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Cancer Science
The official journal of the Japanese Cancer Association

Momordica charantia leaf extract suppresses rat prostate cancer progression *in vitro* and *in vivo*

Pornsiri Pitchakarn,[1,2] Kumiko Ogawa,[1,3] Shugo Suzuki,[1] Satoru Takahashi,[1] Makoto Asamoto,[1] Teera Chewonarin,[2] Pornngarm Limtrakul[2] and Tomoyuki Shirai[1,4]

[1]Department of Experimental Pathology and Tumor Biology, Graduate School of Medical Sciences, Nagoya City University, Nagoya, Japan; [2]Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand; [3]Division of Pathology, National Institute of Health Sciences, Tokyo, Japan

(Received March 3, 2010/Revised May 30, 2010/Accepted June 18, 2010/Accepted manuscript online July 6, 2010/Article first published online July 29, 2010)

Cancer metastasis is a major cause of death in cancer patients, with invasion as a first step greatly contributing to the failure of clinical treatments. Any compounds with an inhibitory influence on this process are therefore of prime interest. *Momordica charantia* (bitter melon) is widely consumed as a vegetable and especially as a folk medicine in Asia. Here, we investigated the anti-invasive effects of bitter melon leaf extract (BMLE) on a rat prostate cancer cell line (PLS10) *in vitro* and *in vivo*. The results indicated that non-toxic concentrations of BMLE significantly inhibited the migration and invasion of cells *in vitro*. The results of zymography showed that BMLE inhibited the secretion of MMP-2, MMP-9 and urokinase plasminogen activator (uPA) from PLS10. Real-time RT-PCR revealed that BMLE not only significantly decreased gene expression of MMP-2 and MMP-9, but also markedly increased the mRNA level of TIMP-2, known to have inhibitory effects on the activity of MMP-2. An EnzChek gelatinase/collagenase assay showed that collagenase type IV activity was partially inhibited by BMLE. In the *in vivo* study, intravenous inoculation of PLS10 to nude mice resulted in a 100% survival rate in the mice given a BMLE-diet as compared with 80% in the controls. The incidence of lung metastasis did not show any difference, but the percentage lung area occupied by metastatic lesions was slightly decreased in the 0.1% BMLE treatment group and significantly decreased with 1% BMLE treatment as compared with the control. Thus, the results indicate for the first time an anti-metastatic effect of BMLE both *in vitro* and *in vivo*. (*Cancer Sci* 2010; 101: 2234–2240)

Prostate cancer is the most common male malignant tumor in Western countries.[1] Androgen ablation therapy is widely used for the initial stage of this disease and may produce favorable outcome, but most patients eventually develop ablation-resistant prostate cancers with metastatic foci. Currently, there is no treatment that is able to cure progressive hormone-refractory metastatic prostate cancer.

Metastasis is a multi-step process, which involves a series of processes including cellular adhesion and invasion through the basement membrane, transfer via the circulation system, extravasation, and proliferation at a distant site.[2,3] Multiple organ failure caused by multiple metastasis is a major cause of death in cancer patients. Therefore, prevention of tumor metastasis is one primary goal. However, cytotoxic agents have generally been applied for tumor metastasis therapy, with serious side-effects that can diminish the quality of life of cancer patients.[4] Recently, many efforts have therefore been made to search for non- or low-cytotoxic agents that can reduce the spread of malignant tumors. One focus is on cell invasion using substances in medicinal plants.[5,6]

Momordica charantia, or bitter melon, has long been frequently used in various Asian traditional medicine systems and commonly consumed as a vegetable.[7,8] Extracts have been reported to possess anti-cancer activity against lymphoid leukemia, lymphoma, choriocarcinoma, melanoma, breast cancer, skin tumor, prostatic cancer, squamous carcinoma of the tongue and larynx, human bladder carcinomas and Hodgkin's disease.[9–14] In addition, antioxidant,[15] antiviral (human immunodeficiency virus [HIV] inhibitor),[16] antidiabetic[17] and immunomodulating properties[18] have been described. A previous study demonstrated that Thai *Momordica charantia* induced Phase II enzymes and repressed monooxygenase, whereas a Chinese variety only affected monooxygenase.[19] Our previous study showed that the bitter melon leaf extract (BMLE) in Thailand reversed the multidrug resistant (MDR) phenotype in cervical carcinoma, KB-V1 cells and increased their sensitivity to a chemotherapeutic agent, vinblastine.[20] However, evidence that BMLE has effects on prostate cancer cell invasion has hitherto been lacking. In this study we therefore examined the inhibitory effects of BMLE against progression of a prostate cancer cell line both *in vitro* and *in vivo*. The androgen-independent, androgen receptor-negative rat prostate cancer cell line (PLS10), which was established in our laboratory from a 3,2′–dimethyl-4-aminobiphenyl plus testosterone-induced carcinoma in the dorsolateral prostate of a male F344 rat,[21] was used in this study.

Materials and Methods

Plant material and extraction. Bitter melon leaves were harvested from the plantation of Lampang Herb Conservation Assembly in Lampang, Thailand and the voucher specimen number was certified by the herbarium at the Flora of Thailand Faculty of Pharmacy, Chiang Mai University.

Fresh leaves of bitter melon were dried at 30–45°C, ground and a sample (1 kg) was extracted exhaustively with 4 L of 80% ethanol by maceration at 37°C for 16 h. The mixture was filtered and re-extracted with 4 L of ethanol. The combined filtrate was bleached with 160 g of active charcoal, filtered and concentrated by rotary evaporation to 120 mL before filtering to remove precipitates. The filtrate was again rotary evaporated and lyophilized then kept at −20°C protected from light. For the *in vitro* study, BMLE was dissolved in a final DMSO concentration adjusted to 0.1% (v/v) in the culture medium.

Cell culture. The PLS10 cell line was cultured in Roswell Park Memorial Institute-1640 Medium (RPMI 1640, Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, Life Technologies Japan Ltd, Japan), 50 U/mL penicillin and 50 μg/mL streptomycin, in a humidified incubator with an atmosphere comprising 95% air and 5% CO_2 at 37°C. When the cells reached 70–80% confluence, they were harvested and plated either for subsequent passage or for treatment.

Animals. The animal experiment was performed under protocols approved by the Institutional Animal Care and Use

[4]To whom correspondence should be addressed.
E-mail: tshirai@med.nagoya-cu.ac.jp

doi: 10.1111/j.1349-7006.2010.01669.x

Committee of Nagoya City University Graduate School of Medical Sciences. Six-week-old male athymic nude mice (KSN strain) were purchased from Nihon SLC (Hamamatsu, Japan) and housed in plastic cages with hardwood chip bedding in an air-conditioned room at 23 ± 2°C and 55 ± 5% humidity with a 12 h light/dark cycle. Oriental MF powder diet (Oriental MF, Oriental Yeast Co., Tokyo, Japan) and distilled water were available *ad libitum*.

Cytotoxicity and growth inhibition assay. PLS10 cells were plated at 2.0×10^3 cells per well in 96-well plates. Twenty-four hours after plating, various concentrations of BMLE were added. The cells were then incubated for 24 and 48 h at 37°C, and cell growth was assessed by WST-1 colorimetric assay (Roche Diagnostics, Mannheim, Germany).[22] In each experiment, determinations were carried out in triplicate.

Invasion and migration assays. For the invasion and migration assays, the cells were seeded in BD Biocoat Matrigel invasion chambers and the BD Falcon 24-multiwell insert system (BD Biosciences, Qume Drive San Jose, CA, USA), respectively, with BMLE (0, 25 and 50 μg/mL) for 24 and 48 h, with 5% fetal bovine serum used as a chemoattractant. Invading or migrating cells were fixed with 100% ethanol for 5 min, then stained with 0.5% crystal violet in 20% methanol for 30 min. The percentage of areas occupied with cells were then determined with ImageJ 1.410 (National Institute of Mental Health, Bethesda, MD, USA).

Zymography. PLS10 cells were maintained in serum-free DMEM for 24 h then treated with various concentrations (0, 25 and 50 μg/mL) of BMLE. Matrix metalloproteinase (MMP)-2 and MMP-9 secretion into the conditioned medium was detected by gelatin zymography as previously described.[23] Samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% acrylamide gel containing 0.1 mg/mL of gelatin (Bio-Rad Laboratories, Hercules, CA, USA) under non-reducing conditions. After electrophoresis, SDS in the gel was washed twice with 2.5% Triton-X 100 then incubated at 37°C in the incubating buffer (50 mM Tris-HCl, 200 mM NaCl, and 10 mM $CaCl_2$, pH 7.4) for 24 h. After incubation, the gel was stained with 0.1% Coomassie brilliant blue R250 (Bio-Rad Laboratories) in 50% methanol/10% acetic acid, and destained with 10% acetic acid/50% methanol. Bands of gelatinolytic activity were analyzed using ImageJ 1.410.

Urokinase plasminogen activator (uPA) secretion from the PLS10 cells into the culture medium was also examined by casein–plasminogen zymography.[24] The culture supernatant was separated by electrophoresis in 10% polyacrylamide gel (PAGE) in the presence of SDS, which was copolymerized with 1 mg/mL β-casein and 10 μg/mL human plasminogen (Sigma-Aldrich, Co., St Louis, MD, USA) under non-reducing conditions. After electrophoresis, the gel was washed twice with 2.5% Triton X 100 and incubated in incubating buffer for 18 h at 37°C, then stained and destained as described above.

Collagenase IV assays. An EnzChek Gelatinase/Collagenase Assay kit (Life Technologies Japan Ltd, Tokyo, Japan) was used for measuring gelatinase or collagenase activity. The substrate, DQ fluorescein-conjugate gelatin, was incubated with collagenase type IV from *Clostridium histolyticum* with BMLE (0, 25, 50 and 100 μg/mL) for 1.5 h and fluorescence signal representing proteolytic activity was measured using a fluorescence microplate reader (480/530 nm).

Real-Time RT-PCR. After treatment with various concentrations (0, 25 and 50 μg/mL) of BMLE, total RNA from cell lysates was extracted with an RNAeasy Mini kit (Qiagen sciences, Germantown, MD, USA) and then 1 μg of RNA was converted to cDNA with avian myoblastosis virus reverse transcriptase (Takara, Shiga, Japan). cDNA samples were subjected to quantitative PCR using SYBR Premix ExTaq (Takara) in a Light Cycler apparatus (Roche Diagnostics). MMP-2, MMP-9 and tissue inhibitor of metalloproteinase (TIMP)-2 mRNA levels were quantified using the following primers: MMP-2 forward, 5′-ACCAGAACACCATCGAGACC-3′ and reverse 5′-GAGTCCACAGCTCATCGTCA-3′; MMP-9 forward, 5′-GTAACCCTGGTCACCGGACTT-3′, and reverse 5′-TTCACCCGGTTGTGGAAACT-3′; TIMP-2 forward, 5′-GCATCACCCAGAAGAAGAGC-3′ and reverse 5′-TCCAGGAAGGGATGTCAAAG-3′. The house-keeping gene, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control to normalize reactions.

Quatitative immunoenzymatic assay (ELISA) for transforming growth factor-β1. PLS10 cells were maintained in serum-free DMEM and treated with various concentrations (0, 25 and 50 μg/mL) of BMLE for 24 h. The levels of total and activated transforming growth factor-β1 (TGF-β1) in the conditioned medium were determined by Mouse/Rat/Porcine/Canine TGF-β1 Immunoassay (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

Metastasis assays in nude mice. After 1 week of acclimation, mice were divided into three groups of 10 mice each and given BMLE (0, 0.1 and 1% w/w) for 3 weeks and a basal diet afterwards. One week after the start of the BMLE treatment, PLS10 cells (1×10^6/animal) were injected into the tail vein. Mice were killed at experimental week 4.

At death, visible tumors and the heart, liver, lung, kidneys, spleen were removed and fixed in 10% formalin. At least one section of each tissue and the largest section from each lobe of the lung were processed for hematoxylin and eosin (HE) and Ki-67 staining. For quantitative analysis of lung metastases, numbers and areas of metastatic foci per total area of section were determined using an Image Processor for Analytical Pathology (IPAP-WIN; Sumika Technos Co., Osaka, Japan). For this experiment, immunostaining of Ki-67 was also used as a subsidiary method.

Immunohistochemistry. For Ki-67 immunostaining, parafilm-embedded specimens were sectioned (5 μm) and stained with Ki-67 antibody (DAKO, Denmark A/S, Glostrup, Denmark) and sequentially with anti-rabbit secondary antibody and avidin–biotin complex (Vectastatin Elite ABC kit; Vector Laboratory, Burlingame, CA, USA), then binding sites were visualized with diamino-benzidine. The sections were then counterstained lightly with hematoxylin for microscopic examination.

Statistical analysis. All *in vitro* experiments were performed at least in triplicate to confirm reproducibility. Statistical analyses were performed with mean ± SD values using one-way ANOVA, the Bonferroni correction and the Student's *t*-test. Statistical significance was concluded with $*P < 0.05$ or $**P < 0.01$.

Results

Effects of BMLE on the cytotoxicity and growth inhibition of PLS10 cells. WST-1 assay (Fig. 1a) showed that BMLE treatment reduced cell growth of PLS10 cells concentration-dependently for 24 and 48 h. At concentrations higher than 100 μg/mL, BMLE treatment significantly inhibited growth, and inhibitory concentration (IC) 50 of BMLE was in the range of 150–200 μg/mL. Treatment with 75 μg/mL BMLE for 24 or 48 h was not cytotoxic to the PLS10 cells. Thus, a range of non-toxic concentrations (25 or 50 μg/mL) was applied for all subsequent experiments.

BMLE inhibition of invasion and migration of PLS10 cells. The data and representative images of invasion and migration studies are presented in Figure 1b,c, respectively. Cell invasion of PLS10 was inhibited by up to 49% and 59% with 25 and 50 μg/mL of BMLE, respectively. The same concentrations of BMLE also inhibited cell migration, by about 38% and 64%, respectively.

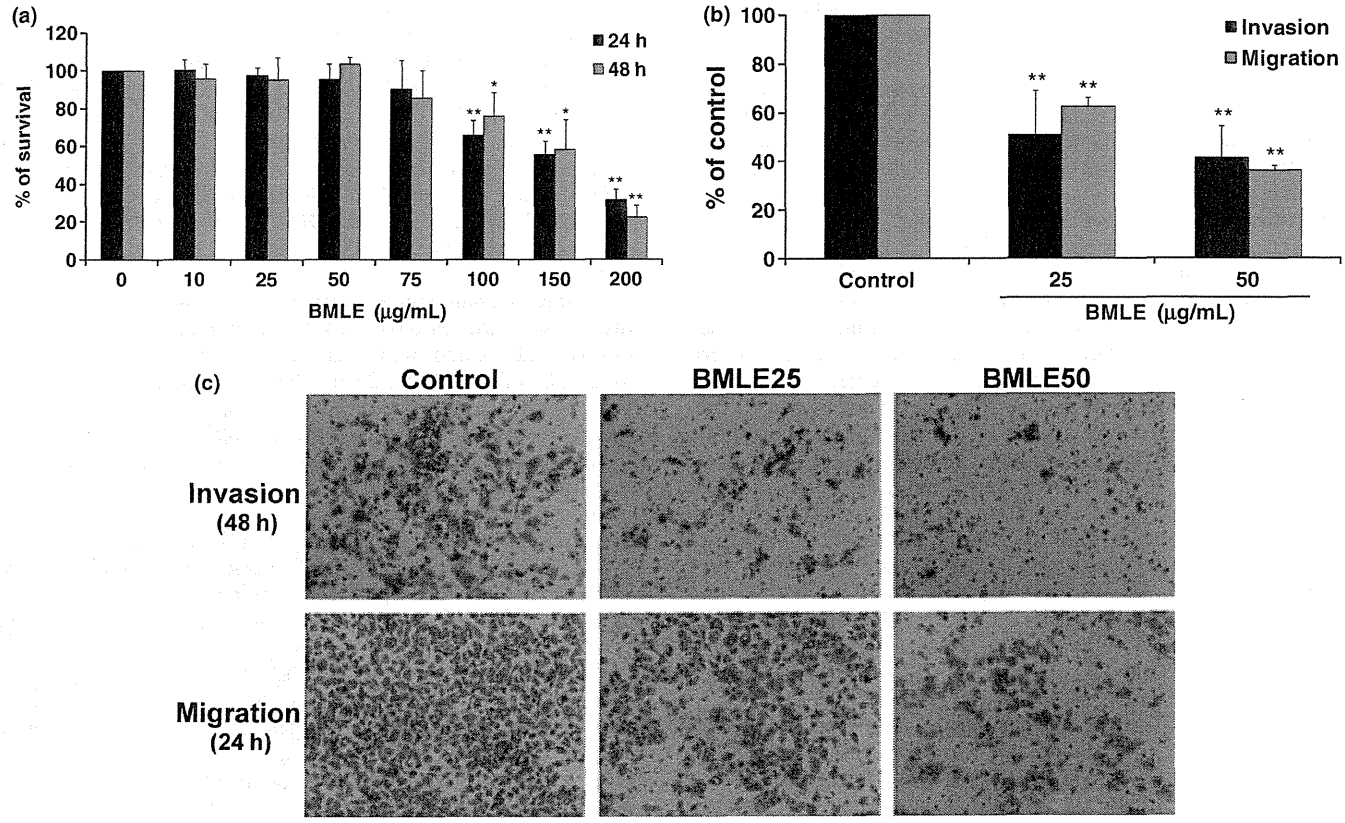

Fig. 1. Bitter melon leaf extract (BMLE) inhibited invasion and migration of PLS10 cells. The cells were incubated with BMLE for 24 and 48 h and then cytotoxicity was assessed by WST-1 assay (a). The cells were treated with 0, 25 and 50 μg/mL BMLE for invasion assays (48 h) and migration assays (24 h) using transwell cell culture chambers, with 5% fetal bovine serum as a chemoattractant. Cells were photographed under phase-contrast microscopy (c) and quantified (b). The results are expressed as percentages of untreated control values. Data are mean ± SD values from three independent experiments. $*P < 0.05$ and $**P < 0.01$.

Reduction of MMP-2, MMP-9 and uPA secretion from PLS10 cells by BMLE. Zymography showed that secretion of MMP-2, MMP-9 and uPA was reduced with BMLE treatment in a concentration-dependent manner. The inhibition of MMP-2 (pro- and active-form) secretion was about 22% and 46% at 24 h, and 19% and 33% at 48 h, with 25 and 50 μg/mL of BMLE, respectively (Fig. 2a,b). As shown in Figure 2c,d, BMLE also inhibited the secretion of MMP-9 (active-form), by approximately 11% and 27% at 24 h, and 17% and 29% at 48 h at the concentrations of 25 and 50 μg/mL, respectively. Moreover, secretion of uPA was significantly reduced by 25 and 50 μg/mL of BMLE, by approximately 26% and 28%, respectively, for the pro-form and 45% and 61% for the active-form (Fig. 2e,f). Collectively, these results indicated secretion of MMP-2, MMP-9 and uPA to be significantly inhibited by BMLE.

BMLE inhibition of collagenase type IV activity. Because of reduction of MMP protein expression by the BMLE treatment, effects on MMP activity were investigated with a Collagenase Assay kit. It was found that proteolytic activity of collagenase type IV was slightly inhibited by 9%, 15% and 17% with 25, 50 and 100 μg/mL of BMLE, respectively (Fig. 2g).

Effects of BMLE on MMP-2, MMP-9 and TIMP-2 mRNA expression. Quantitative real-time RT-PCR results showed that BMLE treatment significantly decreased gene expression of MMP-2, by approximately 31% and 75% at 24 h, and 26% and 62% at 48 h, with 25 and 50 μg/mL of BMLE, respectively (Fig. 3a). Similarly, the expression of MMP-9 was inhibited by approximately 46% and 66% at 24 h, and 18% and 83% at 48 h (Fig. 3b). TIMP-2, which has been known to have an inhibitory effect on the activity of MMP-2 mRNA level, was up-regulated to 152% and 231% at 24 h, and 128% and 222% at 48 h (Fig. 3c).

Effects of BMLE on TGF-β1 secretion. Quantitative ELISA showed that TGF-β1 secretion from PLS10 cells was not altered by 25 and 50 μg/mL of BMLE. TGF-β1 secretion by PLS10 treated with 0 (control), 25 and 50 μg/mL of BMLE was 539 ± 87, 571 ± 108 and 540 ± 109 (pg/mL), respectively, for total TGF-β1, and 32 ± 14, 30 ± 14 and 29 ± 11 (pg/mL) for activated TGF-β1.

Effects of BMLE on lung metastasis of PLS10 cells in nude mice. Four weeks after BMLE treatment, the percentage survival of nude mice in the BMLE-treated groups (100%) was higher than in the controls (80%; Fig. 4a). Administration of BMLE caused no depression in bodyweight (Table 1). All mice developed metastatic nodules in their lungs, which are represented in Figure 4b. Two mice in each group also developed metastasis in the heart and the incidences of metastasis among groups did not differ. The lung weights and the number of tumor in the lung were slightly decreased in the BMLE-treated group, but without significance (Table 1). The calculated tumor metastatic area in the lung was slightly decreased in the 0.1% BMLE group and significantly decreased in the 1% BMLE group, by 35% and 48% relative to the control group, respectively (Table 1). The Ki-67 labeling index was not different among groups (Table 1). There were no metastatic foci apparent in the liver, kidneys and spleen.

Discussion

The present results provided clear evidence that an extract of bitter melon leaves can exert inhibitory potential against

doi: 10.1111/j.1349-7006.2010.01669.x

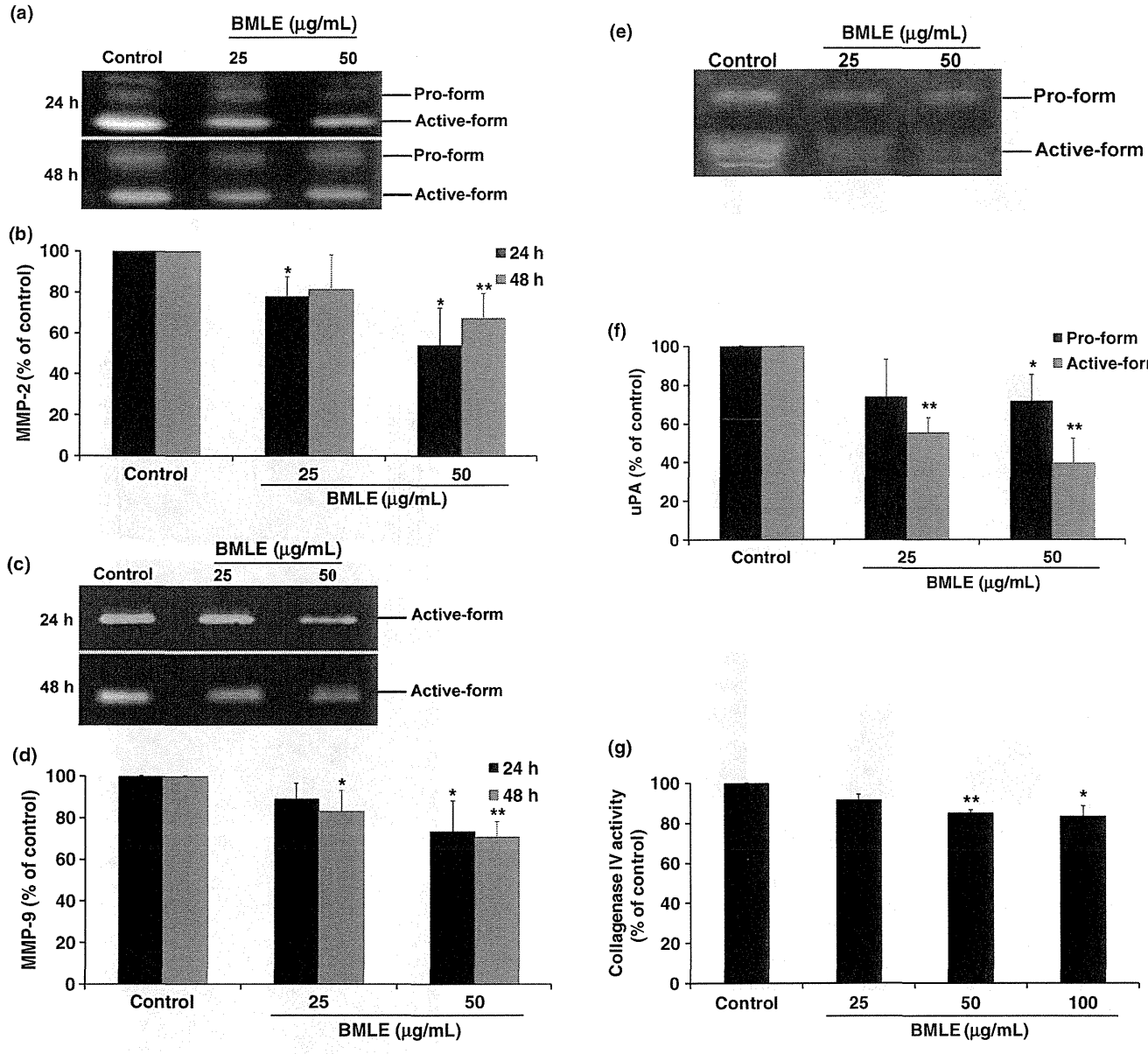

Fig. 2. Bitter melon leaf extract (BMLE) reduction of the secretion of MMP-2, MMP-9 and urokinase plasminogen activator (uPA) from PLS10 and collagenase type IV activity. Cells were treated with 0, 25 and 50 μg/mL BMLE for the indicated time under serum-free conditions. Gelatin zymography was performed for detection of MMP-2 (a) and MMP-9 (c). The uPA secretion was analyzed by casein–plasminogen zymography (e). Levels of MMP-2 (pro- and active-form), MMP-9 (active-form), and uPA (pro- and active-form) were quantified (b,d,f). Gelatinase or collagenase activity was assessed (g). The results are percentages of the control values. Data are mean ± SD values from three independent experiments. $*P < 0.05$ and $**P < 0.01$.

metastatic properties of PLS10 cells in both *in vitro* and *in vivo* experiments. *Momordica charantia*, or bitter melon, has been shown to be safe without any signs of nephrotoxicity and hepatotoxicity and any adverse influence on food intake, growth organ weights and hematological parameters in experimental animals when ingested at low doses for up to 2 months.[25,26] Although animal studies showed toxicity of bitter melon when extracts were injected intravenously or intraperitoneally, with extracts from fruit and seed having relatively higher toxicity than the leaf or aerial parts of the plant,[14,19,27] our results pointed to beneficial effects at non-toxic levels. The BMLE might thus afford an advantageous anti-cancer progression agent especially for tumor metastasis therapy.

An important aspect of high incidence of morbidity and mortality in prostate cancer is tumor invasiveness and metastasis. Metastasis is accompanied by various physiological alterations involved in extracellular matrix (ECM) degradation, which allows cancer cells to invade the blood or the lymphatic system and spread to another tissue or organ. In this study, non-cytotoxic BMLE treatment dramatically reduced migration and invasion properties of androgen-independent rat prostate cancer cells. It has been postulated that cell–ECM interactions are essential for invasion, migration and metastasis of tumors.[28,29] MMP play important roles in ECM degradation for tumor growth, invasion and angiogenesis.[30] Among MMP, MMP-2 and MMP-9 are reported to be the most important for basement membrane type IV collagen degradation,[31,32] and their expression correlates with an aggressive, invasive or metastatic tumor phenotype.[33–35] Therefore, MMP-2 and MMP-9 are candidate molecules as targets for therapeutic anticancer drugs. We

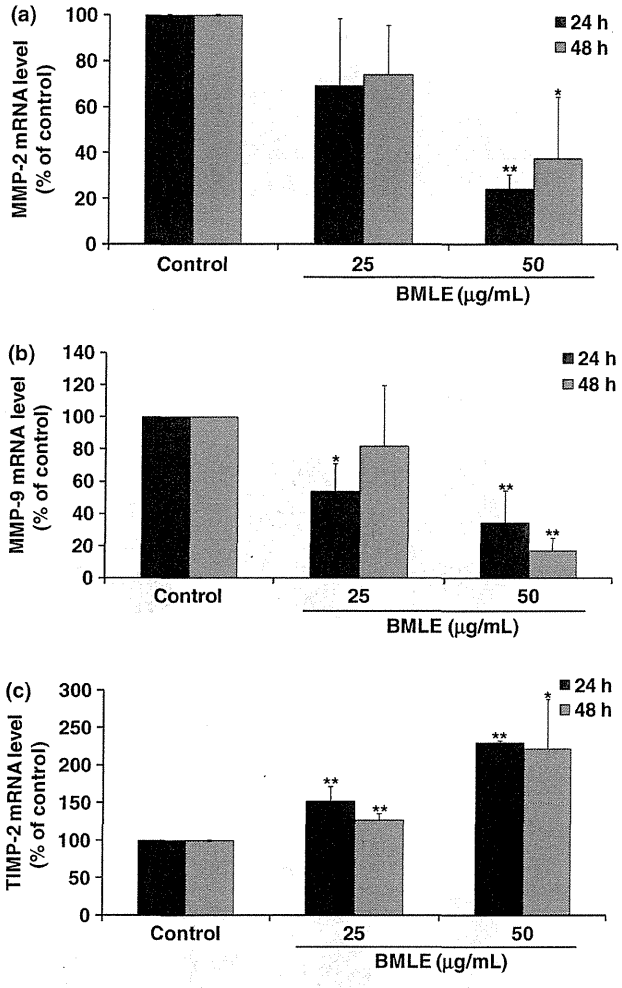

Fig. 3. Effects of bitter melon leaf extract (BMLE) on MMP-2, MMP-9 and TIMP-2 gene expression. PLS10 cells were treated with 0, 25 and 50 μg/mL BMLE for 24 and 48 h and then total RNA samples were extracted and subjected to a real-time PCR, with GADPH used as an internal control. The mRNA expression of MMP-2 (a), MMP-9 (b) and TIMP-2 (c) is presented as percentages of untreated control values. Data are mean ± SD values from three independent experiments. $*P < 0.05$ and $**P < 0.01$.

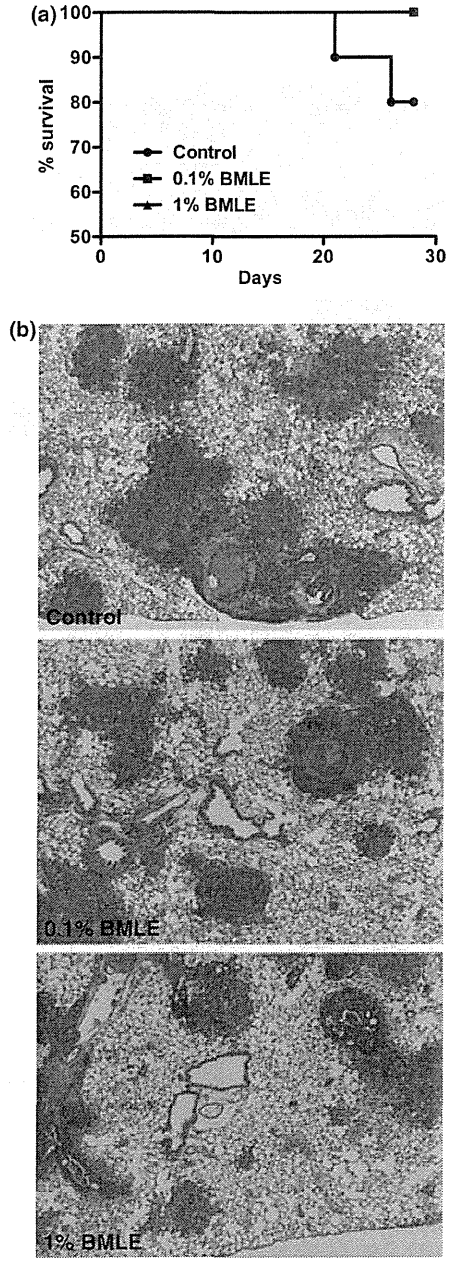

Fig. 4. Bitter melon leaf extract (BMLE) suppression of lung metastasis in an intravenously inoculated mouse prostate tumor model. For the metastasis assay in nude mice, animals were given BMLE (0, 0.1 and 1% w/w) in their diet during the 1–3 week experimental period, 1 week after PLS10 cells (1×10^6/animal) were injected into the tail vein. Mice were killed at week 4. The percentage survival of nude mice represented as a Kaplan–Meier survival curve (a). Representative hematoxylin and eosin staining of lung metastases (b).

showed here that BMLE treatment reduced not only secretion but also expression of MMP-2 and MMP-9 in the PLS10 cell line. Additionally, uPA, which is an upstream enzyme of MMP also implicated in tumor cell invasion, survival and metastasis,[36,37] was similarly reduced by the BMLE treatment. Since TIMP have the ability to form tight 1:1 complexes with the active MMP enzymes, changes in TIMP levels directly affect MMP activity.[38,39] Increase of TIMP-2 protein may inhibit tumor cell invasion,[40,41] and in this study the BMLE treatment induced the expression of TIMP-2. In addition, BMLE slightly reduced proteolytic activity of collagenase type IV. Therefore, BMLE might mainly reduce activity of MMP by suppression of uPA and induction of TIMP-2, and also partially inhibit MMP activity directly.

TGF-β is a member of a superfamily of multifunctional cytokines that has been reported to be associated with enhanced angiogenesis and metastasis in prostate cancer.[42,43] Down-regulation of TGF-β1 reduces tumorigenicity of prostate cancer cells.[44] It has been reported that bitter melon extract induced the secretion of TGF-β in colon cancer cell line, Caco-2 cell,[45] but induction of TGF-β by extract has been not reported in prostate cancer. The present study showed that the secretion of TGF-β1 was not effected by BMLE, indicating that the BMLE did not modulate the prostate cancer cell migration and invasion via TGF-β1 signaling. In addition, a previous study showed that promoter regions of TGF-β receptor type 2 gene were methylated and the expression was downregulated in the PLS10 cell line.[46] Hence, TGF-β signaling might not be the critical mechanisms for the cancer cell metastasis.

In vivo study further showed that dietary BMLE treatment tended to reduce the lung weight and number of lung metastatic tumors. Treatment of BMLE significantly reduced the percentage of tumor area in the lungs in a dose-response manner, at

doi: 10.1111/j.1349-7006.2010.01669.x

Table 1. Effect of bitter melon leaf extract (BMLE) on bodyweight and lung metastasis of PLS-10 cells in nude mice

Group	Bodyweight (g)	Lung weight (g)	Number of tumors (No./mm^2)	Percentage of tumor area (%)	Ki-67 LI in tumors (%)
Control	24.45 ± 2.7	0.5 ± 0.23	2.18 ± 0.43	36.1 ± 11.1	89.4 ± 3.1
0.1% BMLE	25.57 ± 2.9	0.5 ± 0.18	1.66 ± 0.79	23.3 ± 18.0	91.2 ± 1.4
1% BMLE	24.66 ± 2.3	0.4 ± 0.25	1.67 ± 0.72	18.6 ± 9.6*	89.4 ± 3.1

*$P < 0.05$ compared with the control group. Values are expressed as the mean ± SD. LI, labeling index.

least in terms of the lesion size. In the histopathology, excessive numbers of metastatic tumors developed and coalesced in the lungs; therefore, a significant effect was not evident on incidence or multiplicity. Additionally, no difference in cell proliferation was evident among the groups. Further studies might be needed to characterize the active constituents of BMLE and confirm BMLE effects on metastasis *in vivo*.

Recently, several triterpenoids from various plants have been reported to feature anti-proliferative[47–49] and anti-invasive[50,51] bioactive components. More than 50 triterpenoids have been isolated from bitter melon, but their biological activities are yet to be explored in detail. However, it was shown that Cucurbitacin B (cucB), a triterpenoid from Cucurbitaceae vegetables also found in bitter melon seeds, caused cell-cycle arrest and apoptosis induction in human colon adenocarcinoma cancer cells.[49] Moreover, two flavonoids and four phenolic acids, including rutin, naringin, gentistic acid, benzoic acid, *o*-coumaric acid and *t*-cinnamic acid, are present in bitter melon leaves.[52] Rutin, a flavonoid glycoside, has been reported to show growth inhibition of leukemia and ovarian carcinomas, with anti-invasive effects on melanoma.[52–55] Triterpenoids and flavoniods included in BMLE might be promising components with critical roles against cancer cell progression, but active compound(s) remain to be identified.

Taken together, our results provide the first evidence that BMLE exerts inhibitory effects on the progression of rat prostate cancer cells *in vitro* and *in vivo*. The exact nature of the active components of bitter melon leaf extracts that exert anti-invasion effects now needs to be explored, along with further elucidation of the underlying molecular mechanisms.

Acknowledgments

This work was supported by grants from the Royal Golden Jubilee PhD Program of Thailand, the Research Foundation for Oriental Medicine and the Society for Promotion of Pathology of Nagoya, Japan.

Disclosure Statement

The authors have no conflict of interest.

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doi: 10.1111/j.1349-7006.2010.01669.x

Pathology International

Pathology International 2011; **61**: 202–209 doi:10.1111/j.1440-1827.2011.02645.x

Original Article

Hypertension is positively associated with prostate cancer development in the TRAP transgenic rat model

Kentaro Takeshita, Satoru Takahashi, Mingxi Tang,* Azman Seeni,† Makoto Asamoto and Tomoyuki Shirai

Department of Experimental Pathology and Tumor Biology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

Epidemiological data on the relationship between hypertension and prostate cancer development are conflicting. To cast light on this question, we performed animal experiments using hybrid rats generated by crossing the spontaneously hypertensive rat (SHR) or its normotensive control Wistar Kyoto (WKY) rat with a transgenic rat for adenocarcinoma of prostate (TRAP) that features development of adenocarcinoma at high incidence by 15 weeks of age. The number of adenocarcinomatous foci in the lateral prostate of hypertensive (TRAP × SHR)F1 rats was demonstrated to be significantly increased compared with those of normotensive (TRAP × WKY)F1 rats. In the ventral prostate, increase of carcinoma foci was also observed but did not reach significance. The number of cancer foci showing microinvasion in (TRAP × SHR)F1 rats was higher than that of (TRAP × WKY)F1 rats, but again without significance, while treatment with prazosin, an anti-hypertensive agent, tended to decrease microinvasive carcinoma foci in both the ventral and lateral prostate. In conclusion, the present study provided additional evidence that high blood pressure is associated with prostate cancer risk.

Key words: hypertension, prazosin, prostate cancer, SHR, TRAP, WKY

Prostate cancer now has the highest incidence of all non-cutaneous neoplasms in men in Western countries such as Europe and the United States.[1] The prevalence of prostate cancer has also been increasing in Japan,[2] concomitantly with change in life-style. Epidemiologic and genomic studies have provided a great number of information concerning exogenous factors including diet, and environmental agents, as well as endogenous factors such as genetic predisposition for susceptibility, associated with the risk of prostate cancer.[3–5] Dietary factors, particularly fat or well-done meat intake, are considered to strongly contribute in the genesis of prostate cancer[6–8] partly on the evidence that Japanese immigrants to the United States showed an increased risk of prostate cancer.[9,10] Our previous studies demonstrated that 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), one of the heterocyclic amines which are mutagenic compounds found in cooked meats, exerts carcinogenicity in the prostate, mammary gland and colon in rats,[11,12] and that spontaneously hypertensive rat (SHR) showed high susceptibility in PhIP-induced prostate carcinogenesis among the various strains of rats.[13] These findings prompted us to investigate the relationship between hypertension and prostate cancer development. To our knowledge, this is the first study of the effects of high blood pressure on prostate carcinogenesis in an *in vivo* animal model.

MATERIALS AND METHODS

Chemicals and animals

Prazosin hydrochloride, an α1-adrenergic receptor antagonist, was purchased from Sigma (St. Louis, MO, USA). Homozygous transgenic rat for adenocarcinoma of prostate (TRAP) with a Sprague-Dawley genetic background, which were established our laboratory,[14,15] were bred in our animal facility. Male F344 (F344/DuCrj) rats, male SHR (SHR/NCrj) rats, and male offspring from mating of the two strains were obtained from Charles River Japan (Yokohama, Japan). Male SHR/Izm and male normotensive

Correspondence: Satoru Takahashi, MD, PhD, Department of Experimental Pathology and Tumor Biology, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan.
Email: sattak@med.nagoya-cu.ac.jp
Present address: *Department of Pathology, Luzhou Medical College, 319 ZhongShan road, Luzhou, Sichuan, China.
†School of Dental Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia.
Received 14 July 2010. Accepted for publication 4 December 2010.

Wistar-Kyoto (WKY) (WKY/Izm) rats were from Japan SLC Inc. (Hamamatsu, Japan). They were housed 2–3/cage on wood-chip bedding in an air-conditioned animal room at 23 ± 2°C and 50 ± 10% humidity. Food and tap water were available *ad libitum*.

Experimental protocol

Experiment 1: Male F344 rats (n = 5), male SHR/NCrj rats (n = 5), male (F344 × SHR)F1 (n = 10) and male (SHR × F344)F1 rats (n = 10) were killed at 10 weeks of age and their prostate glands were removed and fixed in 10% neutral formalin.

Experiment 2: Homozygous female TRAP rats were mated with male SHR/Izm or male WKY/Izm rats to generate offspring. Eighteen male (TRAP × SHR/Izm)F1 and 18 male (TRAP × WKY/Izm)F1 offspring were randomly divided into two groups each, and designated as groups 1–4, respectively. Rats of groups 2 and 4 continuously received 2 mg/kg/day prazosin hydrochloride in drinking water starting at 10-weeks-old for 15 weeks while groups 1 and 3 served as controls receiving tap water alone. The systolic blood pressure of all rats was measured every week by tail-cuff plethysmography (KN-210, Natsume Seisakusho, Tokyo, Japan). The experiment was terminated when the animals were 25-weeks-old. Prostate glands were removed and fixed in formalin.

The present experiments were performed under protocols approved by the Institutional Animal Care and Use Committee of Nagoya City University Graduate School of Medical Sciences.

Assessment of epithelial hyperplasia in the ventral prostate

We calculated the percentage area occupied by epithelial cells in acini to determine hyperplastic changes in ventral prostate. Quantitative measurement was with an Image Processor for Analytical Pathology (IPAP, Sumika Technos Co., Osaka, Japan).

Evaluation of prostate neoplastic lesion development

Neoplastic lesions in prostate glands of TRAP rats were evaluated as previously described.[16] Briefly, neoplastic lesions were classified into three types, low-grade prostatic intraepithelial neoplasia (LG-PIN), high-grade PIN (HG-PIN) and adenocarcinoma, according to their structural morphologies. Low-grade PINs were characterized by having one or two layers of atypical cells with hyperchromatic nuclei and intact gland profiles. High grade PINs showed increased epithelial stratification, papillary or micropapillary proliferation of atypical epithelial cells but not filling in the acini with proliferative lesions. Adenocarcinomas were characterized by atypical cells filling almost all of the lumen of the ducts with cribriform structures or solid growth in acini (Fig. 1). The relative numbers of acini with the respective histological

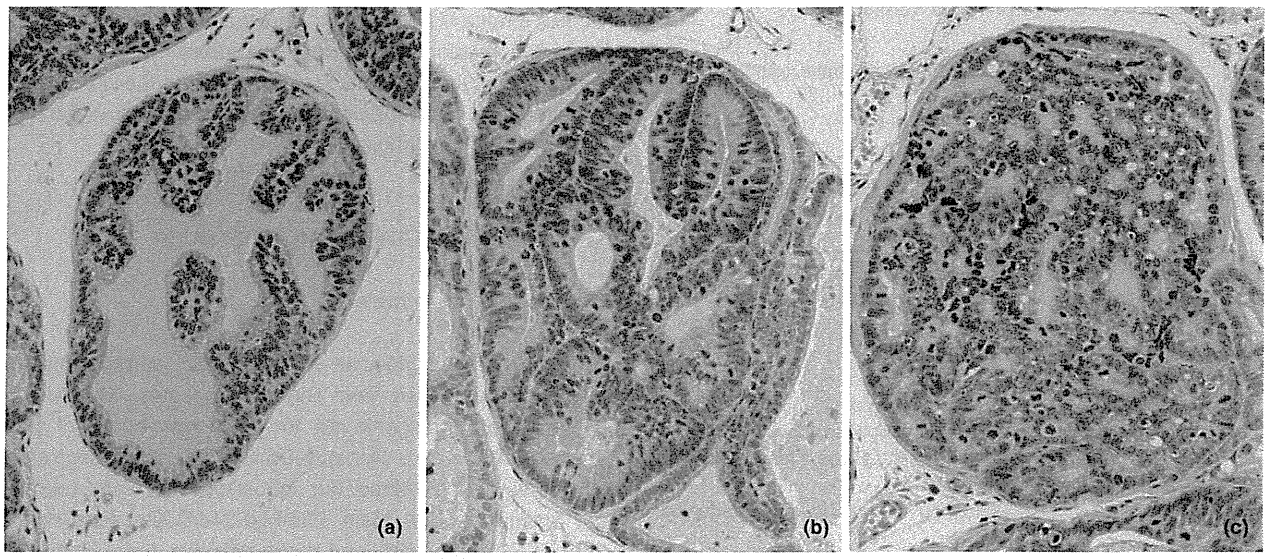

Figure 1 Representative histopathological findings for (**a**) low grade prostatic intraepithelial neoplasia (LG-PIN), (**b**) high grade PIN (HG-PIN) and (**c**) adenocarcinoma in lateral prostates of (spontaneously hypertensive rat (SHR) × TRAP)F1 rats.

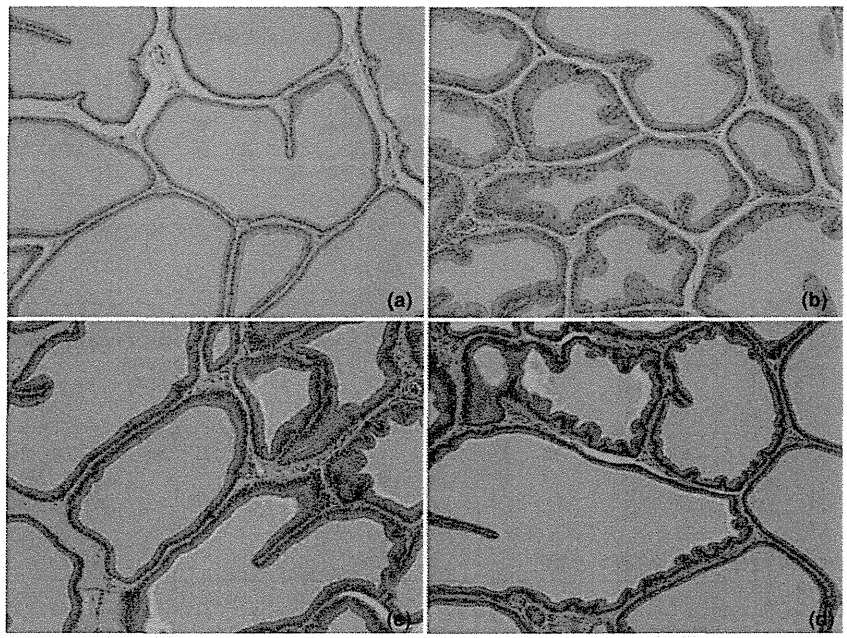

Figure 2 Representative histopathological findings for ventral prostates of (**a**) F344, (**b**) spontaneously hypertensive rat (SHR), (**c**) (F344 × SHR)F1 and (**d**) (SHR × F344)F1 rats.

characteristics were quantified by counting for the total acini in each prostatic lobe.

Immunohistochemistry

For Ki-67 immunostaining, deparaffinized sections were incubated with diluted rabbit polyclonal Ki-67 antibodies (Novocastra, Newcastle, UK). Apoptotic cells were detected using an *In situ Apoptosis Detection Kit* (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method) according to the manufacturer's instructions (Takara Bio Co. Ltd, Otsu, Japan). Labeling indices were counted separately in the ventral, dorsal and lateral prostate and expressed as numbers of Ki-67-positive or TUNEL-positive cells per 100 cells.

Statistical analysis

Differences in means between groups were determined by analysis of variance (ANOVA), followed by the Dunnett's post-hoc test with GraphPad Prism (version 5.0c) (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

Experiment 1

As shown in Fig. 2, both (F344 × SHR)F1 and (SHR × F344)F1 offspring developed epithelial hyperplasia of the ventral prostate as reported previously for SHR rats.[17] Quantitative analysis revealed that degrees of lesion development in offspring were intermediate between those of parental F344 and SHR rats (Table 1). Values for (F344 × SHR)F1 were significantly higher than for their (SHR × F344)F1 counterparts.

Table 1 Quantitative data for epithelial content in ventral prostate of F344, SHR, and their F1 rats

Strain	No. of rats	Ventral prostate epithelium / acinus (%)
F344/DuCrj	5	28.8 ± 4.6
SHR/NCrj	5	51.4 ± 3.8
(F344 × SHR)F1	10	39.2 ± 4.7
(SHR × F344)F1	10	33.4 ± 5.9

*$P < 0.05$, **$P < 0.001$.
SHR, spontaneously hypertensive rat.

Experiment 2

Based on the results of Experiment 1, we generated hybrid rats of female TRAP rats mated with male SHR rats, or with normotensive control male WKY rats, to investigate the effect of hypertension on prostate carcinogenesis. Systolic blood pressure was sequentially monitored and the dose of prazosin was gradually increased up to 5 mg/kg/day as indicated in Fig. 3 because doses of 2 or 3 mg/kg/day proved ineffective. One rat from each of groups 2 and 4 were found dead

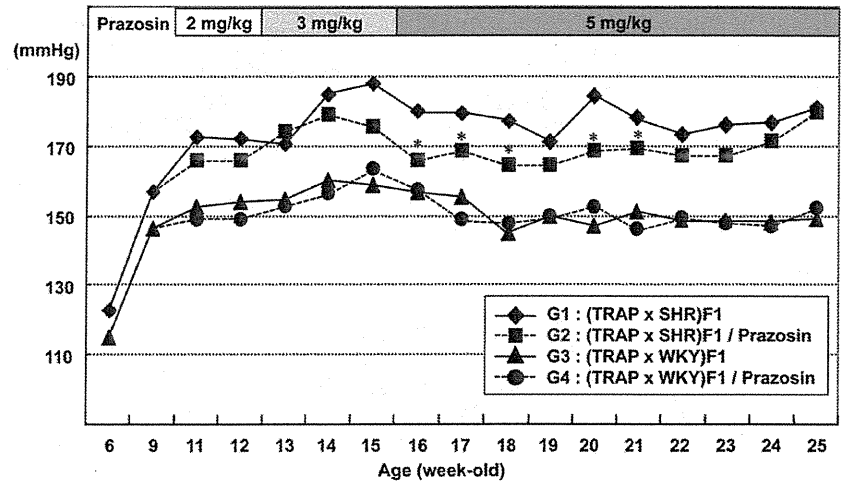

Figure 3 Sequential changes of systolic blood pressure in rats given prazosin. *$P <$ 0.05 vs. G1.

Table 2 Weights of final body, ventral prostate, and serum testosterone levels

Group	Treatment	No. of rats	Body weight (g)	Ventral prostate weight (g)	Serum testosterone (ng/mL)
1	(TRAP × SHR)F1	9	449.2 ± 17.9	0.31 ± 0.03	1.02 ± 0.28
2	(TRAP × SHR)F1 / Prazosin	8	434.9 ± 19.8	0.31 ± 0.04	2.64 ± 1.73*
3	(TRAP × WKY)F1	9	450.4 ± 18.6	0.33 ± 0.03	2.12 ± 1.18
4	(TRAP × WKY)F1 / Prazosin	8	441.9 ± 22.6	0.34 ± 0.05	1.07 ± 0.53

*$P < 0.05$ vs. Group 1.
TRAP, transgenic rat for adenocarcinoma of prostate; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.

during the experiment from unknown causes. The final body weights of rats given prazosin tended to be lower than those of each non-treated control but the differences were not significant. Ventral prostate weights were almost the same among the groups. Effects of prazosin or hypertensive genetic background of SHR were variable on serum testosterone levels, but the values in group 2 were significantly higher than those of group 1 (Table 2).

All rats in the present study harbored adenocarcinoma foci in their prostates regardless of their size. The numbers in the lateral prostates of hypertensive (TRAP × SHR)F1 rats demonstrated a 78% significant increase compared with those of normotensive (TRAP × WKY)F1 rats (Table 3). In the ventral prostate, increase of carcinoma foci was also apparent in (TRAP × SHR)F1 but it did not reach statistical significance. Moreover, the proportions of preneoplastic and neoplastic lesions in the lateral prostate showed that prazosin treatment tended to suppress adenocarcinoma development about 31% in (TRAP × SHR)F1 rats (Table 3). We next focused on the effect of high blood pressure on microinvasive adenocarcinoma development which is age-dependent in our TRAP rats.[15] In the present study, the multiplicity of invasive carcinoma foci in (TRAP × SHR)F1 rats was greater, albeit without significance, than in (TRAP × WKY)F1 rats and a tendency for decrease was found in (TRAP × SHR)F1 rats given prazosin in both of the ventral and lateral prostate (Table 4). No differences in Ki-67 and apoptotic indices were found in any lobe among the groups (Fig. 4).

DISCUSSION

The present experiments provided evidence that prostate cancer risk in (TRAP × SHR)F1 offspring with high blood pressure was significantly higher than that in (TRAP × WKY)F1 rats. Although the anti-hypertensive effects of prazosin were somewhat weak there appeared to be some reduction in the incidence of adenocarcinomas. Since invasive lesions are observed after 35 weeks of age in TRAP rats,[15] it is plausible that associations would become more apparent by extension of the experimental period.

To date, conflicting results have been published regarding possible relationships between hypertension and the risk of prostate cancer. Clear links were reported in a population-based cohort study of 82 098 men in Norway[18] and a case-control study of 498 African-American men in the United States.[19] On the other hand, data from cohort studies in Sweden with 336 159 individuals and the United States with 58 704 men demonstrated no obvious connection.[20,21] With data concerning anti-hypertensive drugs, use of alpha-blockers, beta-blockers or angiotensin-I-converting enzyme (ACE) inhibitors may reduce prostate cancer risk[22–24] although

Table 3 Quantitative evaluation of neoplastic lesions in rat prostate

Group	Treatment	No. of rats	Relative number of acini with histological characteristics (%)									
			Ventral					Lateral				
			LG-PIN	HG-PIN	Adenocarcinoma			LG-PIN	HG-PIN	Adenocarcinoma		
					Non-invasive	Invasive	Total			Non-invasive	Invasive	Total
1	(TRAP × SHR)F1	9	25.6 ± 8.0	57.6 ± 8.8	16.7 ± 6.6	0.2 ± 0.3	16.9 ± 6.7	16.4 ± 5.1	69.1 ± 8.3	14.5 ± 6.3	0.2 ± 0.6	14.6 ± 6.6*
2	(TRAP × SHR)F1 / Prazosin	8	22.2 ± 5.2	62.0 ± 7.0	15.6 ± 9.1	0.2 ± 0.3	15.8 ± 9.2	20.6 ± 11.2	69.4 ± 13.3	9.8 ± 5.6	0.2 ± 0.4	10.0 ± 5.9
3	(TRAP × WKY)F1	9	26.6 ± 7.3	61.7 ± 8.0	11.6 ± 2.4	0.1 ± 0.2	11.7 ± 2.5	21.0 ± 8.7	70.7 ± 9.2	8.2 ± 5.2	0.1 ± 0.2	8.2 ± 5.0
4	(TRAP × WKY)F1 / Prazosin	8	26.8 ± 8.8	61.8 ± 10.0	11.4 ± 4.0	0	11.4 ± 4.0	14.0 ± 3.5	79.4 ± 5.2	6.5 ± 4.1	0.1 ± 0.3	6.6 ± 4.1

*$P < 0.05$ vs. Group 3.

LG-PIN, low grade prostatic intraepithelial neoplasia; HG, high grade; TRAP, transgenic rat for adenocarcinoma of prostate; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.

prospective cohort studies provided no evidence that such medications exerted appreciable protective effects.[25,26]

One complicating factor might be gender and several reports have discussed the relationship between sex hormones and hypertension. Blood pressure in males is higher than in female SHR rats,[27] and young male SHR rats aged 9–10 weeks with chemical castration by flutamide treatment demonstrate delayed development of hypertension.[28,29] Moreover, administration of testosterone to ovariectomized female SHR rats exacerbates the disease.[30,31] There is thus mounting evidence that testosterone might play a promoting role in these animals. Intact (TRAP × SHR)F1 rats, however, showed the lowest level of serum testosterone among the groups and there was no clear association between testosterone levels and hypertension in the present study. Therefore, some factor(s) other than testosterone might be involved in the observed increased risk of prostate cancer development. A possible candidate factor is a genetic polymorphism of the angiotensin-converting enzyme (ACE) gene because this is reported to correlate with serum ACE levels,[32] blood pressure,[33] and the risk of prostate cancer in rats[34–36] and humans.[37,38] Prazosin is reported to not directly affect the renin-angiotensin system in terms of hypertension while inhibiting angiotensin II-induced DNA synthesis in vascular smooth muscle cells.[39] The non-significant tendency for protection observed here might not necessarily be due to lowering of blood pressure because prazosin and doxazosin *per se* have been reported to exert antitumor activity against prostate cancer cells.[40,41]

Spontaneously hypertensive rats are one of the most widely accepted animal models of human essential hypertension, having been characterized as closely paralleling the human disorder in its hemodynamic, endocrine, and end-organ manifestations. Systolic blood pressure in SHR is usually between 180–200 mmHg at around 5–6 weeks of age,[42,43] and (TRAP × SHR)F1 rats in the present study showed 170–190 mmHg. Although this level of blood pressure is much higher than patients with hypertension, it is possible to apply the current data to human patients because the control (TRAP × WKY)F1 rats exhibited 140 to 160 mmHg which is also higher than that in human cases, and the blood pressure levels in the SHR is significantly higher than that of control.

The enhancement of the prostate cancer risk in SHR rats was possibly induced due to the genetic predisposition of SHR rather than hypertension *per se*. Increased RhoA expression in vascular smooth muscle cells was constantly found in SHR compared with WKY rats[44,45] and RhoA activation was significantly reduced by the treatment of valsartan, an angiotensin II receptor type I blocker (ARB), but not by prazosin.[44] Recently, we observed a significant suppressive effect of ARBs on the prostate carcinogenesis in TRAP rats (Takahashi *et al.*, unpublished data). RhoA signaling is reported to be involved in the proliferation, migration or

Table 4 Incidence and multiplicity of microinvasive adenocarcinoma

Group	Treatment	No. of rats	Ventral		Lateral		Anterior	
			Incidence	Multiplicity (/rat)	Incidence	Multiplicity (/rat)	Incidence	Multiplicity (/rat)
1	(TRAP × SHR)F1	9	4	0.56 ± 0.73	1	0.33 ± 1.00	8	1.11 ± 0.60
2	(TRAP × SHR)F1 / Prazosin	8	2	0.37 ± 0.74	2	0.25 ± 0.46	7	1.00 ± 0.53
3	(TRAP × WKY)F1	9	2	0.22 ± 0.44	1	0.11 ± 0.33	8	1.11 ± 0.60
4	(TRAP × WKY)F1 / Prazosin	8	0	–	2	0.25 ± 0.46	6	1.25 ± 0.89

TRAP, transgenic rat for adenocarcinoma of prostate; SHR, spontaneously hypertensive rat; WKY, Wistar Kyoto.

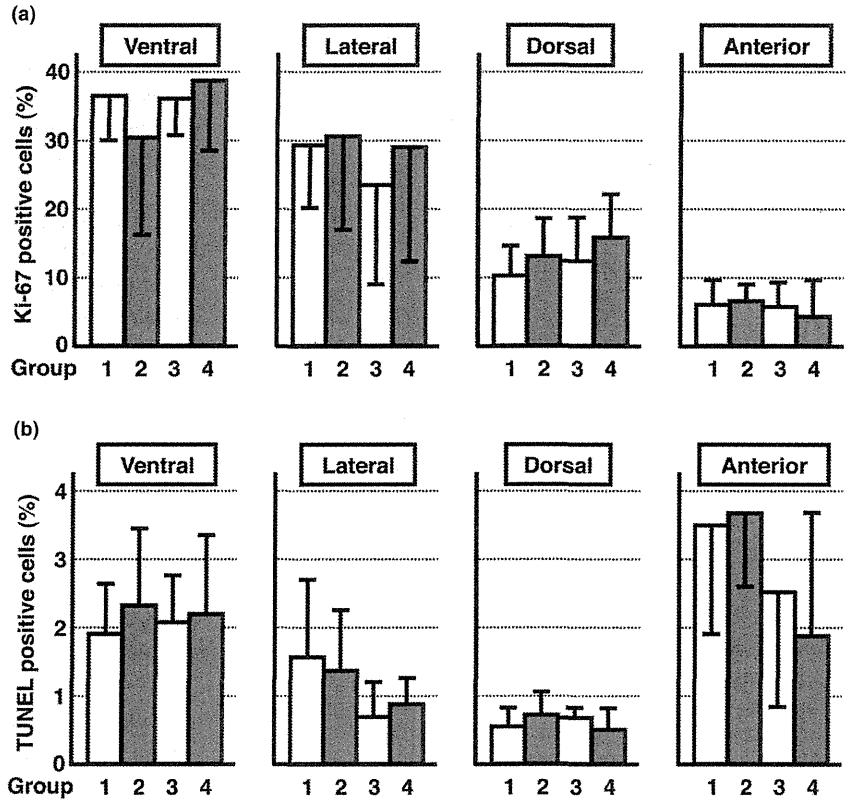

Figure 4 Labeling indices for (**a**) Ki-67 and (**b**) apoptotic cells (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)) in individual prostatic lobes in Experiment 2.

metastasis of prostate cancer cells.[46,47] Therefore, it is speculated that RhoA restrictedly contributed to the progression from HG-PIN to adenocarcinoma but not from LG-PIN to HG-PIN in the present experimental condition because the RhoA signaling pathway is involved in relatively late-stage of prostate carcinogenesis.

In conclusion, the present animal experiment provided some evidence supporting the conclusion that development of hypertension contributes to increased prostate cancer risk.

ACKNOWLEDGMENTS

This work was supported by Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare of Japan and a grant from the Society for Promotion of Pathology of Nagoya, Japan.

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Cancer Letters 306 (2011) 142–150

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Cancer Letters

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Induction of G1 arrest and apoptosis in androgen-dependent human prostate cancer by Kuguacin J, a triterpenoid from *Momordica charantia* leaf

Pornsiri Pitchakarn [a,d], Shugo Suzuki [a], Kumiko Ogawa [a,b], Wilart Pompimon [c], Satoru Takahashi [a], Makoto Asamoto [a], Pornngarm Limtrakul [d,*], Tomoyuki Shirai [a,*]

[a] *Department of Experimental Pathology and Tumor Biology, Nagoya City University, Graduate School of Medical Sciences, Nagoya, Japan*
[b] *Division of Pathology, National Institute of Health Sciences, Tokyo, Japan*
[c] *Laboratory of Natural Products, Department of Chemistry, Faculty of Science, Lampang Rajabhat University, Lampang, Thailand*
[d] *Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Chiang Mai 50200, Thailand*

ARTICLE INFO

Article history:
Received 18 October 2010
Received in revised form 5 February 2011
Accepted 28 February 2011

Keywords:
Prostate cancer
Apoptosis
Cell cycle
Bitter melon
Kuguacin J

ABSTRACT

In this study, we focused on the effects of a bitter melon (*Momordica charantia*) leaf extract (BMLE) and a purified component, Kuguacin J (KuJ), on androgen-dependent LNCaP human prostate cancer cells. Both treatments exerted growth inhibition through G1 arrest and induction of apoptosis. In addition, KuJ markedly decreased the levels of cyclins (D1 and E), cyclin-dependent kinases (Cdk2 and Cdk4) and proliferating cell nuclear antigen, and caused an increase in p21 and p27 levels. Its induction of apoptosis was accompanied by an increase in cleavage of caspase-3 and poly (ADP-ribose) polymerase, attributable to augment of Bax/Bcl-2 and Bad/Bcl-xL and reduction of survivin levels. BMLE and KuJ also reduced the expression of androgen receptor (AR), prostate-specific antigen (PSA) while induced P53 protein level. Down-regulation of p53 by RNA interference indicated that BMLE and KuJ inhibited cell growth partly through p53-dependent cell cycle arrest and apoptotic pathways. Both BMLE and KuJ caused less toxicity in a normal prostate cell line, PNT1A. Our results suggest that BMLE and a purified component, KuJ, from its diethyl ether fraction could be promising candidate new antineoplastic and chemopreventive agents for androgen-dependent prostate cancer and carcinogenesis.

1. Introduction

Prostate cancer is the most common male malignant tumor in Western countries [1]. The substantial associated mortality and morbidity and its limited treatment options have led to a search for novel means for prevention. Contributing risk factors include age, ethnicity, genetic factors, and diet; in fact, diet-based agent for prevention and therapy is an attractive option based on significant findings of various epidemiologic studies, which have directly linked dietary habits to various forms of cancers, including prostate cancer [2]. Therefore, identification of bioactive compounds present in diet with potential anti-cancer efficacy has been an attractive research area in recent years [3].

Generally, deregulation of cell growth and resistance to apoptosis are major defects of cancer cells, so that approaches to induce cell cycle arrest and stimulate the apoptotic machinery could be effective. Recently, dietary phytochemicals have shown promise in this regard [4,5].

The fruit and/or leaves of *Momordica charantia*, or bitter melon, are widely consumed as a vegetable and especially as a folk medicine in Asia [6,7]. Anti-cancer activity of extracts against lymphoid leukemia, lymphoma,

* Corresponding authors. Address: Department of Experimental Pathology and Tumor Biology, Nagoya City University, Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan, Department of Biochemistry, Faculty of Medicine, Chiangmai University, 110 Intawaroros Rd., T. Suthep, A. Maung, Chiangmai 50200, Thailand. Tel.: +81 52 853 8154; fax: +81 52 842 0817 (T. Shirai), tel.: +66 53 945325 6x218; fax: +66 53 217144/894031 (P. Limtrakul).
E-mail addresses: plimtrak@med.cmu.ac.th (P. Limtrakul), tshirai@med.nagoya-cu.ac.jp (T. Shirai).

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doi:10.1016/j.canlet.2011.02.041

choriocarcinoma, melanoma, breast cancer, skin tumor, prostatic cancer, squamous carcinoma of the tongue and larynx, human bladder carcinomas and Hodgkin's disease has been reported [6,8–12], possibly linked to antioxidant [13], antiviral (human immunodeficiency virus [HIV] inhibitor) [14], antidiabetic [15] and immunomodulating properties [16]. A previous study demonstrated that Thai *M. charantia* induced phase II enzymes and repressed monooxygenase whereas a Chinese variety only affected monooxygenase [17]. We have reported that a bitter melon leaf extract (BMLE) in Thailand increased the sensitivity to a chemotherapeutic agent, vinblastine, in cervical carcinoma KB-V1 cells [18] and inhibited invasion and migration of an androgen-independent rat prostate cancer cell line (PLS10) *in vitro* while decreasing lung metastasis *in vivo* [19]. Recently, fresh bitter melon extract demonstrated growth inhibitory effects on a breast cancer cell by modulating cell cycle regulatory genes and enhancing apoptosis [20]. Our previously study, bioguided fractionation was used to identify the active component of BMLE, which is able to modulate the function of P-glycoprotein (ABCB1) and the multidrug resistance (MDR) phenotype. We found that Kuguacin J (KuJ) inhibited P-glycoprotein-mediated MDR and increased sensitivity to vinblastine and paclitaxel in KB-V1 cells [21]. In the present study, for the first time, effects of BMLE against an androgen-dependent human prostate cancer cell line, LNCaP, *in vitro* were examined. Since BMLE is crude extract, we also focused on KuJ, a purified component.

2. Materials and methods

2.1. Plant material and extraction [21]

Bitter melon leaves were collected in Lampang province, Thailand, in March 2009. A voucher specimen (BKF No. 15602) has been deposited at the Forest Herbarium, Department of National Park, Wildlife and Plant Conservation, Ministry of Natural Resources and Environment, Bangkok, Thailand. Fresh leaves were dried at 30–45 °C, ground and powdery plant samples (1 kg) were extracted exhaustively with 4 L of 80% ethanol by maceration at 37 °C for 16 h. The mixture was filtered and re-extracted with 4 L of ethanol. The combined filtrate was bleached with 160 g of active charcoal, filtered and concentrated by rotary evaporation to 120 mL before filtering to remove precipitates. The filtrate was rotary evaporated and lyophilized twice to obtain BMLE (65.37 g) then kept at −20 °C protected from light. BMLE was dissolved in a final DMSO concentration adjusted to 0.1% (v/v) in the culture medium.

For isolation of KuJ, 100 g of BMLE was re-dissolved in 1 L of 50% methanol and successively partitioned with hexane (0.58 g), diethyl ether (13.32 g), chloroform (4.63 g), ethyl acetate (3.65 g) and an aqueous layer. Each fraction was dried under reduced pressure and then submitted to bioassays. A portion of the diethyl ether fraction (15 g) was chromatographed in a silica gel 60 (Merck KGaA, Darmstadt, Germany) column, eluting in sequence with an increasing concentration of hexane in ethyl acetate. Further separation by silica gel 60 F254 preparative thin layer chromatography using hexane in ethyl acetate (90:10) as a mobile phase led to isolation of KuJ (1.23 g). The compound was identified as the previously described triterpenoid, KuJ, by comparison of its physical and HPLC, IR, MS and NMR data with those reported in the literature [21–25]. The purified KuJ ($C_{30}H_{46}O_3$, purity >99.99% by high-resolution mass spectrometry) with a molecular weight of 454 (Fig. 2B) was dissolved in DMSO as a stock solution, and used directly in the cell culture treatments. The percentage composition of KuJ included in BMLE and the organic fraction (diethyl ether) was analyzed by HPLC.

2.2. Cell culture

An androgen-dependent human prostate cancer cell line, LNCaP, was purchased from The American Type Culture Collection (ATCC, Manassas, VA, USA) and a human normal prostatic epithelial cell line, PNT1A, from the Health Protection Agency Culture Collections (Salisbury, United Kingdom). The cells was cultured in Roswell Park Memorial Institute-1640 Medium (RPMI 1640, Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, Life Technologies Japan Ltd., Japan), 50 U/ml penicillin and 50 μg/ml streptomycin, in a humidified incubator with an atmosphere comprising 95% air and 5% CO_2 at 37 °C. When the cells reached 70–80% confluence, they were harvested and plated either for subsequent passage or for treatment. DMSO was used as a vehicle control in all experiment and adjusted to the final concentration at 0.1% in culture medium. The following experiments were performed in triplicate.

2.3. Cytotoxicity and growth inhibition assays

LNCaP cells were plated at 5.0×10^3 cells per well in 96-well plates. Twenty-four hours after plating, various concentrations of BMLE or KuJ were added. The cells were then incubated for 24 and 48 h at 37 °C, and cell growth were assessed by WST-1 colorimetric assay (Roche Diagnostics, Mannheim, Germany) [26].

2.4. Cell cycle analysis

The cells were treated with BMLE or KuJ at various concentrations for 24 or 48 h then suspensions were prepared and stained with propidium iodide (Guava® cell cycle reagent, Guava Technologies) according to the Guava® Cell Cycle Assay protocol. Cell cycle phase distributions were determined on a Guava® PCA Instrument using CytoSoft Software.

2.5. Apoptosis assays

Apoptosis was assessed using the Guava® Nexin reagent and the Guava® PCA system (Guava Technologies, Hayward, CA, USA). The Guava® Nexin assay utilizes two stains (annexin V and 7-amino actinomycin D [7-AAD]) to quantitate the percentage of apoptotic cells. It was performed according to the manufacturer's protocol. After treatment with BMLE or KuJ for 24 or 48 h, cell suspensions were

prepared and stained with Guava® Nexin reagent for 20 min and immediately processed with the Guava PCA system. Cells were gated based on forward scatter (size), and results reported as the percentage of gated cells positive for both annexin V and 7-AAD.

2.6. Immunoblot analyses

After treatment, cells were washed twice with ice-cold phosphate buffer saline (PBS), scraped with a cell scraper into ice-cold PBS and centrifuged at 1500 rpm for 10 min. The supernatant was removed and cell pellets were lysed with a RIPA buffer (Pierce Biotechnology, Rockford, Illinois, USA) containing a protease inhibitor (Pierce Biotechnology) for 15 min on ice. The insoluble matter was removed by centrifugation at 12,000 rpm for 20 min at 4 °C and supernatants were collected. Protein concentrations were determined with a Coomassie Plus™-The Better Bradford Assay Kit (Pierce Biotechnology).

Samples were mixed with 2× sample buffer (Bio-Rad Laboratories, Hercules, California USA) and heated for 5 min at 95 °C and then subjected to 10% SDS–PAGE. The separated proteins were transferred onto nitrocellulose membranes followed by blocking with 5% nonfat milk powder (w/v) in 1× TBS [10 mmol/L Tris–HCl (pH7.5), 100 mmol/L NaCl, 0.1% Tween-20] for 1 h at room temperature. Membranes were probed with antibodies for AR, Bax, cyclin D1, cyclin E, p21, p27, proliferating cell nuclear antigen (PCNA) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Bcl-2, prostate-specific antigen (PSA) (DAKO, Denmark A/S, Glostrup, Denmark), CDK2, CDK4 (BD Biosciences, San Jose, CA, USA), Bad, Bcl-xL, caspase-3, cleaved caspase-3, poly(ADP-ribose) polymerase (PARP), p53, and survivin (Cell Signaling, Technology Inc., Danvers, Massachusetts, USA) in 5% nonfat dry milk, 1X TBS, 0.1% Tween-20 at 4 °C overnight, followed by exposure to peroxidase-conjugated appropriate secondary antibodies and visualization with an enhanced chemiluminescence detection system (GE Healthcare Bio-sciences, Buckinghamshire, NA, UK). To confirm equal protein loading, each membrane was stripped and reprobed with anti-β-actin (Sigma–Aldrich, Co., St. Louis, MO, USA) or anti-tubulin antibodies (Santa Cruz Biotechnology). Band density of Bad, Bax, Bcl-2, Bcl-xL and tubulin were then determined with ImageJ 1.410 (National Institute of Mental Health, Maryland, USA).

2.7. RNA interference

Cells were transfected with 20 nMol/L of control siRNA or two clones of siRNA for human p53 (Invitrogen, Carlsbad, CA, USA) using Lipofectamine™ RNAiMAX reagent (Invitrogen) for 24 h. p53 siRNA2 was used for subsequent experiments with BMLE or KuJ treatments.

2.8. Statistical analysis

All data are presented as mean ± S.D. values. Statistical analyses were conducted with Prism version 5.0 using one-way ANOVA, the Dunnett test or the Tukey's test. Statistical significance was concluded with *, **, ***: $P < 0.05$, 0.01 or 0.001, respectively.

3. Results

3.1. BMLE inhibits growth of LNCaP cells

LNCaP cells were treated with the varying concentrations (0–250 μg/ml) of BMLE for 24 h and 48 h, and its effects on cell growth were assessed by WST-1 assay. Cell growth of LNCaP was significantly inhibited by BMLE at between 25 and 250 μg/ml in a concentration-dependent manner. Inhibitory concentration (IC) 50 values were approximately 100 μg/ml and 50 μg/ml for 24 h and 48 h treatment, respectively (Fig. 1A).

3.2. BMLE induces cell cycle arrest in LNCaP cells

To explore the underlying mechanism of BMLE-induced growth suppression, cell cycle analysis was performed by Guava® cell cycle assay. BMLE-treated cells appeared to dramatically accumulate in G1 phase, especially at 150 (65%, $p < 0.01$), 200 (70%, $p < 0.001$) and 250 μg/ml (65%, $p < 0.01$), compared to control (52%), with concomitant decrease in the percentage of cells in the G2/M phase (Fig 1B and C). Cell cycle arrest began to occur from 24 h of treatment (data not shown). These results thus suggest that BMLE inhibited cell proliferation by arresting cells in the G1phase.

3.3. BMLE causes apoptotic death of LNCaP cells

In addition to cell cycle arrests, morphologic observation of BMLE-treated LNCaP cells indicated floating cells which are associated with cell death (data not shown). Guava® Nexin assays showed the treatment of LNCaP with 250 μg/ml of BMLE for 48 h resulted in significant increase in both the early and late stages of apoptosis (Fig. 1D): control (6% and 5%) and 250 μg/ml (24%, $P < 0.01$ and 34%, $P < 0.05$). It was shown that BMLE at high concentration caused inhibition of LNCaP cell growth by apoptosis induction.

3.4. Effects of BMLE on expression of cell cycle- and apoptosis-related proteins

LNCaP is an AR-positive prostate cancer cell line [27] expressing wild-type p53 [28]. As shown in Fig. 1E, BMLE (200 and 250 μg/ml) reduced its expression of AR and PSA proteins as assessed by western blotting analysis. Additionally, it was found that p53 protein expression was up-regulated after treatment with 250 μg/ml of BMLE, the concentration which caused both cell cycle arrest and induction of apoptosis.

As BMLE caused G1 arrest and apoptotic induction in LNCaP, we next examined the effect of BMLE on the expression of G1 cell cycle and apoptosis-regulatory proteins. Treatment with BMLE for 48 h also resulted in a marked reduction in the expression of cyclin D1 and PCNA in a concentration-dependent manner, as well as decrease in Bcl-2, with no effect on Bax. Besides, BMLE treatment led to the decrease of pro-caspase-3 and the increase of cleaved caspase-3 in a concentration-dependent manner (Fig. 1F). The cell cycle data shown in Fig. 1B and C coupled with the reduced expression of cyclin D1 and PCNA indicate that BMLE causes G1 arrest of LNCaP cells and the apoptosis data shown in Fig. 1F coupled with the decrease in Bcl-2 and the increase in cleaved caspase-3 indicate that BLME also induces LNCaP cells to undergo apoptosis. These data confirmed BMLE caused the inhibition of LNCaP cell growth via cell cycle arrest and apoptosis induction.

3.5. Identification of an active component in BMLE

According to Section 2, partition–extraction of BMLE using organic solvents (hexane, diethyl ether, chloroform and ethyl acetate) with increasing polarity provided four organic fractions. WST-1 assays showed growth inhibitory effects on LNCaP cells with all (Fig. 2A). The diethyl ether fraction (DEF) showed the strongest inhibitory effect on LNCaP cell growth (Fig. 2Ac), which led us to further isolate purified compound using DEF as a starting material.

The active compound in DEF was identified as KuJ (Fig. 2B), which we previously show its anti-cancer effect to modulate P-glycoprotein function and reverse cancer multidrug resistance [21,23], and this compound was further tested for influence on the growth of LNCaP cells (Fig. 2C). Treatment with KuJ for 24 h slightly decreased LNCaP cell growth, whereas treatment for 48 h significantly decreased cell growth and viability of the cancer cells with an IC50 of 15 μM.