A BRIEF COMMUNICATION

Hepatocytes Detoxify *Atuna racemosa* Extract

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A number of traditional medicine plants are hepatotoxic. Thus, while the traditional uses of *Atuna racemosa* suggest little indication for toxicity, it is nonetheless important to examine the potential for this extract to target the liver. Using Jurkat T cells and HepG2 hepatocytes as a model, the potential hepatotoxicity of this extract was evaluated. The results of a conditioned media experiment suggest that *A. racemosa* extract would likely be detoxified by the liver. These results provide the necessary background to initiate an *in vivo* toxicology investigation. Exp Biol Med 231:1739–1743, 2006

Key words: detoxify; hepatotoxicity; natural product; liver; plant; extract

Introduction

Despite a continuing loss of biodiversity (1), plants (2) and traditional medicines (3) are still reasonable sources of new drugs. Since plants continue to generate useful arrays of secondary metabolites (4), these resources will likely continue to be a source of new drugs in the future (5, 6). Compounding the loss of biodiversity, drug discovery involving traditional healers is becoming less feasible because of the generational loss of traditional knowledge (7). Nonetheless, throughout history herbal texts have been written that provide a record of previous medicinal practices involving plants (8). Using a bioinformatics system (9) we have developed a technique to mine these historic herbal texts for drug leads to help resurrect this lost traditional medicine knowledge (2). Through this work we identified *Atuna racemosa* as a potential novel antibacterial lead. After collecting this plant in Samoa, we showed that this purported medicinal property was correct (10).

In the examined historic herbal text (11) the kernel of *A. racemosa*, or "The Atun Tree," was described as a particularly strong treatment for diarrhea. In the text there is little indication of toxic effects through use of this plant, and, similar to many traditional medicines, there is not a clear delineation between the Atun tree being used as a food or medicine (12). The kernel taste is reported to be "...dry, tart, hard and with an astringent taste," with no reports of overdoses. Furthermore, the text states that "this fruit...is used... in the preparation of a strange dish, which the Natives call *Gou-Gou*... [by] adding or mixing in with this oft mentioned Atun kernel, after they have been grated; and they use this food as a side dish to whet one's appetite..." Additionally, *A. racemosa* has a single report in the current literature of medicinal use: on the islands of Samoa the kernel is used in a salve preparation for massages (13). While the antibacterial properties of this plant are exciting, a number of medicinal plants, such as kava (14) and chaparral (15), are hepatotoxic at higher doses, and thus the ability of the *A. racemosa* extract to induce hepatotoxicity required examination.

Using HepG2 cells as a model system (16), the potential hepatotoxicty of the *A. racemosa* extract was examined. Here I show that at extract concentrations inhibiting bacterial growth (~30 μg/ml; Ref. 10) the extract of *A. racemosa* is toxic to neither T cells nor hepatocytes. However, at higher concentrations the extract is toxic to T cells but not hepatocytes. This toxicity may be due to perturbation of cell cycle progression. Furthermore, the lack

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of hepatocyte toxicity is due to the ability of hepatocytes to detoxify the extract.

Materials and Methods

Cell Culture. Jurkat T lymphocytes (ATCC, Manassas, VA) were maintained in RPMI-1640 (Cellgro, Herndon, VA). HepG2 cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM; Cellgro). Both media were supplemented with 10% heat-inactivated fetal bovine serum (Cellgro) and 1% penicillin and streptomycin (Gibco, Grand Island, NY) at 37°C in 5% CO_2. Jurkat cells were maintained between 0.1×10^6 and 1×10^6 cells/ml, and HepG2 cells were passed every third day at 4:1.

Cell Death by Dye Exclusion and Cytometry. Propidium iodine (PI) dye exclusion assays and cytometry were performed as previously described (17). In this viability assay, PI was excluded from live cells, whereas dead cells stained positive for PI. HepG2 cells and Jurkat cells were treated with extract (0.001, 0.01, 0.05, 0.1, 0.2, 0.5, and 1 mg/ml) for 24 hrs and were analyzed by cytometry. Briefly, Jurkat cells were washed twice with phosphate-buffered saline (PBS). PI (Sigma-Aldrich, St. Louis, MO) was added to achieve a final concentration of 10 ng/ml. Cells were incubated at room temperature for 15 mins and assayed by flow cytometry. HepG2 cells were washed once with PBS, and supernatant was collected. Cells were treated with trypsin (Cellgro) and combined with collected supernatant. Cells were washed once and suspended in PBS without calcium and magnesium. PI was added to a final concentration of 10 ng/ml, and samples were incubated at room temperature for 15 mins and assayed by flow cytometry.

Cell Cycle Analysis. For cell cycle analysis HepG2 cells and Jurkat cells were treated with 100 μg/ml extract for 24 hrs. This value was selected because it induced death in ~20% of the Jurkat cells. For cell cycle analysis cells were washed twice with PBS and suspended in a volume of 300 μl. While gently vortexing, ice-cold ethanol was added dropwise at a rate of 1 drop every 15 secs. Samples were then stored on ice for 1 hr. Samples were rehydrated with 1 vol PBS and washed three times. Samples then were resuspended in 200 μl RNAse A (Sigma-Aldrich) in 0.1 *M* citrate buffer (Fisher Scientific, Pittsburgh, PA) for 15 mins, followed by 15 mins with 100 μg/ml PI in 0.1 *M* citrate buffer. Cells then were analyzed by flow cytometry with a FACScan flow cytometer (BD Biosciences, Mountain View, CA). Data analysis was performed using WinMDI 2.8 (Joseph Trotter, Scripps, San Diego, CA) and ModFit LT (Verity Software, Topsham, ME).

HepG2 cells were analyzed by cell cycle following the same protocol; however, the cells were treated with trypsin to produce a single-cell suspension prior to the initial washing.

Conditioned Media Experiment. HepG2 cells were plated in 6-well plates at 50% confluence and incubated for 24 hrs. HepG2 cells were washed once with PBS and treated with 200 μg/ml extract in 1 ml RPMI-1640 for 24 hrs. This concentration was selected because it induced death in ~50% of the Jurkat cells. Supernatant from the cultures was removed from the HepG2 cells and spun at 600 *g* for 10 mins to pellet and remove dead HepG2 cells (typically ~10%, determined by cell cycle). This supernatant was the HepG2-conditioned media. Jurkat cells were suspended in the conditioned media at a final concentration of 1×10^7 cells/ml and were incubated for 24 hrs. The percentage of dead cells then was examined by PI exclusion and analyzed by flow cyctometry as described above. Extract alone also was incubated without HepG2 cells in tissue culture incubator. This extract was used as the mock conditioned extract.

Extract Preparation. Extraction of the *A. racemosa* kernel was performed as previously described (10). Briefly, samples were collected in Samoa (voucher specimen HEJ 1858, deposited at the National Tropical Botanical Gardens, Kalaheo, HI) and were preserved in 70% ethanol for 1 week prior to processing. Samples were macerated in a blender and shaken for 1 hr at room temperature. Samples were sedimented by centrifugation, and supernatant was removed. Finally, the solvent was removed under vacuum at room temperature. Prior to application in cell culture the resuspended samples were 0.2-μm sterile filtered (Millipore, Billerica, MA).

Statistics. The results are from at least three replicates and are reported as mean ± SEM. Differences were determined by Mann-Whitney *U* test using SigmaStat (Systat, Richmond, CA). Values of $P < 0.05$ were regarded as significant.

Results

Toxicity of the *A. racemosa* Extract. To assess the toxicity of the *A. racemosa* extract, HepG2 cells and Jurkat cells were treated with extract for 24 hrs and were analyzed by cytometry. Figure 1 illustrates the dose-dependent toxicity of the Jurkat cells compared with the lack of toxicity in HepG2 cells.

Altered Cell Cycle Through Treatment with *A. racemosa* Extract. As HepG2 cells are resistant to many types of apoptosis-inducing agents, such as TRAIL (18), I examined cell cycle progression of the extract-treated cells to assess whether the extract could alter cell cycle in treated cells without inducing cell death. Figure 2 shows that Jurkat cells treated with 100 μg/ml extract for 24 hours exhibit an increased number of cells with reduced DNA, whereas the cell cycle progression of the HepG2 cells is unaffected. Furthermore, while the HepG2 cells did not experience any change in the relative populations of cells in different stages of the cell cycle, the Jurkat cells experienced a significant change ($P < 0.05$). The percentage of Jurkat cells in G_1 phase remained similar with drug treatment; however, with

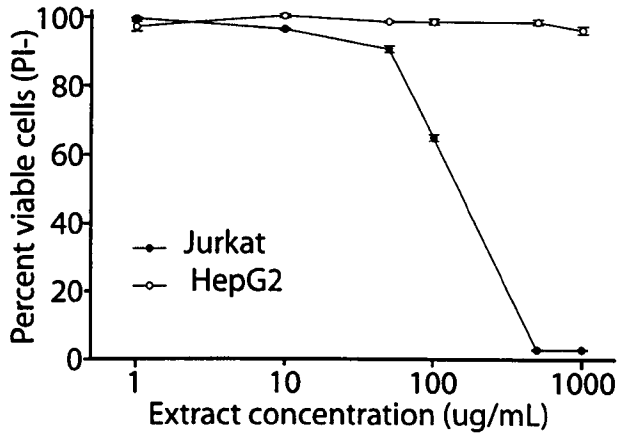

Figure 1. *Atuna racemosa* toxicity dose response curve for Jurkat T cells and HepG2 hepatocytes. As measured by PI exclusion and cytometry, treatment for 24 hrs with the extract of *A. racemosa* induced a dose-dependent toxicity in Jurkat cells. Importantly, no significant death of HepG2 cells was observed at any dose ($n = 3$).

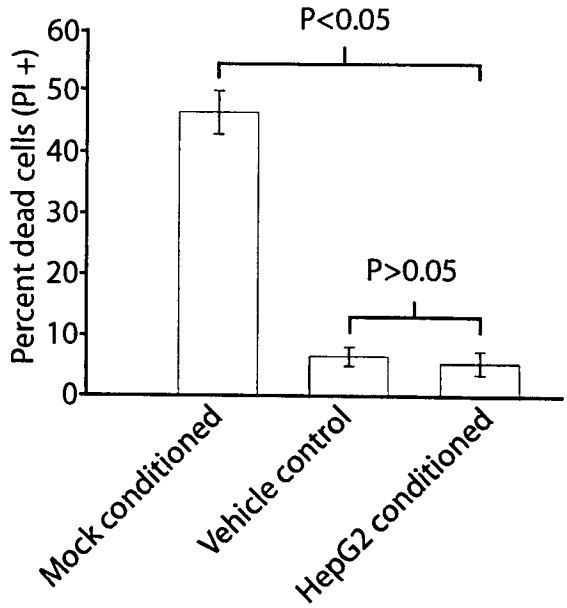

Figure 3. HepG2 cells detoxify the extract of *A. racemosa*. HepG2 cells were plated at 50% confluence and incubated for 24 hrs with 200 μg/ml extract, a concentration shown to induce death in ~50% of the Jurkat cells. This conditioned media supernatant was removed from the HepG2 cells. Jurkat cells were suspended in this media and incubated for 24 hrs. The percentage of dead Jurkat cells in mock-conditioned (extract incubated for 24 hrs without HepG2 cells), HepG2-conditioned (extract incubated for 24 hrs with HepG2 cells), and control (vehicle treatment without extract) samples were determined by PI exclusion. Incubation of *A. racemosa* extract with HepG2 cells for 24 hrs mitigated the toxicity of the extract to Jurkat cells ($n = 3$).

treatment there was a shift in the S-phase population from 10.9% ± 0.1% to 20.2% ± 1.2% and a shift in the G_2-phase population from 36.7% ± 1.4% to 28.4% ± 0.5%.

HepG2 Cells Detoxify *A. racemosa* Extract. A conditioned media experiment was performed in order to determine whether the HepG2 cells were able to detoxify the *A. racemosa* extract. There was a significant difference ($P < 0.05$) between the mock-conditioned media and the HepG2-conditioned media (Fig. 3). Importantly, the HepG2-conditioned media compared with the mock-conditioned media induced death in Jurkat cells at a percentage similar ($P > 0.05$) to vehicle-treated levels. The ability of the HepG2 cells to detoxify the *A. racemosa* extract was confirmed by forward scatter analysis (Fig. 4).

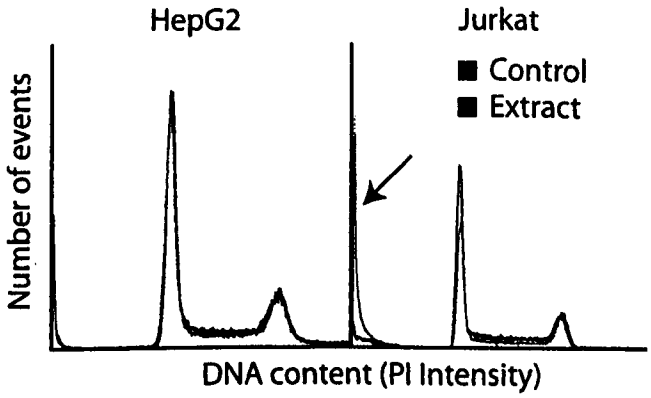

Figure 2. Treatment with *A. racemosa* extract and cell cycle. Treatment for 24 hrs with 100 μg/ml *A. racemosa* extract did not affect the cell cycle progression of HepG2 cells. However, treatment did alter the cell cycle progression of Jurkat cells. The percentage of Jurkat cells in G_1 phase remained similar with drug treatment; however, with treatment there was a shift in the S-phase population from 10.9% ± 0.1% to 20.2% ± 1.2% and a shift in the G_2-phase population from 36.7% ± 1.4% to 28.4% ± 0.5%. Arrow indicates increased number of sub-G_1 Jurkat cells ($n = 3$).

Discussion

Nearly 400 years ago, the extract of the *A. racemosa* was identified as a potential antibiotic (2, 9). Our group has shown that this extract can function as an antibiotic (10); however, the potential toxic effects of this extract *in vivo* are not known. Here I show that at concentrations greater than the minimal inhibitory concentration this extract causes perturbation of the cell cycle, but hepatocytes are able to detoxify this extract.

A number of natural product antimicrobials have been shown to alter cell cycle progression. Currently, the most intensely investigated of these is rapamycin (19) from the island of Rapa Nui in the Pacific (20). Through targeting mTOR (21), this compound and the synthetic analogs inhibit G_1- to S-phase progression and have great potential as a chemotherapeutic (19). While I have shown an altered cell cycle progression and a notable dose necessary to kill Jurkat cells (~50% death at 200 μg/ml), the unchanged G_1 phase does not particularly suggest this compound to be a strong candidate for examination as a chemotherapeutic. Furthermore, the ~30 μg/ml minimal inhibitory concentration for gram-positive bacteria suggests a lucrative therapeutic index as an antibiotic (10).

A conditioned media experiment was performed to examine whether the HepG2 cells were able to clear the toxic agent in the *A. racemosa* extract. Interestingly, *A.*

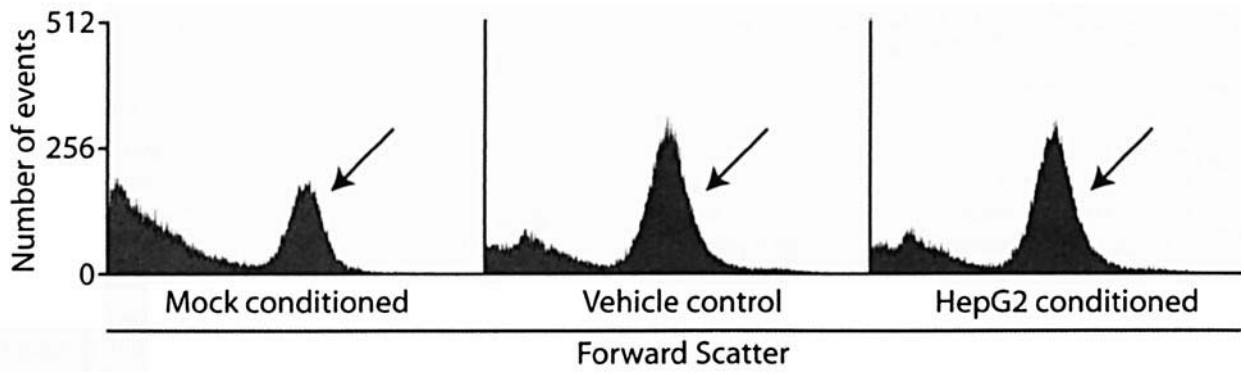

Figure 4. HepG2 cells detoxify the extract of *A. racemosa* by light scatter analysis. Comparing the relative size of the cells from the conditioned media experiment by light scatter analysis revealed that incubation of *A. racemosa* extract with HepG2 for 24 hrs mitigated the toxicity of the extract to Jurkat cells. Arrows indicate viable cells ($n = 3$).

racemosa extract conditioned with HepG2 cells resulted in complete reduction in toxicity of the extract. These results indicate that the hepatocytes are able to clear the toxic agent in the *A. racemosa* extract. The effective ability of the HepG2 cells to detoxify the *A. racemosa* extract was striking. While the inducible phase I and phase II enzymes of HepG2 cells are known to effectively detoxify xenobiotics (22), chemical carcinogens (23), and ethanol (24), the extent of the reduced toxicity was unexpected. Future work will focus on determining whether the detoxified extract still maintains the antibacterial properties.

The use of this plant as a traditional medicine suggests there is little risk of toxicity when used in physiologically appropriate doses (13). Nonetheless, it is important to examine the potential target organs in the event of a toxic dose. This work suggests that the extract of *A. racemosa* would likely be detoxified by the liver and sets the stage for an *in vivo* toxicity investigation.

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