Original Study

Tumor Cell Selective Cytotoxicity and Apoptosis Induction by an Herbal Preparation from *Brucea javanica*

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Abstract

The plant *Brucea javanica* has shown impressive efficacy for treating various diseases including cancer. However, the mechanism by which *B. javanica* acts is poorly understood. We have established tissue culture assays to study the effects of *B.* javanica on cervical and several other cancer cells. Our results demonstrated that the aqueous extract from *B. javanica* is selectively toxic to cancer cells. Induction of apoptosis by *B. javanica* appears to be a possible mechanism by which it kills cancer cells. Interestingly, a significant increase of p53 protein level was observed in these apoptotic cells. Our studies indicated that both p53-dependent and p53-independent activities contributed to herb-induced cell death. These results imply that further studies with *B. javanica* may lead to the development of novel anti-cancer drugs.

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Introduction

For several millennia, herbal preparations and natural remedies have been shown to be effective in treating many types of maladies. Approximately half of the drugs currently in clinical use are of natural origin.[1,2] Some of the herbs tend to possess functional groups (providing hydrogen bond acceptor/donors, etc).[3] Although herbal therapies are becoming increasingly popular worldwide, we know little about the molecular mechanisms and active ingredients in many of those therapeutic herbs.

Brucea javanica (L.) MERR. is a herb that is distributed throughout South East Asia and South China and its fruits have been used in traditional Chinese Medicine to treat malaria, and amoebiasis as well as cancer.[4,5] Interestingly, *B. javanica* has also been successfully used for treating genital warts.[6]

Several efforts have been made to identify the bioactive components from *B. javanica*. The major anti-amoebic properties were found to be a group of quassinoids mainly present in methanol/chloroform extracts.[7] An aqueous extract was found to be much less active against amoebae. Similarly, anti-malarial and anti-leukemic quassinoids were also identified from an organic extract of *B. javanica*.[8] A series of quassinoid glycosides have been isolated from the extracts of *B. javanica* and have exhibited cytotoxicity in melanoma and ovarian cancer cell lines.[9] Among these, Bruceoside C has shown excellent cytotoxic activity (50% effective dose (ED50) <0.1 g/ml) in KB (a human epidermoid carcinoma of the nasopharynx) and RPMI-7951 (human malignant melanoma) cell lines. To date, none of the bruceosides have progressed to drug development.

During the preparation of this manuscript, apoptosis-inducing activity from an aqueous extract of *B. javanica* has been demonstrated on four human cancer cell lines, i.e., breast, hepatocytes, lung, and esophageal.[10] The *B. javanica*-induced apoptosis proceeded through a mitochondrial dependent pathway associated with caspase-3 activation. However, a direct comparison of the effect of aqueous extract of *B. javanica* on appropriate normal cells has not been examined. The effect of aqueous *B. javanica* extract on other cancer cells remains to be examined. The molecular basis for *B. javanica* induced apoptosis remains undefined. The present study is designed to address some of these issues.

Materials and Methods

Preparation of B. javanica extract

The *B. javanica* fruit powders were obtained from KPC Products, Inc. (CA, USA) and Mayway Corp. (CA, USA). The product from Mayway was GMP certified. In the first step, the powder was suspended in phosphate-buffered saline (PBS) in an eppendorf tube and incubated with modest agitation at room temperature for 48 hrs. After centrifugation for 2 min at 12,000 rpm, the supernatant was passed through a 0.22 µm sterile filter. Aliquots were stored at –20˚C. The protein concentration of the extract was determined by the BCA protein assay kit (Pierce, Rockford, IL). An ethanol extract of *B. javanica* was similarly prepared.

Cell culture

All cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (base medium) unless otherwise indicated. The cells include the human epidermoid carcinoma cell line A431, human fibrosarcoma cell line HT1080, the African green monkey kidney fibroblast cell line CV-1, prostate carcinoma LNCaP, mammary adenocarcinoma cell line MCF-7, the HPV 18-postive human cervical epithelial cancer cell line HeLa, the HPV 16-postive human cervical epithelial cancer cell lines Caski and SiHa, the colon cancer cell line HCT116 (both p53 WT and $p53^{-/-}$), the breast carcinoma cell line HTB-126 and its matched control cell line HTB-125 derived from normal breast tissue peripheral to the carcinoma. LNCaP cells were maintained in RPMI 1640 medium supplemented with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% fetal bovine serum. HTB-126 cells were cultured in base medium supplemented with 0.01 mg/ml bovine insulin. HTB-125 cells were cultured in ATCC Hybri-Care medium supplemented with 30 ng/ml mouse EGF and 1.5 g/L sodium bicarbonate. PHKs were obtained from Bio Whittaker (Walkersville, MD) and Tufts-NEMC and maintained in Keratinocyte Growth Medium BulletKit (KGM-2 BulletKit). Experiments were performed using PHKs within 3 passages. The culture of HMEC strain 76N from reduction mammoplasty in DFCI-1 medium has been described previously.[11] Experiments were performed using HMEC cells within 18 passages.

Colorimetric MTT assays for cell survival

Cells were seeded in 96-well plates at a density of 2 x 10^3 cells per well. The following day, the media were changed to media containing an extract of B. javanica (treated cells) at 2 g/ml or regular media plus PBS (untreated cells) for 48 hours unless otherwise specified. Following treatment with *B. javanica*, viable cells were measured using a quantitative colorimetric MTT assay kit (Chemicon International Inc., Temecula, CA) according to the manufacture's protocol. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) is cleaved by living cells to yield a dark blue formazan product. Plates were analyzed in an ELISA plate reader at 570 nm with a reference wavelength of 655 nm. Cytotoxicity was calculated as the percent ratio of the difference in absorbance between PBS-treated and herb-treated cells with respect to the PBS-treated control.

CellTiter-Glo Luminescent Viability Assay

This assay is based on the measurement of ATP, which signals the presence of metabolically active cells. The assay uses luciferase as the detection enzyme because of the absence of endogenous luciferase activity in mammalian cells. An equal volume of CellTiter-Glo™ Reagent is added to the cell culture and luminescence is measured. The light signal is proportional to the amount of ATP present, which correlates with the number of viable cells present. The Veritas™ Microplate Luminometer used detects as little as $1.5X10^{-15}$ moles ATP and values are linear from 760 fg to 5.1 ng of ATP. The supplier's (Promega Corporation) instructions were followed in our studies reported here.

Cell Death Detection ELISA^{Plus}

Cells were seeded in 96-well plates at a density of 2 x 10^3 cells per well. The following day, the media was changed to or media containing an extract of B. javanica (treated cells) at 2 µg total protein/ml or regular media plus PBS (untreated cells) and incubated for 48 hours. Of 200 µl extract collected from each well, 20 µl were used for analysis of nucleosomes in cytoplasmic fractions by the Cell Death Detection ELISA^{plus} kit (Boehringer Mannheim Corp., Indianapolis, IN) according to the manufacturer's protocol. Enrichment factor represents the absorbance measured at 405 nm with a reference of 492 nm of treated cells divided by that of the corresponding untreated cells.

Protein Preparation and Immunoblot Analysis

Exponentially growing cells were treated with extract of *B. javanica* for various time periods, harvested and lysed in lysis buffer (250 mM NaCl, 20 mM Tris-HCl (pH7.4), 0.1% Nonidet P-40, 1 mM EDTA, 2 mM DTT, 0.1% nonfat dry milk, and 1 mM PMSF). After removing cell debris by centrifugation, proteins were fractionated on a SDS-polyacrylamide gel and blotted simultaneously with antibody against p53 (Pab 1801, Santa Cruz Biotechnology) and an anti-tubulin β antibody (Sigma). The antigen-antibody complexes were detected by SuperSignal Substrate (Pierce).

Statistical Analysis

The differences between means were assessed by Student's *t* test. A $p \leq 0.05$ was considered statistically significant.

Results

Selective cytotoxicity of B. javanica on cervical cancer cells

We have examined the anti-cancer activity and selectivity of *B. javanica* on cervical cancer using cell-based assays. To this end, a set of established human cervical cancer cells lines were used. These include the cervical squamous cell carcinoma CaSki, SiHa, C33A, and the cervical adenocarcinoma HeLa. For control, primary human keratinocytes (PHKs) were used. Cells were treated with *B. javanica* extracts and cell viability was determined quantitatively by the CellTiter-Glo Luminescent Viability Assay. As shown in **Figure 1A**, the aqueous extract of *B. javanica* specifically inhibited the growth of cervical carcinoma SiHa cells, but had a much less effect on the normal PHKs over a wide range of concentrations ($P < 0.0001$).

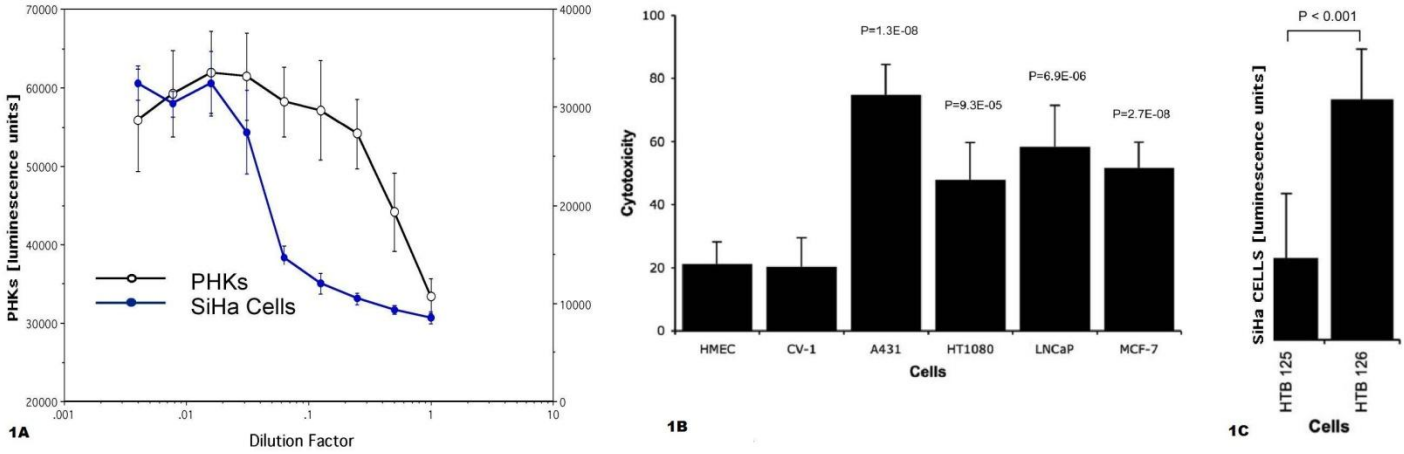

Figure 1. Tumor cell specific cytotoxicity by an aqueous extract from *B. javanica*. **A.** Selective cytotoxicity of *B. javanica* on cervical cancer cells. SiHa cells or PHKs were incubated with medium containing PBS or *B. javanica* extract at multiple concentrations (Extract dilution 1 = 400 µg total original powder/mL or 35 µg total protein/mL). Cell viability was determined 48 hours later by quantitative CellTiter-Glo Luminescent Viability Assay and is represented by number of luminescence units. All values represent the mean of six determinations. Error bars reflect the standard deviations of the mean. **B.** Cytotoxicity of *B. javanica* in multiple cancer cell types. Cells were treated with *B. javanica* extract at 2 µg total protein/ml for 48 hours and cell viability was determined by analysis of MTT conversion. All values represent the mean of at least two independent experiments, each done in triplicate. P values were obtained after compared with HMEC data. **C.** Cytotoxicity of *B. javanica* on breast cancer cells and matched normal control cells from the same patient. Cells were treated and analyzed as described in B. All values represent the mean of two independent experiments, each with six determinants.

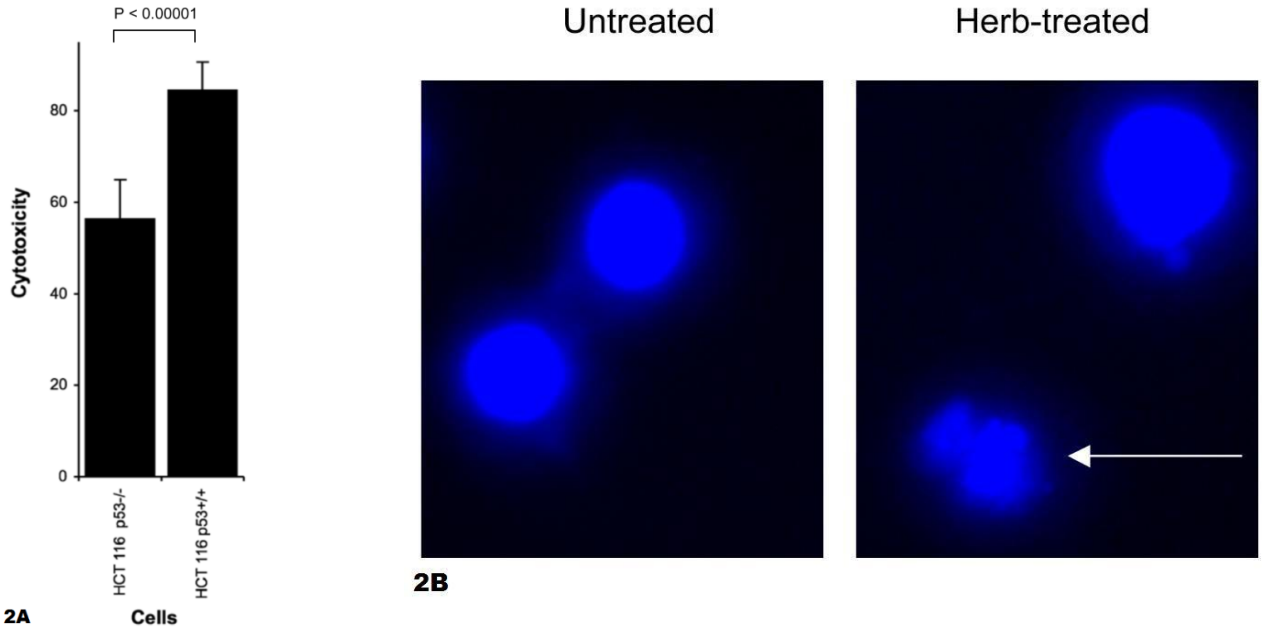

Figure 2. Induction of apoptosis in cervical cancer cells by an aqueous extract of *B. javanica*. **A**. Sub-confluent proliferating cervical cancer HeLa and breast cancer MCF-7 along with control HMEC and CV-1 cells were treated with medium containing extract from *B. javanica* or medium with PBS. Cell lysates were analyzed for nucleosomes in cytoplasmic fractions by the Cell Death Detection ELISA^{plus} kit. Relative apoptosis shows the enrichment factor of treated cells divided by that of the corresponding control PBS-treated cells. Values represent the mean of at least two independent experiments, each done in duplicate. Error bars reflect the standard deviations of the mean. **B**. HeLa cells treated with *B. javanica* for 48 hours were stained with Hoechst 33342. Representative images were shown. Arrow indicates a condensed and fragmented chromatin.

The aqueous extract of *B. javanica* also showed significant differences in cytotoxicity between other cervical cancer cells (CaSki, C33A, and HeLa) and PHKs ($P < 0.05$, not shown). In contrast, ethanol extracts of *B. javanica* did not work as efficiently as aqueous extracts, as demonstrated in HeLa cells (not shown). We have obtained consistent results with two batches of *B. javanica* preparations from the same source (KPC Products, Inc) as well as preparations from a different source (Mayway Corp.).

B. javanica has a broad cytotoxic effect on cancer cells

Next we examined the cytotoxicity of the *B. javanica* aqueous extracts on several other cancer cell lines. These included the mammary adenocarcinoma cell line MCF-7, the prostate carcinoma cells LNCaP, the human epidermoid carcinoma cell line A431, and the human fibrosarcoma cell line HT1080. For controls, the normal human mammary epithelial cells (HMECs) and the normal epithelial cell line CV-1 from African green monkey kidney were used. CV-1 cells proliferate as efficiently as cervical cancer cells in culture (not shown) and have served as an indicator cell line for normal epithelial cells.[12] As shown in **Figure 1B**, *B. javanica* extract at 2 µg total protein/ml inhibited the growth of all tumor cells but caused less inhibition of the growth in the normal control HMEC or CV-1 cells. While on average more than 58% of cancer cells were killed by *B. javanica*, relatively mild cytotoxicity (~20%) was observed for the control cells. We also compared the herb-induced cytotoxicity between breast cancer cell line HTB-126 and normal fibroblast cell line HTB-125 established from the same patient. *B. javanica* treatment lead to 70% cell death in breast cancer cells, whereas only about 24% of normal control cells were killed (**Figure 1C**).

All the differences in cytotoxicity between cancer and normal cells are highly significant ($P < 0.001$).

Apoptosis is a putative mechanism by which B. javanica kills cancer cells

The CellTiter-Glo Luminescent Viability Assay or MTT assay measure cell survival and cytotoxicity but do not differentiate between cells dying from apoptosis or other mechanisms. To determine if the cytolysis of cells after *B. javanica* treatment is apoptotic, we performed the Cell Death Detection ELISA^{plus} assay (Boehringer Mannheim Corp., IN), which measures cytoplasmic histone-associated-DNA-fragments resulting from DNA degradation that occurs specifically in apoptotic cells. As shown in **Figure 2A**, treatment of HeLa and MCF-7 cells with *B. javanica* at 2 µg total protein/ml resulted in a specific enrichment of mono- and oligonucleosomes released into cytoplasm. An approximate 2-fold and 4-fold enrichment of nucleosomes in cytoplasm was observed in HeLa and MCF-7 cells respectively as compared with control HMECs ($P<0.0001$). These results indicate that apoptosis is a possible mechanism by which *B. javanica* kills cancer cells. To strengthen this observation, we examined cell morphology following Hoechst 33342 stain of herb-treated cells. Consistent with results from the Cell Death Detection ELISA^{plus} assay, an increased number of cells showing chromatin condensation and/or fragmentation were found in treated HeLa cells (~22%) as compared with untreated cells (~1%). Representative images of Hoechst 33342 stained cells are shown in **Figure 2B**.

A role for p53 in B. javanica-induced cell death

As an initial step to explore the mechanism by which *B. javanica* induces apoptosis of cancer cells, we examined the steady-state level of the tumor suppressor p53, which is an important regulator of apoptosis.[13] In HPV-positive cervical cancers, p53 is targeted for degradation by HPV oncoprotein E6 and p53 level is reduced. Significantly, the levels of p53 proteins were considerably increased in HeLa cells after treatment with *B. javanica* extract at 2 µg total protein/ml (**Figure 3A**). This result is consistent with *B. javanica* induced apoptosis in HeLa cells. To determine the role for p53 in *B. javanica*–induced apoptosis of cancer cells, the colon cancer HCT116 cell lines with and without p53 were tested. While up to 84% of *B. javanica*–treated HCT116 p53 WT cells were dead, p53-null HCT116 cells were much less sensitive (56% cytotoxicity, **Figure 3B**). The differences in cytotoxicity between p53 WT and p53-null HCT116 cells after herb treatment are highly significant ($P<0.00001$). Nonetheless, there was still more than 50% p53-null HCT116 cell death in response to *B. javanica* treatment. Notably, the p53-mutated A431 cells exhibited significantly higher cytotoxicity than normal controls cells (**Figure 1B**). Taken together, these results indicate that both p53-dependent and p53-independent activities contribute to the herb-induced cell death.

Discussion

In this study, we demonstrated that the aqueous extract from *B. javanica* was selectively toxic to several cancer cell lines but not normal cells. Induction of apoptosis by components of *B. javanica* is an indicated mechanism by which it kills cancer cells. Interestingly, a significant increase of p53 level was observed. Both p53-dependent and p53-independent activities contributed to *B. javanica*-induced cell death.

An ideal therapeutic goal for cancer is to trigger tumor-selective apoptotic cell death. Apoptosis is believed to be deregulated in cancers. Therefore, drugs that restore the apoptotic pathways have the potential for effectively treating tumors.[14] Our studies suggest that induction of apoptosis in cancer cells is a mechanism by which *B. javanica* kills the cells.

Significantly, we found that effective ingredients from *B. javanica* were in the PBS-soluble fraction and not in the alcohol soluble fraction. We consider this to be the key difference between our findings and those of the majority of previous studies. Herbal medicines were normally prepared by water instead of organic solvent extraction in traditional Chinese medicine. Notably, the apoptosis-inducing activity of *B. javanica* on human carcinoma cells was also identified from the water-soluble fraction in a recent report.[10] Future studies should be aimed at isolating the bioactive ingredients from the aqueous fraction of *B. javanica*.

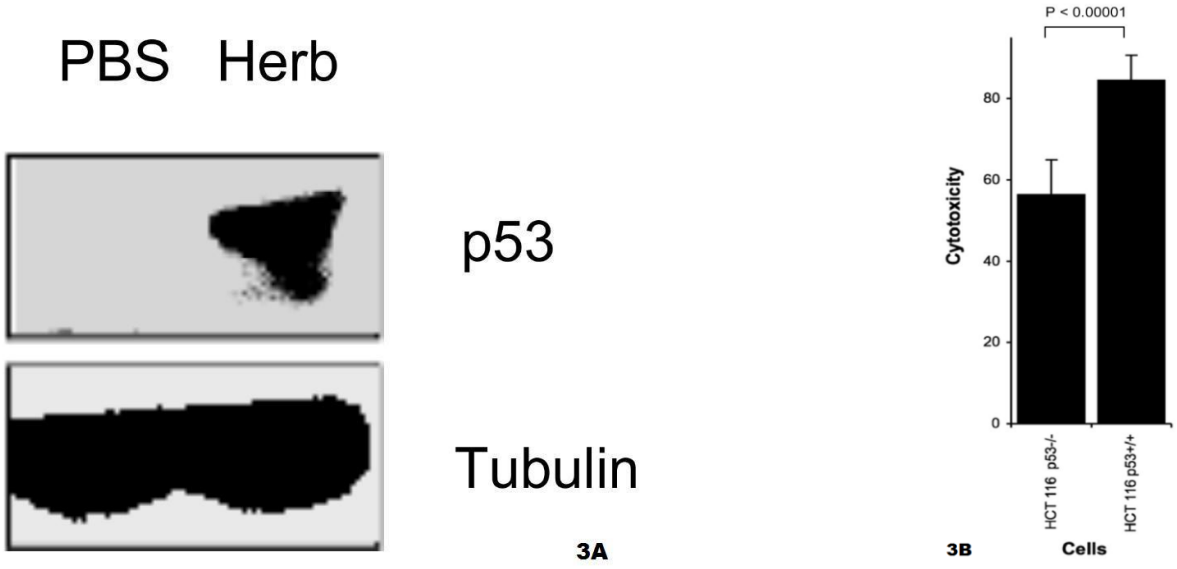

Figure 3. A role for p53 in *B. javanica*-induced cell death. **A**. Up-regulation of p53 in cervical cancer cells after treatment with *B. javanica*. Exponentially growing HeLa cells were treated with medium containing *B. javanica* extract at 2 □g total protein/ml or medium plus PBS for 48 hours. Cell extract was fractionated on SDS-PAGE and blotted with antibody against p53. The antigen-antibody complexes were detected by SuperSignal Substrate. b tubulin blot was included as a loading control. **B**. Absence of p53 significantly reduces herb-induced cytotoxicity. HCT 116 p53+/+ and HCT 116 p53-/- cells were treated with B. javanica extract for 24 hours. Cell viability was determined by MTT conversion. All values represent the mean of two independent experiments with a total of nine determinants. Error bars reflect the standard deviations of the mean.

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