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Cytotoxic and Antitumor Activity of Momilactone B from Rice Hulls

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The cytotoxic and antitumor activity of methanolic extract of rice hulls (MERH) were evaluated by the MTT-dye reduction assay against human colon cancer cells and the colonic aberrant crypt foci (ACF) assay in 1,2-dimethylhydrazine (DMH)-injected F344 male rats, respectively. MERH was found to be highly cytotoxic, with IC_{50} values of 0.5 μg/mL in vitro. Forty weeks of MERH supplementation (50 mg/kg of body weight/day) reduced colonic pre-neoplastic ACF formation by 35% ($p < 0.01$). An active compound, momilactone B, was isolated from MERH by silica gel chromatography, Sephadex LH-20 chromatography, and HPLC. The cytotoxic activity of momilactone B was evaluated by the MTT-dye reduction, lactate dehydrogenase (LDH), and colony-forming ability assays in human colon cancer HT-29 and SW620 cells. The results indicated that momilactone B from rice hulls might be a new candidate for chemotherapeutic agent against human colon cancer.

KEYWORDS: Momilactone B; rice hulls; cytotoxic; antitumor; anticancer; human colon cancer

INTRODUCTION

Cancer chemotherapy has gradually improved with the development of novel antitumor drugs. While treatment of certain malignancies with chemotherapy has been successful and encouraging, the effectiveness has often been limited by drug resistance of tumors and by side effects on normal tissues and cells (*1*). In fact, many therapeutic anticancer agents exhibit intrinsically and potently cytotoxic activity against normal cells (*2*, *3*). This serious problem has meant that much effort has been expended to develop novel anticancer agents with few side effects and low cytotoxicity on normal tissues and cells in cancer chemotherapy (*4*). Many plant products have pharmacological applications and have the potential for use in cancer chemotherapy because of their low toxicity and great medicinal value (*5*, *6*). Much research has concentrated on different plant extracts' abilities to induce cytotoxic effects in cancer cells (*7*, *8*).

Rice is one of the principal cereals in Asia, some countries of Africa, and Latin America (*9*). Rice hulls are an agricultural byproduct that poses a very serious problem for the global environment (*10*). In the case of South Korea, more than 1 million tons of rice hulls are produced annually from the processing of rice. The rice hull comprises 20% of the rice grain kernel and, similar to other plant biomass, contains a high percentage of organic substances (*10*). It is therefore recognized as a potential source of energy and organic chemicals (*9*, *11*). Rice hulls have recently been reported to contain antioxidant substances that protect rice seed from oxidative stress (*12*). In our previous study (*13*), we identified several phenolic compounds including *o*-methoxycinnamic acid, 4,7-dihydroxybenzoic acid, and *p*-coumaric acid from methanolic extract of rice hull (MERH) and induced the high antioxidant activity against scavengers of singlet oxygen and free radicals. Furthermore, we recently reported that MERH showed significant scavenging activity toward reactive oxygen species and also inhibited hydrogen peroxide-induced damage to cellular DNA in human lymphocytes, providing a protective effect against oxidative damage (*14*). The objective of this study was to elucidate cytotoxic and antitumor activity of MERH and to identify (a) specific compound(s) possessing those activities from MERH.

MATERIALS AND METHODS

Materials. Rice hull from rice cultivar (*Oriza sativa* L.), a Japonica type rice, was obtained from Kimcheon, South Korea. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), methylene blue, and 1,2-methylhydrazine was provided by Sigma Chemical Co. (St. Louis, MO). A lactate dehydrogenase (LDH) release assay kit was purchased by Wako Pure Chemical Industries, Ltd. (Osaka, Japan). HT-29 and SW620 cells, which were originally derived from human colon cancer, were purchased from ATCC (Rockville, MD). RPMI 1640 medium, fetal bovine serum, and penicillin/streptomycin were obtained from Gibco BRL (Grand Island, NY). All organic solvents and other chemicals were of analytical grade or complied with the standards needed for cell culture experiments.

Preparation of Methanolic Extracts of Rice Hulls (MERH) and Isolation of Momilactone B. Each 30 g of rice hulls was extracted with 1 L of methanol in a shake incubator overnight at room temperature and filtered through Whatman No. 1 filter paper. After removal of methanol by a rotary evaporator, the residues were used as methanolic extracts of rice hulls (MERH). For isolation of momilactone B, MERH

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was further extracted with ethyl acetate three times and concentrated in vacuo. The solvent layer was dried over Na_2SO_4 and concentrated to give an oily residue. The residue was fractionated by a silica gel column using $CHCl_3$−MeOH (20:1) as a mobile phase. The active fraction was collected and concentrated under reduced pressure and then was applied on a Sephadex LH-20 column. MeOH was used as a mobile phase, and the active fraction was collected and concentrated in the same manner described above. Finally, momilactone B was obtained by high-performance liquid chromatography (HPLC) using a CLIPEUS C^{18} column 5 μm (4.6 i.d. × 150 mm, Higgins Analytical, Inc., USA) eluted with 70% MeOH. Detection was accomplished with a UV−Vis detector and chromatograms were recorded at 210 nm.

Cell Culture and Treatments. HT-29 and SW620 cells were maintained in the RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 mg/mL), and 2 g/mL $NaHCO_3$. These cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. MERH and momilactone B were dissolved in DMSO and added to the culture medium so that the final concentration of DMSO was <1%.

MTT Reduction Assay for Cell Viability. Cell viability was measured with blue formazan that was metabolized from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial dehydrogenase, which are active only in live cells. HT-29 and SW620 cells were preincubated in a 96-well plate at a density of 1.5×10^4 cells per well for 24 h in a humidified atmosphere of 5% CO_2 in air at 37 °C. Cells were pretreated with various concentrations of MERH and momilactone B. After incubation for 24, 48, and 72 h, respectively, MTT reagent (5 mg/mL) was added to each of the wells, and the plate was incubated for an additional 1 h at 37 °C. The media were then removed, and the intracellular formazan product was dissolved in 100 μL of dimethyl sulfoxide (DMSO). The absorbency of each well was then measured at 540 nm using the ELISA reader (BioRad, Model 680, USA), and the percentage viability was calculated.

Lactate Dehydrogenase Release Assay. Cytotoxicity was determined by measuring the release of lactate dehydrogenase (LDH). HT-29 and SW620 cells were pretreated with various concentrations of momilactone B for 72 h and the supernatant was used to assay LDH activity. The reaction was initiated by mixing 50 μL of cell-free supernatant with potassium phosphate buffer containing nicotinamide adenine dinucleotide (NADH) and sodium pyruvate in a final volume of 100 μL to 96-well plate. A colorimetric assay was applied, according to which the amount of formazan salt is proportional to LDH activity in the sample. The intensity of red color formed in the assay and measured at a wavelength of 490 nm was proportional to LDH activity and to the number of damaged cells. Data were normalized to the activity of LDH released from vehicle-treated cells (100%) and expressed as percentage of the control (obtained separate plating).

Colony Formation Assay. HT-29 and SW620 cells were seeded at 1×10^5 in a 24-well plate, cultured overnight, and treated with various concentrations of momilactone B for 18 h. For the colony formation assay, the cells were then diluted in a fresh medium lacking momilactone B, reseeded at 1×10^3 cells per well in a 6-well plate, and cultured under normal growth conditions for 7 or 8 days to form colonies. Formed colonies were fixed with 10% formaldehyde, stained with 0.01% crystal violet, and counted. Cell survival (mean values with 95% confidence intervals from triplicate determinations) was calculated by setting the survival of control cells as 100%. IC_{50} values (concentration required for 50% inhibition of colony formation) were determined from the dose−response curves of colony formation inhibition.

Animals and Diets (in Vivo Test). Five-week-old male F344 rats ($n = 24$) were purchased from Koatec Inc. (Pyeongtaek, Korea) and were housed individually in hanging wire cages in a room controlled for humidity (55%), temperature (25 °C), and light (12/12 h light−dark cycle). The animal care followed the *Guide for Care and Use of Laboratory Animals* (Department of Health, Education and Welfare, 1985). The experimental protocol was approved by a local Ethical Committee for Animal Experimentation. The rats were allowed free access to water and fed a commercially prepared pelleted diet (2018 Teklad Global diet 18% protein, Harlan, Madison, WI). After 1 week of adjustment, the rats were randomly allocated to two groups; control group was fed basal diet and rice hull supplemented group received MERH in the drinking water (50 mg/kg body weight/day) with basal diet for 40 weeks. Five days after beginning feeding of the experimental diets, all animals received the carcinogen 1,2-methylhydrazine (DMH) s.c. once a week for 6 weeks at a dose of 180 mg/kg body weight. Animals were monitored daily for general health, and body weights were recorded every week for the duration of the study.

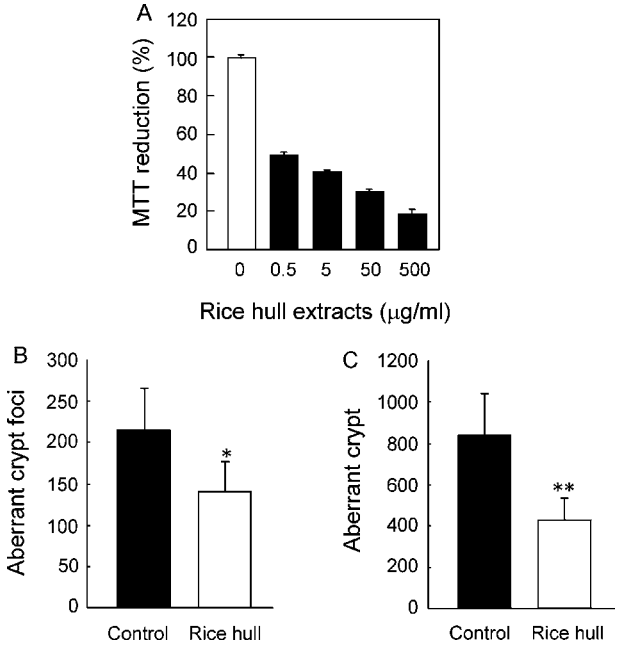

Figure 1. Effect of methanolic extract from rice hulls against human colon cancer cells in vitro and in vivo. Cells were treated with various doses of methanolic extract from rice hulls for 24 h (**A**). Cell viability was determined using the MTT assay. Effect of rice hull supplementation on number of aberrant crypt foci (ACF) (**B**) and total number of aberrant crypts (ACs) (**C**) in distal colon. Bars represent mean ± S.D. of triplicate determination. Significantly different from the control group: *: $p < 0.05$; **: $p < 0.01$ (student's t-test).

Aberrant Crypt Foci (ACF) Assay. At the end of the experimental period, the rats were anesthetized with ethyl ether and the entire colon was collected for aberrant crypt analysis. The determination of aberrant crypts was performed on a 5-cm-long segment corresponding to the distal part of the colon. The segment was washed with physiological saline (0.9% NaCl), cut open, and fixed in 10% buffered formalin solution for at least 24 h. Later, the colon samples were stained with 0.2% methylene blue for 5 min; the mucosal side was then placed on a glass slide and examined microscopically using the 10× objective for assessment of the number of aberrant crypts, following a procedure described by Bird (*15*).

Statistical Analysis. The results are reported as mean ± S.D. Data were analyzed using the SPSS package for Windows (Version 10). The in vitro data were evaluated by one-wa y analysis of variance (ANOVA) followed by Scheffe's test. The mean values of in vivo experiments were compared using the Student's *t*-test. The differences were considered significant at a level of $P < 0.05$.

RESULTS

Cytotoxic and Antitumor Activity of MERH. The inhibitory effect of MERH on the proliferation of HT-29 cells was dose-dependent (**Figure 1A**). HT-29 cells were treated with various concentrations of MERH, and the cytotoxic effect was evaluated with the MTT reduction assay. A 24 h exposure to MERH decreased cell viability by 50−70% compared to non-exposed control. The cell viability rate decreased to 30% when cells were treated with 500 μg/mL of MERH. The HT-29 cells were sensitive to MERH in the dose range of 0.5−500 μg/mL with an IC_{50} value of 0.5 μg/mL.

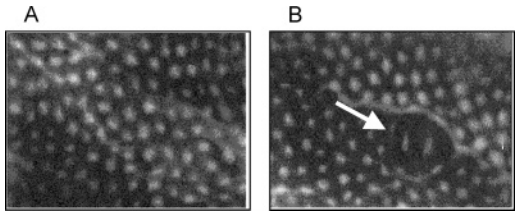

Figure 2. Aberrant crypt foci (Preneoplastic lesion of colon) surrogate intermediate biomarker of colon cancer. **A**: Normal colon without any ACF; rats were treated with a vehicle (saline); magnification 10×. **B**: Colon with two AC (arrow), rats were treated with DMH (carcinogen of colon). Magnification 10×.

Table 1. Effects of Rice Hull Supplementation on Final Body Weight and Organ Weights in F344 Male Rats[a]

	control[b]	rice hull
final body weight (g)	393.5 ± 24.3^{ns}	385.0 ± 65.3
organ weight (g/100 g of BW)		
liver	2.96 ± 0.26^{ns}	2.76 ± 0.24
spleen	0.21 ± 0.02^{ns}	0.21 ± 0.05
heart	0.24 ± 0.03^{ns}	0.25 ± 0.03
kidney	0.52 ± 0.04^{ns}	0.56 ± 0.09

[a] Values represent mean ± S.D. for 12 animals in each group. [b] Superscript "ns" indicates not significant by student's t-test.

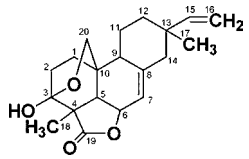

Figure 3. Structure of momilactone B.

All rats injected with DMH developed abnormal and hyperplastic crypts in the colon as shown in **Figure 2B**, while rats were treated with saline showed normal colon without any ACF (**Figure 2A**). The frequency of preneoplastic ACF in the rat colon was 35% less (significant at $p < 0.05$) in the group supplemented with MERH than the control (**Figure 1B**). Each ACF contained 1−10 ACs (aberrant crypts). The total number of ACs in the colon was 49% lower (significant at $p < 0.01$) in the rice hull supplemented group than the control group (**Figure 1C**). During the experiment, no signs of treatment-related adverse effects were observed in the clinical appearance of the animals. No statistical differences in final body weight and organ weight were found between the two groups (**Table 1**).

Purification of Momilactone B from MERH. The cytotoxic and antitumor compound was isolated and purified from MERH. MERH was re-extracted with ethyl acetate, and the active compound was purified using silica gel column chromatography, Sephadex LH-20 chromatography, and preparative HPLC.

The molecular formula of the active compound was found to be $C_{26}H_{20}O_4$ using FAB-MS spectroscopy (data not shown). The structure of the active compound was determined on the basis of its physicochemical properties and various NMR spectroscopic methods. Through a database and literature searches, the active compound was identified as 3,20-epoxy-3-hydroxy-9-pimara-7,15-dien-19,6-olide (momilactone B; **Figure 3**).

Momilactone B was originally isolated from rice husks as a growth inhibitor and later was also found in rice leaves and straws as a phytoalexin (*16*, *17*). Momilactone B acts as an allelopath, inhibiting growth of neighboring plants (*18*, *19*). However, cytotoxic and antitumor activity of momilactone B against human colon cancer cells has not been reported up to now.

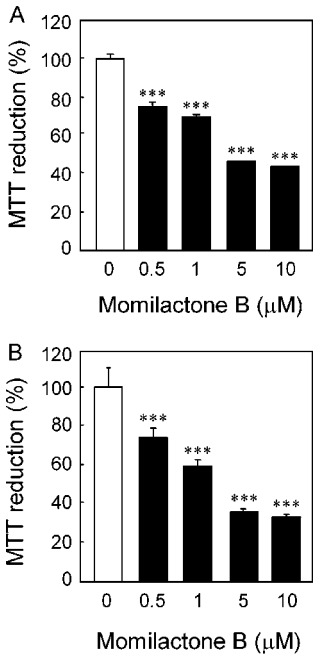

Figure 4. Effect of momilactone B on cell viability of HT-29 and SW620 cells. These cells were treated with various concentrations of momilactone B (**A**, HT-29 cells; **B**, SW620 cells). Cell viability was determined using the MTT assay and expressed as a percentage of the mean absorbance measured in untreated control cultures. The values represented means ± S.D. of triplicate determinations. ***: Significant vs control untreated cells ($p < 0.001$).

Cytotoxic Effect of Momilactone B on HT-29 and SW620 Colon Cancer Cells. The cytotoxic effects of momilactone B on HT-29 and SW620 human colon cancer cells are shown in **Figures 4**, **5**, and **6**. HT-29 and SW620 cells, originally derived from human colon cancer, showing strong tolerance to anticancer agents in vitro and in vivo, were chosen as representative hypovascular tumors (*20*). Cell viability was measured by the MTT reduction assay.

The effect of momilactone B on the proliferation of HT-29 and SW620 cells was dose-dependent. After 24 h of exposure to momilactone B, varying concentrations of momilactone B decreased cell viability by 20−50% when compared to the viability of control HT-29 and SW620 cells (**Figures 4A** and **4B**). The cytotoxic effects of momilactone B on cultured HT-29 and SW620 human colon cancer cells were determined by measuring the extent of LDH leakage into the medium. The loss of intracellular LDH and its release into the culture medium is an indicator of irreversible cell death due to cell membrane damage. HT-29 and SW620 cells were exposed to momilactone B (0, 0.5, 1, and 5 μM) for 72 h. Momilactone B increased cell damage by 20−30% in HT-29 and SW620 cells compared to controls, as measured by LDH release (**Figures 5A** and **5B**).

The effects of momilactone B on cell viability was determined by the colony forming assay. Momilactone B was highly toxic in the culture medium to HT-29 and SW620 cells with the momilactone B level required for 50% inhibition of colony formation (IC_{50}) being less than 1 mM (**Figures 6A** and **6B**). These results clearly indicated that momilactone B had cytotoxic activity on HT-29 and SW620 human colon cancer cells.

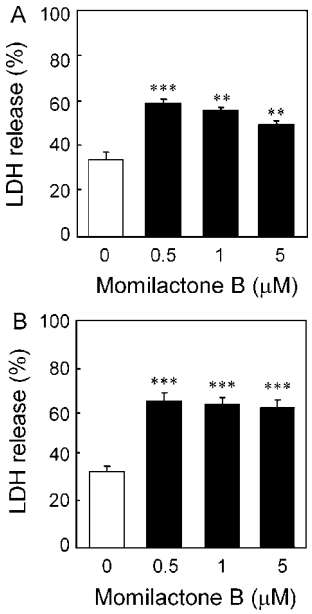

Figure 5. Effect of momilactone B on cytotoxicity of HT-29 and SW620 cells. These cells were treated with various concentrations of momilactone B (**A**, HT-29 cells; **B**, SW620 cells). Cell death was determined using LDH release assay. Data were normalized to the activity of LDH released from vehicle-treated cells (100%) and expressed as percentage of the control (obtained separate plating). The values represent means ± S.D. of triplicate determinations. **: $p < 0.01$; ***: $p < 0.001$.

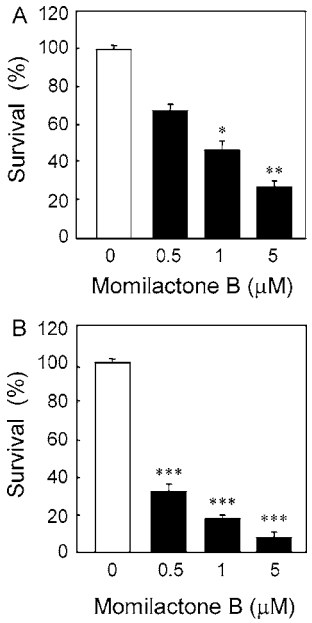

Figure 6. Cytotoxic effect of momilactone B on HT29 and SW620 cells. The cells were exposed to the indicated concentrations of momilactone B for 18 h. After a colony formation assay (**A**, HT-29 cells; **B**, SW620 cells), the survival rate (means ± S.D. of triplicate determinations) was calculated by setting each of the control survival rates. After 7–8 days, colonies were fixed with 10% formaldehyde, stained with 0.01% crystal violet, and counted. The values represent means ± S.D. of triplicate determinations. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

DISCUSSION

The results of this study clearly demonstrate cytotoxic and antitumor activity of MERH both in HT-29 human colon cancer cells and in F334 rats without causing any adverse effects on body weight or organ weight (**Figure 1**, **Table 1**). HT-29 cells were sensitive to MERH in the dose range of 0.5−500 μg/mL with an IC_{50} value of 0.5 μg/mL (**Figure 1A**). The colonic carcinogen DMH causes a high incidence of precancerous lesions, referred to as ACF, in rats. ACF also has been identified in the human colon and is associated with carcinomas (*21*, *22*). The study of premalignant hyperproliferative lesions and aberrant crypts is crucial for the understanding of the pathogenesis of colon cancer because of the potential progression of early changes to malignancy. When MERH (50 mg/kg of body weight/day) was supplemented in rats, MERH effectively suppressed the occurrence of colonic ACF induced by DMH (**Figures 1B** and **1C** and **Figure 2**).

Momilactone B (**Figure 3**), isolated from MERH, was identified as the active compound having cytotoxic and antitumor activity against human colon cancer cells. Momilactone B was cytotoxic to HT-29 and SW620 human colon cancer cells. As shown in **Figure 4**, cell viability was reduced by momilactone B. The cytotoxicity study also confirmed that the cytotoxicity of momilactone B was dose-dependent (**Figures 5** and **6**). The molecular and biochemical mechanisms of the cytotoxicity of momilactone B cannot be fully understood from the results of this study, but for the reasons given above it is likely that it acts by inhibiting human colon cancer cell survival. Together with these consistent observations, momilactone B and MERH are potently cytotoxic to human colon cancer cells in vitro and in vivo.

In conclusion, our results suggest that momilactone B and MERH may be potential candidates for novel therapeutic agents to induce cell death in human colon cancer cells.

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