°C in a 5% CO₂ incubator. One day before treatment with kavalactones and alkaloids, HepG2 cells were trypsinized and seeded at a density of 5 × 10⁴ cells/well in clear-view white, 96-well CulturPlate (cat. 6005181; Packard BioScience, Meriden, CT). All assays were performed in 96-well plates.

**Test compounds.** PM (CAS 375797-99-2) was isolated from stem peelings of Piper methysticum cv. Isa (Dragull et al., 2003), and the kavalactones DHM (CAS 19902-91-1) and DMY (CAS 15345-89-8) were from commercial root powder. Preparative liquid chromatography was performed on silica gel (40 μm; Mallinckrodt Baker Inc., Phillipsburg, NJ); all solvents used were HPLC grade (Fisher Scientific, Fair Lawn, NJ). EtOAc extracts of plant materials were chromatographed with a gradient of EtOAc/hexane mixtures (Shao et al., 1998) running from a ratio of 1:9 to 1:0 (Dragull et al., 2003). Crude target compounds were further purified by repeated isocratic flash chromatography using the same packing material and EtOAc/hexane (1:1). PM was obtained as an oil (>98% pure by GC-FID) and kavalactones were obtained as crystals (>98% pure by GC-FID). The identities of the compounds were confirmed by GC-MS and HRMS high resolution mass spectroscopy.

**Treatments.** HepG2 cells were treated with the kavalactones and alkaloid at concentrations of 1, 10, 25, 50, 100, and 200 μM for up to 24 h. Based on brief, 4 h, without weights, the kavalactones and alkaloid were dissolved in DMSO and added to the culture media. The final concentration of DMSO in the media was below 0.1%. All compounds were visually soluble in the media and did not precipitate over time.

Long-term toxicity was measured up to 15 days by changing the media with fresh test compounds every 2 days. Controls were treated with equivalent amounts of DMSO. Media were harvested to measure the release of LDH and cells were harvested to determine caspase-3, mitochondrial membrane potential, cellular ATP content, and production of reactive oxygen species (ROS). All the assays were read using the Perkin Elmer multiplate reader, Wallac Victor² (Perkin Elmer Life Sciences, Boston, MA).

**Cytotoxicity and apoptosis assay.** Cell viability was assayed fluorimetrically using the release of lactate dehydrogenase (LDH) from cells with a damaged membrane. Briefly, the released LDH was measured with a 10-min coupled enzymatic assay that resulted in the conversion of resazurin into resoruin, using the commercial CytoTox-ONE assay kit (Promega, Madison, WI) with an excitation wavelength of 560 nm and an emission wavelength of 590 nm. Apoptosis was measured using a Homogenous Caspase-3 assay kit (Roche Applied Science, Indianapolis, IN), according to the manufacturer’s protocols. Briefly, after treatment of HepG2 cells for 24 h with test compounds, the cells were washed twice with PBS. Cells were then incubated for 2 h at 37°C in the incubation media along with the caspase substrate, rhodamine 110 (R110), supplied by the manufacturer. After washing excess substrate with PBS, fresh PBS was added and the free R110 was measured fluorimetrically, at an excitation wavelength of 475 nm and an emission wavelength of 560 nm, using a Perkin Elmer multiplate reader, Wallac Victor² (model 1420-011, multilabel counter, Perkin Elmer Life Sciences).

**Mitochondrial membrane potential (Δψm).** Δψm was measured by staining the cells with the lipophilic cationic probe 5’,6,6’,3’,3’-tetrathylbenzimidazolylcarbocyanine iodide (JC-1; Molecular Probes, Eugene, OR), according to published protocols (Mukherjee et al., 2002) with slight modifications. Briefly, cells were washed twice in phosphate-buffered saline (PBS) and stained with 10 μM JC-1 for 30 min in the dark at 37°C. After the final wash, PBS was added to the wells and fluorescence was measured at 530 and 590 nm using the multiplate reader, Wallac Victor². The ratio of 590/530 nm was considered as the relative Δψm value. Valinomycin (Molecular Probes) at a final concentration of 100 μM was used as a positive control.

**Cellular ATP concentrations.** Cellular ATP levels were measured using the ATPLite-M kit from Packard BioScience (Meriden, CT). Briefly, 1 × 10⁴ HepG2 cells/well were plated in white 96-well CulturPlate (cat. 6005181) obtained from Packard BioScience. Then, 24 h after plating, cells were treated with test compounds for 24 h. Cells were washed twice with PBS and assayed for ATP according to the manufacturer’s directions. The principle of the assay is based on production of light when the enzyme luciferase is oxidized to oxyluciferin, using ATP. The emitted light is proportional to the ATP con-
centration within experimental limits. Luminescence was measured using a Perkin Elmer multiplate reader, Wallac Victor \(^2\). ATP was calculated from a standard curve generated with each set of experiments and was expressed as mmol/10^5 cells.

**ROS production.** HepG2 cells were treated with PM, DHM, and DMY for 15 min, 30 min, and 1, 2, 4, 6, and 24 h. The cells were then washed with PBS and further incubated with 20 mM of 2',7'-dihydrodichlorofluorescein diacetate in PBS (H\(_2\)-DCFDA, Molecular Probes) for 30 min at 37°C (Osseni et al., 2000). The dye was washed once with PBS. Fluorescence was measured at 485 and 535 nm after adding 200 µl of fresh PBS to the wells, using a Wallac Victor\(^2\) fluorimeter. H\(_2\)-DCFDA is a nonfluorescent, cell-permeant compound that is cleaved by endogenous esterases and the product 2',7'-dichlorofluorescein (DCF) is oxidized by ROS to generate dichlorofluorescein that is fluorescent. The fluorescence is directly proportional to ROS production.

**Statistical analysis.** All data are presented as average ± SD. Three different sets of experiments were performed in triplicate and data were analyzed using the Student’s \(t\)-test.

**RESULTS**

Chemical structures of the three test compounds are shown in Figure 1. The daily doses of kava total extracts involved in case reports of hepatotoxicity generally ranged from 60 to 240 mg kavalactones. Initial studies with the kavalactones kavain, dihydromkavain, methysticin, DHM, yangonin, and DMY at concentrations less than 500 nM failed to show any toxic effects in HepG2 cells for up to 2 weeks (data not shown). Based on these observations, we chose DHM, DMY, and PM at 1, 25, 50, 100, and 200 µM concentrations for 48 h. At 200 µM, PM caused cell death within the first 6 h (data not shown). The morphological and cytotoxic effects of kavalactones and PM on HepG2 are shown in Figure 2. In contrast to untreated control (Fig. 2A) and DMY-treated HepG2 cells (Fig. 2B), treatment with 100 µM of PM for 24 h showed a characteristic rounded morphology of HepG2 cells before detachment (Fig. 2C). About 80–90% HepG2 cells treated with 100 µM PM sloughed off from the surface of culture dishes after 36–48 h, while those treated with 50 µM PM detached after 72 h (data not shown). As shown in Figure 2D, 50 and 100 µM PM caused significant (65 and 90%, \(p < 0.001\)) cell death within 24 h, as measured by the release of LDH into the medium. HepG2 cells were treated with varying concentrations of PM, DHM, and DMY for 24 h. Each value is the average of three different experiments in triplicate. Data are represented as percentages of control values and as averages ± SD; *\(p < 0.001\).

To further characterize probable mechanisms of cellular toxicity, we measured the effects of PM, DHM, and DMY on cellular ATP levels, changes in \(\Delta\psi\)m, generation of ROS, and release of caspase-3 as a measure of apoptosis. Both DHM and DMY at 100 µM had no effect on cellular ATP content, \(\Delta\psi\)m, ROS production, or apoptosis (data not shown). In contrast, 50 and 100 µM PM significantly depleted ATP levels by 70–90% within 24 h (\(p < 0.001\), Fig. 3A), with a simultaneous increase in caspase-3 activity by 250 and 575%, respectively (\(p < 0.001\), Fig. 3B). We further investigated whether changes in \(\Delta\psi\)m were involved in PM-induced cell death. As shown in Figure 3C, 24 h treatment of HepG2 cells with 50 and 100 µM PM resulted in a significant (35–40%) decrease in \(\Delta\psi\)m (\(p < 0.001\)). Studies now indicate that the generation of ROS can lead to the onset of mitochondrial permeability transition causing necrosis and apoptosis in hepatocytes (Kim et al., 2003). In our study, only PM at 100 µM concentration demonstrated an insignificant increase (125%, \(p < 0.5\)) in ROS production after

![Chemical structures of the three test compounds](http://toxsci.oxfordjournals.org/content/early/2016/05/01/toxsci.2016.03.011/F1.large.jpg)

**FIG. 1.** Structures of the major lipophilic compounds from kava that were tested, pipermethystine (PM), dihydromethysticin (DHM), and desmethoxyyangonin (DMY).
24 h (data not shown), suggesting that earlier time points need to be analyzed.

**DISCUSSION**

After the first report of fulminant liver failure surfaced in the medical literature (Strahl *et al.*, 1998), several studies have been conducted to elucidate potential mechanisms of kava toxicity. Mechanisms such as glutathione depletion (Whitton *et al.*, 2003), *in vitro* formation of electrophilic metabolites such as quinones (Johnson *et al.*, 2003), and genetic polymorphisms for CYP2D6 (Russmann *et al.*, 2001) have been proposed for kavalactone toxicity. However, if kava-associated toxicities arose from kavalactones alone, consumption of beverages among Pacific Islanders would also be expected to cause toxicities. Kava-associated hepatic toxicities are most prevalent in supplement-consuming populations. These observations would indicate a role for compounds other than kavalactones. A review of 36 Swiss and German cases noted great variations in both the time of onset of symptoms of liver toxicity, ranging from 2 weeks to 2 years, and the cumulative dose necessary to induce hepatotoxicity expressed in kavalactone equivalents (Stickel *et al.*, 2003).

Recently, it has been noted that increased use of aerial material for kava preparations may lead to the inclusion of alkaloids such as PM (Dragull *et al.*, 2003), which would only be present in negligible quantities in traditional drinks that are prepared mainly from underground parts. In general, plant alkaloids are the leading plant toxins associated with human and animal hepatotoxicity (Prakash *et al.*, 1999), but no toxicological data was available for PM. Therefore, we tested the *in vitro* toxicities of kavalactones and an alkaloid in the human hepatoma cell line HepG2.

In contrast to primary human hepatocytes, HepG2 cells contain low levels of P450 isozymes (Feierman *et al.*, 2002; Wilkening *et al.*, 2003). However, since kavalactones apparently do not need to be metabolized by the liver to exert their physiological effects (Blasche *et al.*, 1994; Klohs, 1967), HepG2 cells offer a convenient and reproducible *in vitro* system to test their initial cytotoxicity. No data are available on the metabolism of PM. This lipophilic and major alkaloid in aerial kava (Dragull *et al.*, 2003; Smith, 1979, 1983) is a relatively stable oil, but it may decompose partially at room temperature upon standing for 3–4 months (Smith, 1979). Our study indicates that PM is toxic to HepG2 cells at concentrations of 50 and 100 μM (Fig. 2D), as measured by the release of LDH into the media. However, the concentrations of PM used in our study (50 and 100 μM) were 25- to 100-fold higher than that of the classical hepatotoxin aflatoxin B1 (AFB1), which has been demonstrated to significantly increase LDH release at 0.5 μM after 24 h of incubation in HepG2 cells (O’Brien *et al.*, 2000).

The alkaloid and kavalactones at the concentrations used gave clear solutions, suggesting samples were well below their saturation point. Our study indicates that PM is considerably more toxic to HepG2 cells than the kavalactones DHM and DMY. Low toxicity of kavalactones is consistent with previous observations and clinical trials with kava total extracts (Blasche, 1994) and the recent observations that rats fed 200 or 500 mg of kavalactones/kg/day for 2 or 4 weeks did not demonstrate any changes in ALT, AST, alkaline phosphatase (ALP), and LDH (Singh and Devkota, 2003). Recently, Russmann *et al.* (2003) demonstrated that 23 of 27 heavy kava drinkers in New Caledonia had elevated γ-glutamyl transferase (GGT), while 8 of 27 had minimally elevated transaminases. In our study, structural instability could not account for the low toxicity of kavalactones, since >90% of kavalactones were recovered from the media that were incubated under identical experimental conditions without any cells (data not shown). In contrast, the recovery of PM from the media was lower than that of the kavalactones. Based on the structure of PM, it is expected that the 3,4 double bond would be relatively reactive...
since PM is found naturally in epoxidized form (Dragull et al., 2003).

A malfunction in mitochondrial bioenergetics due to the loss of membrane potential (Δψm) is known to uncouple mitochondria and decrease cellular ATP (Gergely et al., 2002). In our study, 50 and 100 μM PM significantly decreased cellular ATP content as well as Δψm (Figs. 3A and 3B). Loss of Δψm is a common event following toxicant exposures leading to cellular necrosis or apoptosis (Lemasters et al., 1998). Alternatively, permeability changes to the outer mitochondrial membrane and collapse of Δψm are known to be associated with the release of cytochrome c from mitochondria to cytosol, ultimately initiating the mitochondrial death pathway (Regula et al., 2003). There are two major pathways through which apoptosis is known to be induced: one involves death receptors and is exemplified by Fas-mediated caspase-8 activation and another is the stress- or mitochondria-mediated caspase-9 activation pathway. Both pathways converge on caspase-3 activation, resulting in nuclear degradation and cellular morphological change (Ueda et al., 2002). As shown in Figure 3C, PM-associated toxicity could be due to increased caspase-3 activity at 50 and 100 μM.

It has been observed that, when administered individually, kavalactones do not exhibit the same degree of pharmacological activity as the whole extract (Blasche et al., 1994; Keller and Klohs, 1963). The same could be true for toxicity. Therefore, an initial necessity, but major limitation of our study, is testing the effects of kavalactones and alkaloids individually rather than in combination. Overall, our findings suggest that the alkaloid PM, rather than kavalactones, is toxic to human hepatoma cells HepG2 in vitro and thus may be involved in kava-associated hepatotoxicity. Studies are in progress to address the long-term toxicity at lower concentrations as well as the additive effects of kavalactones and alkaloids.

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