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

Pratibha V. Nerurkar, Klaus Dragull and Chung-Shih Tang

**Affiliation:** Department of Molecular Biosciences and Bioengineering, College of Tropical Agriculture and Human Resources, University of Hawaii at Manoa, Honolulu, HI 96822. pratibha@hawaii.edu, dragull@hawaii.edu, tangcs@hawaii.edu

**Running Title:** *In vitro* toxicity of pipermethystine

**Corresponding Author:** Pratibha V. Nerurkar, Ph.D., Laboratory of Metabolic Disorders and Alternative Medicine, Department of Molecular Biosciences and Bioengineering, College of Tropical Agriculture and Human Resources, 1955 East-West Road, Agriculture Science Building, Room 218, Honolulu, Hawaii 96822, U.S.A. Telephone: (808) 956-9195; Fax: (808) 956-3542; E-mail: pratibha@hawaii.edu
Abstract

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 employ primarily the underground parts of the shrub to prepare the kava beverage. However, some kava herbal supplements may contain ingredients from aerial stem peelings. The aim of this project 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), desmethoxyyangonin (DMY) that are abundant in the roots. Exposure of human hepatoma cells, HepG2, to 100 μM of 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 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.
INTRODUCTION

In the past two years, 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 a recreational and ceremonial drink for some two thousand years (Lebot *et al.* 1997) and at present it is consumed extensively in Vanuatu, Samoa, Tonga, and Fiji. Traditionally, the underground 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 infections and other physical conditions (Bilia *et al.* 2002). Kava has been compared to benzodiazepines such as diazepam (valium) or alprozolam (xanax) and recognized as a “natural alternative” to these anti-anxiety 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.

Pathophysiological 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 a number of countries (2002a; 2002b; Currie and Clough 2003) or advisories have been issued (2002b; 2003) due to suspected hepatotoxicity, including cases requiring liver transplants and death (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;
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 transferases (GGT) and alkaline phosphatases (ALP), but normal ALT have been noted among heavy kava drinkers of aboriginal communities in Australia (Currie and Clough 2003; Mathews et al. 1988). These observations do not suggest acute inflammation, as opposed to hepatotoxicity cases involving kava herbal supplements that demonstrated high levels of ALT. 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, that 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). In brief, the general disparity in toxicity patterns could arise from genetic differences in human metabolism (Russmann et al. 2001), differences in extraction procedures (2003), and/or from differences in kava raw material use (Dragull et al. 2003).

Public records (2002b) 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 (1998a; 1998b; 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
In this report, we tested the above hypothesis using human hepatoma cell line, HepG2, which provides a reproducible in vitro model system to identify potential liver toxins. To date, no data is 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 studies were conducted using DHM and DMY as “representative” lactones, to test the potential of kavalactones and alkaloids to induce apoptosis as 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 and 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 X 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:** Pipermethystine (PM; CAS# 375797-99-2) was isolated from stem peelings of *Piper methysticum* cv. Isa (Dragull et al. 2003), the kavalactones, 7,8-dihydromethysticin (DHM; CAS# 19902-91-1) and desmethoxyyangonin (DMY; CAS# 15345-89-8) from commercial root powder. Preparative liquid chromatography was performed on silica gel (40 µm, Mallinckrodt Baker Inc.), all solvents used were HPLC grade (Fisher Scientific). EtOAc extracts of plant materials were chromatographed with a gradient of EtOAc/n-hexane mixtures (Shao et al. 1998) running from ratio 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/n-hexane (1:1). PM was obtained as an oil (> 98% pure by GC-FID) and kavalactones as crystals (> 98% pure by GC-FID). The identities of the compounds were confirmed by GC-MS and HRMS.
**Treatments:** HepG2 cells were treated with kavalactones and alkaloids at concentrations of 1, 10, 25, 50, 100 and 200 µM for up to 24 h. Based on their molecular 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 two days. Controls were treated with equivalent amounts of DMSO. Media was 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 by measuring the release of lactate dehydrogenase (LDH) from cells with a damaged membrane. In brief, the released LDH is measured with a 10-minute coupled enzymatic assay that results in the conversion of resazurin into resorufin, using the commercial CytoTox-ONE™ Assay kit (Promega, Madison, WI), using an excitation wavelength of 560 nm and emission wavelength of 590 nm.

Apoptosis was measured using “Homogenous Caspase-3 Assay” kit, (Roche Applied Science, Indianapolis, IN), according to manufacturer’s protocols. In brief, 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 excitation 475 nm and emission at 560 nm using a Perkin Elmer multiplate reader, Wallac Victor² (Model number 1420-011, multilabel counter).

**Mitochondrial Membrane Potential (Δψₘ):** Δψₘ was measured by staining the cells with the lipophilic cationic probe 5,5’,6,6’-Tetrachloro-1,1’,3,3’-tetraethylbenzinidazolylcarbocyanine 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). In brief, 1 X 10⁵ HepG2 cells/well were plated in white 96-well CulturPlate™ (Cat. # 6005181) obtained from Packard BioScience. Twenty-four hours after plating, cells were treated with test compounds for 24 hr. Cells were washed twice with PBS and assayed for ATP according to the manufacturer’s direction. The principle of the assay is based on production of light when the enzyme luciferase is oxidized to oxyleuciferin, using ATP. The emitted light is proportional to the ATP concentration within...
In vitro toxicity of pipermethystine
Nerurkar et al, 2003

Experimental limits. Luminescence was measured using a Perkin Elmer multiplate reader, Wallac Victor². ATP was calculated from a standard curve, generated with each set of experiments, and was expressed as µmol/10⁵ cells.

**Reactive Oxygen Species (ROS) Production**: HepG2 cells were treated with PM, DHM, and DMY for 15 min, 30 min, 1, 2, 4, 6 and 24 h. The cells were then washed with PBS and further incubated with 20 µM of 2’,7’-dihydrodichlorofluorescin diacetate in PBS (H₂-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 Wallac Victor² fluorimeter. H₂-DCFDA is a non-fluorescent 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 depicted 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, dihydrokavain, methysticin, dihydromethysticin, yangonin, desmethoxyyangonin 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 demonstrated in Figure 2. In contrast to untreated control (Figure 2A) and DMY-treated HepG2 cells (Figure 2B), treatment with 100 μM of PM for 24 h showed a characteristic rounded morphology of HepG2 cells before detaching (Figure 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 seen in Figure 2D, 50 and 100 μM PM caused significant, 65 and 90% (p<0.001) cell death within 24 h, as measured by release of LDH into the medium, while higher concentrations of the kavalactones DHM and DMY (200 μM), caused only 30% cell death after 48 h (p<0.01, data not shown). Lower concentrations of DHM and DMY (100 and 50 μM) did not show any toxicity at 24 h (Figure 1D) as well as up to 8 days of treatment (data not shown).

To further characterize probable mechanisms of cellular toxicity, we measured the effects of PM, DHM, and DMY on cellular ATP levels, changes in Δψm, 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 $\mu$M of PM significantly depleted ATP levels by 70-90%, within 24 h ($p<0.001$, Figure 3A) with simultaneous increase in caspase-3 activity by 250 and 575%, respectively ($p<0.001$, Figure 3B). We further investigated whether changes in $\Delta \psi_m$ were involved in PM-induced cell death. As observed in Figure 3C, 24 h treatment of HepG2 cells with 50 and 100 $\mu$M PM resulted in a significant 35-40% decrease in $\Delta \psi_m$ ($p<0.001$). Studies now indicate that 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 $\mu$M concentration demonstrated an insignificant increase (125%, $P<0.5$) in ROS production after 24 h (data not shown), suggesting that PM-induced apoptosis may not involve ROS.

**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), 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 develop toxicities. However, kava-associated hepatic toxicities are most prevalent in supplement-consuming populations. These observations would indicate a role for compounds other than kavalactones. Reviews of 36 Swiss and German cases noted great variation in both, the time of onset of symptoms of liver toxicity, ranging from 2
In vitro toxicity of pipermethystine  
Nerurkar et al, 2003

weeks to 2 years, and also the cumulative dose necessary to induce hepatotoxicity expressed in kavalactone equivalents (2003; Stickel et al. 2003; Stoller 2000).

Recently it has been noted that increased use of aerial material for kava preparations may lead to 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. We therefore tested the in vitro toxicities of kavalactones and alkaloids in 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 do not need to be metabolized by the liver to exert their physiological effects (1994), HepG2 cells offer a convenient and reproducible in vitro system to test their initial cytotoxicity. No data are available on the metabolism of PM. Pipermethystine (PM), the lipophilic and major alkaloid in aerial kava (Smith, 1979; Smith, 1983; Dragull et al., 2003), is a relatively stable oil, but 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 (Figure 2) as measured by the release of LDH into the media. However, the concentrations of PM used in our study (50 and 100 µM) are 25- to 100 fold higher than the classical hepatotoxin, aflatoxin B1 (AFB1) which have 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
concentration 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 total kava extracts (1994) and the recent observations that rats fed 200 or 500 mg of kavalactones/kg for two or four weeks did not demonstrate any changes in ALT, AST, alkaline phosphatases (ALP) and LDH (Singh and Devkota 2003). Recently, Russmann et al. demonstrated that 23/27 heavy kava drinkers in New Caledonia had elevated gamma glutamyl transferases (GGT), while 8/27 had minimally elevated transaminases (Russmann et al. 2003). In our study, structural instability could not account for the low toxicity of kavalactones, since >90% of kavalactones were recovered from the media that was 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 loss of membrane potential (Δψm) is known to uncouple mitochondria and decrease cellular ATP (Gergely et al. 2002). In our studies, 50 and 100 µM PM significantly decreased both, cellular ATP content as well as Δψm (Figure 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 and 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 observed 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 observed after administration of whole extract (1994; Klohs 1967; 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. Collectively, our findings suggest that, under these experimental conditions, the alkaloid PM, rather than kavalactones are toxic to human hepatoma cells, HepG2 in vitro, and may be involved in kava-associated hepatotoxicity. Studies are currently in progress to address the long-term toxicity at lower concentrations as well as additive effects of kavalactones and alkaloids.

ACKNOWLEDGEMENTS

Plant material used for PM isolation was a generous gift from Dr. H.C. Bittenbender (TPSS/CTAHR) and Pakielo Kaufusi (MBBE/CTAHR), both of UH. Kavalactones were isolated by standard procedures by Dr. Wen-Jing Tang. We acknowledge the assistance of Dr. Bernard Fromenty (Institut National de la Santé et de la Recherche Médicale (INSERM) Unité –481, Paris, France), Dr. Vivek Nerurkar (Retrovirology Research Laboratory, Pacific Biomedical
In vitro toxicity of pipermethystine
Nerurkar et al, 2003

Research Center, UH) and Dr. Naoky Tsai (JABSOM, UH) in reviewing this article. This work was supported partially by T-STAR grant 2002-34135-12724 and partially by U.S. Public Health Service grants (G12RR003061 and P20RR011091) from the Research Centers in Minority Institutions Program, National Center for Research Resources, NIH.

REFERENCES


**Figure 1:** Structures of major lipophilic compounds from kava tested; pipermethystine (PM), dihydromethysticin (DHM), and desmethoxyyangonin (DMY).

**Figure 2:** Phase-contrast photomicrographs and cytotoxicity curves after 24 h treatment with kavalactone and alkaloid. As compared to the untreated control (Figure 2A), or cells treated with 200 µM of kavalactone, dihydromethysticin (DHM, Figure 2B), HepG2 cells treated with 100 µM kava alkaloid pipermethystine (PM) demonstrate an abnormal rounded morphology (Figure 2C). Magnification of × 100. Figure 2D demonstrates the levels of lactate dehydrogenase released into the medium. HepG2 cells were treated with varying concentrations of PM, DHM and desmethoxyyangonin (DMY) for 24 h. Each value is the average of three different experiments in triplicate. Data are represented as percentage of control values and as average ± SD. * = p<0.001

**Figure 3:** HepG2 cells were treated with varying concentrations of pipermethystine (PM) for 24 h. Figure 3A represents the cellular ATP content while apoptosis was measured by the release of caspase-3 (Figure 3B). The loss of mitochondrial membrane potential (Δψm) is represented as the ratio of fluorescence at 590 and 530 nm, measured by JC-1 staining (Figure 3C). Valinomycin (Val) was used as a positive control to inhibit Δψm. Each value is the average of three different experiments in triplicate. Data is represented as percentage of control values and as average ± SD. *p<0.001
In vitro toxicity of pipermethystine

Nerurkar et al, 2003

3A

3B

3C

Con 1 10 25 50 100

Pipermethystine (µM)

Con 1 10 25 50 100

Pipermethystine (µM)

Con Val 1 10 25 50 100

Pipermethystine (µM)