RESEARCH ARTICLE

Dietary rice bran component γ-oryzanol inhibits tumor growth in tumor-bearing mice

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Scope: We investigated the effects of rice bran and components on tumor growth in mice.
Methods and results: Mice fed standard diets supplemented with rice bran, γ-oryzanol, Ricetrienol®, ferulic acid, or phytic acid for 2 weeks were inoculated with CT-26 colon cancer cells and fed the same diet for two additional weeks. Tumor mass was significantly lower in the γ-oryzanol and less so in the phytic acid group. Tumor inhibition was associated with the following biomarkers: increases in cytolytic activity of splenic natural killer (NK) cells; partial restoration of nitric oxide production and phagocytosis in peritoneal macrophages increases in released the pro-inflammatory cytokines tumor necrosis factor-α, IL-1β, and IL-6 from macrophages; and reductions in the number of blood vessels inside the tumor. Pro-angiogenic biomarkers vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX-2), and 5-lipoxygenase-5 (5-LOX) were also significantly reduced in mRNA and protein expression by tumor genes. ELISA of tumor cells confirmed reduced expression of COX-2 and 5-LOX up to 30%. Reduced COX-2 and 5-LOX expression downregulated VEGF and inhibited neoangiogenesis inside the tumors.
Conclusion: Induction of NK activity, activation of macrophages, and inhibition of angiogenesis seem to contribute to the inhibitory mechanism of tumor regression by γ-oryzanol.

Keywords:
Angiogenic / Inflammatory / γ-Oryzanol / Rice bran / Tumor growth inhibition

1 Introduction

Rice bran from whole grain brown rice and rice bran oil are rich sources of a large number of bioactive secondary metabolites [1–7] and volatile aroma compounds [8]. In previous studies, we found that aqueous/ethanolic bran extracts from several black rice cultivars exhibited potent antioxidative, antimutagenic, anti-inflammatory, and anticarcinogenic effects in chemical and cell assays [9–13] and that a rice hull smoke extract exhibited anti-inflammatory and antimicrobial effects in mice [14,15]. Reviews by Lerma-Garcia et al. [16] and Islam et al. [17] describe beneficial effects of rice bran-derived phytochemicals and suggest the need for further studies to establish their protective and curative effects in human disease.

Rice bran followed by raw rice seeds and brown rice are good sources of γ-oryzanol (Fig. 1) and phenolic antioxidants [18, 19]. γ-Oryzanol has been shown to exhibit anti-inflammatory, antihyperlipoproteinaemic, and cholesterol-lowering and platelet aggregation lowering properties [16,20]. Germination increased the γ-oryzanol content and cancer cell-inhibiting effects of brown rice [21]. Fermented brown rice and rice bran prevented induced colorectal carcinogenesis [22] and lung tumorigenesis [23] in mice. Ferulic acid from brown rice inhibited the growth of human breast and colon cancer cells [24]. Phytic acid from rice bran induced apoptosis on human colorectal adenocarcinoma cells [25]

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Abbreviations: CM, complete medium; **COX-2**, cyclooxygenase 2; **dNTP**, 2′-deoxyribonucleoside triphosphate; **FBS**, fetal bovine serum; **5-LOX**, 5-lipoxygenase; **iNOS**, inducible nitric oxide synthase; **LTB4**, leukotriene B4; **NK**, natural killer cells; **NO**, nitric oxide; **PGE2**, prostaglandin E2; **R.E.**, relative expression of genes and proteins; **rIFN-γ**, recombinant interferon-γ; **TNF-α**, tumor necrosis factor-α; **VEGF**, vascular endothelial growth factor

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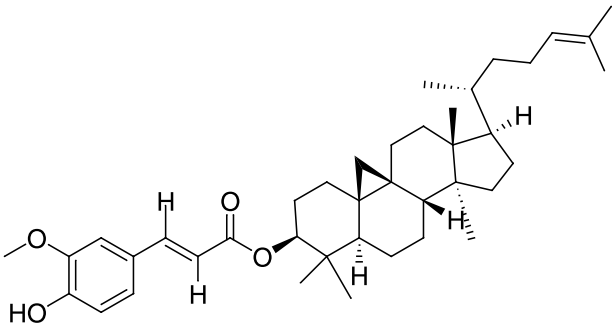

Figure 1. Structure of cycloartenyl ferulate, the main component of γ-oryzanol (a mixture of closely related plant sterol ferulate esters or triterpene alcohol ferulate esters).

and inhibited colon carcinogenesis in rats [26]. Rice bran-derived tocotrienol inhibited tumor cell-induced angiogenesis in the mouse dorsal air sac (DOS) assay [27]. γ-Tocotrienol promoted tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) through upregulation of death receptors in leukemic, kidney, and pancreatic cells [28].

To further demonstrate the potential therapeutic value of rice bran ingredients, the main objective of the present study was to investigate the ability of the bran ingredients γ-oryzanol, Ricetrienol®, ferulic, and phytic acids, and of rice bran to reduce tumor size and associated biomarkers in mice intracutaneously transplanted on their backs with CT-26 colon cancer cells.

2 Materials and methods

γ-Oryzanol (a mixture of sterol ferulate esters [29]), Ricetrienol®, ferulic, and phytic acids, >98% pure, were kindly provided by Tsuno Rice Fine Chemicals (Wakayama, Japan). Rice bran was obtained from the College of Agriculture and Life Science, Seoul National University (Seoul, Republic of Korea). Ricetrienol®, a yellowish-brown colored oily substance prepared from rice bran, is reported to contain α-tocopherol, tocotrienol, and phytosterols [30]. According to the supplier, the Ricetrienol® used in the present study contained 2.0–3% total tocopherols and 2.0–2.5% total tocotrienols.

RPMI 1640 medium, Hanks' balances salt solution (HBSS), fetal bovine serum (FBS), and other cell culture reagents were purchased from Hyclone Laboratories (Logan, UT). Calcein-AM was purchased from Calbiochem (San Diego, CA). Hematoxylin, eosin Y, LPS, recombinant murine interferon-γ (rIFN-γ), and other reagents were obtained from Sigma-Aldrich (St. Louis, MO). The AMV reverse transcriptase and 2′-deoxyribonucleoside triphosphate (dNTP) mixture were obtained from Takara Bio (Kyoto, Japan). PCR primers were custom synthesized and purified by Bioneer (Daejon, Republic of Korea).

2.1 Animals and diets

Pathogen-free female BALB/c mice (6 weeks old), weighing 20–25 g, were purchased from Orient Bio (Seoul, Republic of Korea). The mice were individually housed in a stainless steel cage under a 12-h light/dark cycle with a temperature range of 20–22°C and relative humidity of 50 ± 10%. They were fed a pelletized commercial chow diet purchased from Orient Bio (Cat. No. 5L79) and sterile tap water ad libitum for 1 week after arrival. The mice were then randomly divided into groups ($n = 10$). Each group was fed a standard diet supplemented with 10% rice bran or with 0.2% of ferulic acid, phytic acid, Ricetrienol®, or γ-oryzanol and a control without supplementation. Mice remained on their respective diet throughout the rest of the experimental period, as described in our previous studies with black rice bran [31] and liquid rice hull smoke [15]. γ-Oryzanol, which was active at the 0.2% dietary level, was further tested with 0.2, 0.5, and 1% levels of supplementation along with controls.

2.2 Dietary treatment with γ-oryzanol and transplantation of CT-26 murine colon cancer cells

The protocol for the mice studies was approved by the Ethics Committee for Animal Care and Use, Ajou University, Republic of Korea. All experiments were performed in compliance with the relevant laws and institutional guidelines.

After feeding a normal diet for 1 week, BALB/c mice were fed an oryzanol-supplemented diet for 2 weeks. They were then intracutaneously transplanted with 1×10^6 of CT-26 mouse colon cancer cells in 200 μL PBS into the lateral side of the back. The mice were then fed for another 2 weeks with the same supplemented diet. Control group mice were administered the same volume of PBS only. Mice were sacrificed by CO_2 inhalation at the end of the treatments for the isolation of peritoneal macrophages and excision of tumor masses and organs.

2.3 Tumor growth

To evaluate the time-dependence effects of each treatment, removed tumor masses from the control and experimental groups of mice were weighed in an analytical balance. Figure 2 shows the time dependence of tumor growth.

2.4 Splenic natural killer (NK) cell cytolysis assay

Spleen cells were isolated and blood cells removed as described by Trop et al. [32]. Spleens were crushed through a stainless mesh (size 60) in complete medium (CM) consisting of RPMI 1640 medium and 10% FBS plus antibiotics. NK activity was evaluated as follows. Briefly, splenic mononuclear

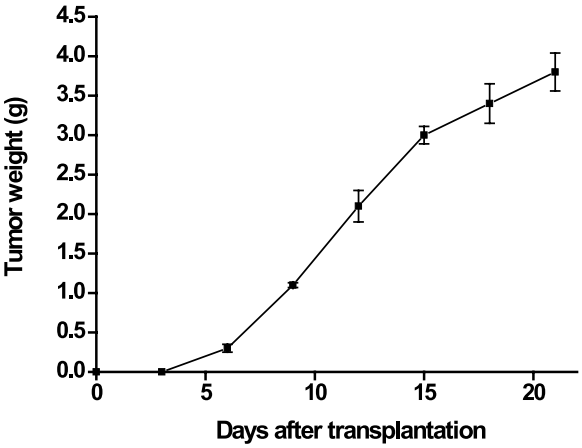

Figure 2. Tumor growth after transplantation of CT-26 colon cancer cells into lateral side of back of BALB/c mice. Results are expressed as mean ± SD ($n = 5$).

cells were obtained by centrifuging the spleen cell suspension on 2 mL of histopaque-1077 (Sigma Diagnostics, St. Louis, MO) to recover the cells in the interface, which were then washed three times with CM. The cells were resuspended in CM at 1×10^6 cells/mL. Yac-1 cells, used as the target cell, were labeled with calcein-AM ester according to the method of Roden et al. [33]. Labeling of the cells (1×10^6 cells/mL) was in all cases performed at a final calcein-AM concentration of 25 μM for 30 min. Purified mononuclear effector cells and labeled Yac-1 target cells were added to a 96-well plate and co-cultured for 3 h at 37°C (20:1 effector:target ratio). Following centrifugation at 400 × *g*, the supernatant (100 μL) from each well were harvested for measuring fluorescence released into medium using a spectrofluorometer (Model RF-5301, Shimadzu, Kyoto, Japan) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. Spontaneous fluorescence release was determined by culturing the labeled target cells in CM without effector cells. Maximum fluorescence was obtained from wells where target cells were incubated with a lysis buffer (50 mM sodium borate, 0.1% triton X-100, pH 9.0). Specific lysis was calculated according to the following formula:

$$\%\text{lysis} = [1 - (\text{experimental fluorescence} - \text{background fluorescence})/(\text{maximum fluorescence} - \text{background fluorescence})] \times 100 \quad (1)$$

2.5 Nitric oxide (NO) generation assay

Isolation and purification of peritoneal macrophage cells from tumor-bearing mice orally administered with γ-oryzanol were performed according to the method of Narumi et al. [34]. Cells exudated through lavaging with HBSS reagent were plated onto 60-mm tissue culture dishes ($1–5 \times 10^6$ cells/dish) to produce macrophage cells firmly adhered onto the dishes. NO was measured by determining the concentration of its stable oxidative metabolite nitrite, using the microplate method described by Xie et al. [35] with slight modification. Briefly, isolated peritoneal macrophages were cultured in a 96-well plate (1×10^5 cells/well) with rIFN-γ (10 U/mL) and LPS (100 ng/mL) for 48 h. To measure nitrite concentrations, culture medium (100 μL) was mixed with an equal volume of Griess reagent [1% sulfanilamide and 0.1% *N*-(naphthyl)-ethylenediamine dihydrochloride in 5% phosphoric acid] at room temperature for 15 min. The absorbance at 570 nm was determined with a microplate reader (model 550, Bio-Rad, Hercules, CA) using a standard calibration curve for sodium nitrite.

2.6 Phagocytotic uptake assay

The phagocytotic activity of peritoneal macrophage cells was measured following the method of Duperrier et al. [36] with slight modification. Briefly, isolated peritoneal macrophages were cultured in a 60-mm culture dish (1×10^5 cells) with rIFN-γ (10 U/mL) and LPS (100 ng/mL) for 48 h. After stimulation, cells (1×10^4 cells) were resuspended in PBS (1 mL) containing FBS (5%), and cultured at 37°C for 15 min. They were then incubated with Dextran-FITC (1 mg/mL) at 37°C for 1 h. The reaction was stopped with cold PBS containing FBS (5%) and sodium azide (1%). The cells were then washed three times with cold PBS, and analyzed on a FACSVantage instrument (Becton-Dickinson, Franklin Lakes, NJ).

2.7 Histology and assessment of tumor vascularity

For histological analysis, the tumor tissue of the mice was fixed with paraformaldehyde (4%) in phosphate buffer (0.5 M, pH 7.4). The tissues were rinsed with water, dehydrated with ethanol, and embedded in paraffin. The samples were sectioned into 4 μm sizes and mounted onto glass slides. The sections were then dewaxed using xylene and ethanol, and stained with hematoxylin and eosin Y (H&E). Blood vessels were counted in six blindly chosen random fields under the microscope at 200× magnification, and the microvessel density was recorded.

2.8 RT-PCR of cellular RNA

Total cellular RNA was prepared from tumor tissues following acid phenol guanidium thiocyanate-chloroform extraction [37]. For reverse transcription, total RNA (1 μg) was incubated with AMV reverse transcriptase (5 U) and oligo (dT18) as primer (100 ng). DNA amplification was then primed in a reaction mixture containing dNTP mix (400 μM), Taq polymerase (2.5 U), and primer sets (20 μM each) representing target genes (Table 1). PCR was conducted using a

Table 1. Primer sets representing four target genes and the internal control β-actin gene

Primer	Sequence
Vascular endothelial growth factor (VEFG) sense	5′-TGACAAGCCAAGGCGGTGAG-3′
VEGF antisense	5′-CCTCCTCCCAACACAAGTCC-3′
Cyclooxygenase-2 (COX-2) sense	5′-TCTCAACCTCTCCTACTAC-3′
COX-2 antisense	5′-GCACGTAGTCTTCGATCACT-3′
Inducible nitric oxide synthase (iNOS) sense	5′-ATGTCCGAAGCAAACATCAC-3′
iNOS antisense	5′-TAATGTCCAGGAAGTAGGTG-3′
5-Lipoxygenase (5-LOX) sense	5′-ATGAGCTGTTTCTAGGCATGTACC-3′
5-LOX antisense	5′-GAATAAAGTACCCCTGACCCAGCC-3′
β-Actin sense	5′-GTGGGGCGCCCCAGGCACCA-3′
β-Actin antisense	5′-GTCCTTAATGTCACGCACGATTTC-3′

thermocycler (model PTC-200, MJ Research Inc., Reno, NV) with one cycle for 5 min at 94°C, followed by 30 cycles for 30 s at 94°C, 45 s at 58°C, 45 s at 72°C, and finally one cycle for 5 min at 72°C. All amplified PCR products were subjected to 1.5% agarose gel electrophoresis and visualized with a UV illuminator. The intensity of the separated bands of DNA was quantified using a gel documentation system (mode LAS-1000CH, Fuji Photo Film Co., Tokyo, Japan).

2.9 Western blot analysis of cell proteins

The tumor tissues were lysed and extracted in a homogenizer with RIPA buffer (50 mM Tris. Cl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, pH 7.4). Protein concentrations were determined according to the Bradford method using a Bio-Rad Protein Kit. BSA was used as standard. The tissue extracts containing proteins (30 μg) were separated on 10% polyacrylamide gels and transferred onto nitrocellulose membrane (Millipore, Billerica, MA).

The following primary antibodies were used for Western blot analysis: goat antimouse cyclooxygenase-2 (COX-2) polyclonal antibody (Santa Cruz, Delaware, CA), rabbit antimouse inducible nitric oxide synthase (iNOS) polyclonal antibody (Cell signaling Tec., Danvers, MA), goat antimouse 5-LOX polyclonal antibody (Abcam, Cambridge, MA), rabbit antimouse vascular endothelial growth factor (VEGF) polyclonal antibody (Santa Cruz), and antimouse β-actin monoclonal antibody (Millipore). After blocking with 5% skim milk, membranes were incubated with each primary antibody, followed by HRP-conjugated anti-IgG antibodies. Blots were developed using the ECL detection kit (Pierce, Rockford, IL). The intensity of the separated protein bands was quantified using a gel documentation system (model LAS-1000CH, Fuji Photo Film Co.). At least three separate replicates were determined for each experiment.

2.10 ELISA of cytokines and eicosanoids

For quantitation of cytokines, peritoneal macrophages from each mice group were stimulated with rIFN-γ (10 U/mL) and LPS (100 ng/mL) followed by recovery of the culture medium and storage at −70°C until analysis. For quantitation of eicosanoids, tumor tissues were extracted to produce tissue fluid following the method of Shin et al. [38]. Briefly, tumor tissues from tumor-bearing mice treated with various doses of γ-oryzanol were homogenized in a phosphate buffer (pH 7.0) containing 0.4 M NaCl, 0.05% Tween-20, 0.5% BSA, 0.1 mM PMSF, and 10 mM EDTA. The homogenates were microcentrifuged at 14 000 × *g* for 15 min at 4°C to recover the supernatant and stored at −70°C until analysis. Cytokines tumor necrosis factor-α (TNF-α), IL-1β, and IL-6 in the culture medium and eicosanoids leukotriene B4 (LTB4) and prostaglandin E2 (PGE2) in the homogenized tissue supernatants were determined by ELISA (Biosource International, Camarillo, CA) following the manufacturer's instructions. The absorbance of the final solution at 420 nm was measured in a microplate reader (Model 550, Bio-Rad).

2.11 Statistical analysis

Results are expressed as the mean ± SD of three independent experiments. Significant differences between means were determined by ANOVA test using the Statistical Analysis Software package SAS (Cary, NC). The $p < 0.05$ is regarded as significant.

3 Results

3.1 Effect of γ-oryzanol on tumor growth after intracutaenous transplantation of CT-26 colon cancer cells

The objective of the present study was to measure the inhibitory effect of representative components of rice bran (oryzanol, Ricetrienol®, ferulic, and phytic acids) and of rice bran on the growth of induced tumors in mice, and to determine several biomarkers that may be associated with the mechanism of tumor growth inhibition by γ-oryzanol. To accomplish this objective, CT-26 mouse colon cancer cells

Table 2. Effect of rice bran and γ-oryzanol-supplemented diets on body and organ weights in tumor-induced mice

Sample	Final body weight, g	Organ weight (g/20 g body weight)				
		Heart	Lung	Stomach	Liver	Spleen
Vehicle	33.4 ± 1.5	0.17 ± 0.01	0.26 ± 0.02	0.25 ± 0.03	1.05 ± 0.11	0.19 ± 0.01
10% rice bran	34.3 ± 0.7	0.17 ± 0.01	0.27 ± 0.01	0.24 ± 0.04	1.01 ± 0.14	0.21 ± 0.03
0.2% γ-oryzanol	33.4 ± 0.7	0.20 ± 0.02	0.28 ± 0.04	0.27 ± 0.05	1.04 ± 0.18	0.18 ± 0.01
0.5% γ-oryzanol	33.6 ± 1.1	0.18 ± 0.02	0.24 ± 0.03	0.26 ± 0.02	0.94 ± 0.15	0.22 ± 0.03
1% γ-oryzanol	33.8 ± 1.3	0.17 ± 0.02	0.24 ± 0.03	0.27 ± 0.04	1.04 ± 0.08	0.20 ± 0.03

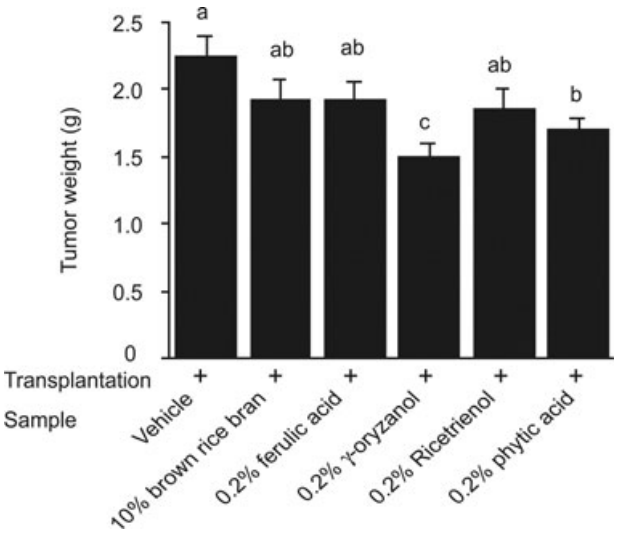

Figure 3. Effects of rice bran, ferulic acid, γ-oryzanol, Ricetrienol®, and phytic acid on tumor growth in vivo. BALB/c mice were intracutaneously transplanted with CT-26 mouse colon cancer cells (1×10^6 cells, 200 μL) and then subjected to oral administration with each compounds (0.2%, w/w) supplemented diets. Rice bran was used as internal control. After 2 weeks, mice were sacrificed to measure tumor weight. Results are expressed as mean ± SD ($n = 10$). Bars with the same letters are not significantly different between groups at $p < 0.05$.

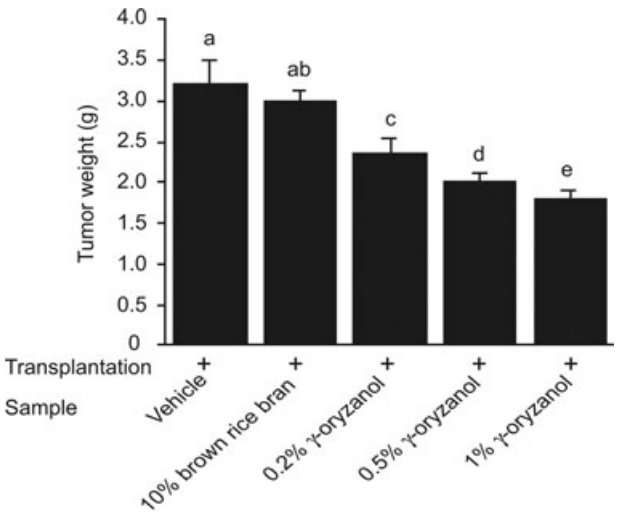

Figure 4. Dose-dependent antitumor effect of γ-oryzanol in vivo. BALB/c mice were intracutaneously transplanted with CT-26 mouse colon cancer cells (1×10^6 cells, 200 μL) and then subjected to oral administration with 0.2, 0.5, and 1% γ-oryzanol-supplemented diets (w/w). Rice bran was used as internal control. After 2 weeks, mice were sacrificed to measure tumor weight. Results are expressed as mean ± SD ($n = 10$). Bars with the same letters are not significantly different between groups at $p < 0.05$.

were transplanted intracutaneously onto the backs of the mice. Figure 2 shows that tumor growth in the mice with transplanted CT-26 cells continually increased with time during the 20-day test periods, reaching a value of approximately 3.5 g/mouse. Based on an average tumor weight of approximately 3.5 g shown in Fig. 2 and body weights shown in Table 2, the tumor accounts for approximately 10.5% of body weight.

Figure 3 shows that dietary administration of 0.2% γ-oryzanol (w/w) or 0.2% phytic acid significantly suppressed tumor growth. By contrast, the inhibition of tumor growth by Ricetrienol®, ferulic acid, and rice bran were not significant.

To further confirm the antitumor activity of γ-oryzanol, we determined the effect of three concentrations of γ-oryzanol on tumor regression. These were selected on the basis of a published study on the dose effect of oryzanol in the treatment of hyperlipoproteinanemias [39] and the preliminary result with 0.2% oryzanol mentioned above.

Figure 4 shows that oral administration of the mouse diet supplemented with 0.2, 0.5, and 1% γ-oryzanol for 2 weeks resulted in a dose-dependent reduction of tumor growth by 26, 37, and 44%, respectively. By contrast, 10% supplementation of rice bran used as internal control induced only a not significant 7% reduction in tumor weight. We then carried out several bioassays of biomarkers associated with carcinogenesis and its prevention in order to define a possible mechanism of tumor growth inhibition by γ-oryzanol.

3.2 Biosafety of γ-oryzanol

Oral administration of the diet supplemented with 0.2–1% γ-oryzanol did not affect various organ weights in both

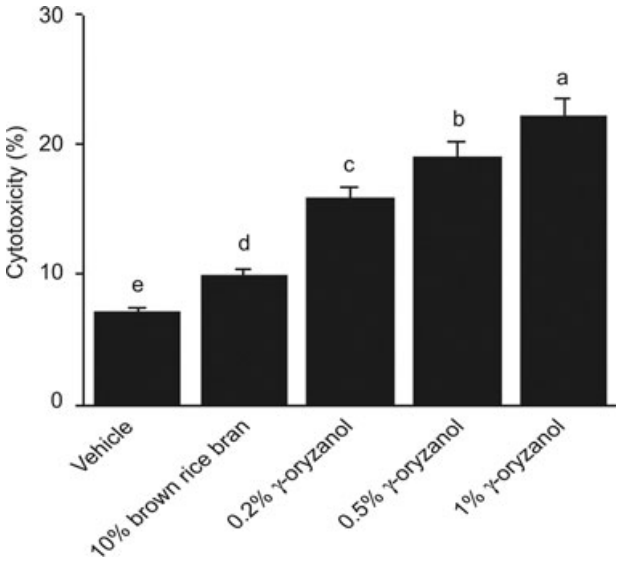

Figure 5. Effect of γ-oryzanol on NK cytolytic activities. Splenic NK cells from tumor-induced mice fed 0.2, 0.5, and 1% γ-oryzanol-supplemented diets (w/w) were incubated with Yac-1 target cells labeled with calcein-AM for 3 h (20:1 effector:target ratio). NK cytolytic activity was evaluated by measuring calcein-AM release from Yac-1 target cells using a fluorometer. Results are expressed as mean ± SD ($n = 10$). Bars with the same letters are not significantly different between groups at $p < 0.05$.

normal and tumor-bearing mice (Table 2). This finding indicates that γ-oryzanol is not toxic to mice within the concentration range employed in this study.

3.3 Splenic NK cell activity

To find out whether NK cells were activated by γ-oryzanol treatment, we determined the dietary administration-triggered activity changes in NK cytolysis. The spleens from the sacrificed mice were removed to separate NK cells (effector cells). To measure the level of calcein-AM released from the target cells caused by cytotoxic action of the effector cells, separated NK cells were incubated with a calcein-AM labeled YAC-1 cell line (target cells). Figure 5 shows that compared with vehicle-treated tumor-bearing mice, the 0.2, 0.5, and 1% γ-oryzanol treatments increased NK cell activities 2.3-, 2.7-, and 3.2-fold, respectively. These results indicate that γ-oryzanol can increase the cytotoxicity of NK cells.

3.4 Macrophage activity

Published studies indicate that physiological activities of macrophage cells are suppressed in tumor-bearing animals [40]. It was therefore of interest to examine whether γ-oryzanol can restore suppressed macrophage activity to the level observed in normal mice. NO generation and phagocytosis were employed as parameters of macrophage activation [41] (Fig. 6). Peritoneal macrophages from tumor-induced mice were primed with rIFN-γ and stimulated with LPS to mimics bacterial infection.

Figure 6A shows that compared to the vehicle treatment, NO generation of the macrophages from the 0.2, 0.5, and 1% γ-oryzanol-treated tumor-bearing mice was increased by about 44, 63, and 80%, respectively. Rice bran also elevated NO production, but activity was lower than that shown for γ-oryzanol (16% increase compared with vehicle-treated control).

Similar results were obtained for changes in phagocytosis of the macrophages. Figure 6B shows that dietary administration of 0.2, 0.5, and 1% γ-oryzanol elevated phagocytotic activities of macrophages by 21, 31, and 42%, respectively, compared with vehicle-treated tumor-induced mice, which amounts to activities which are 81, 88, and 95% of normal, respectively. Administration of 10% rice bran showed only a 7% increase in phagocytotic activity compared with vehicle-treated mice.

3.5 Pro-inflammatory cytokine secretion

As another parameter for macrophage activation, we also measured the stimulation of pro-inflammatory cytokines (TNF-α, IL-1B, and IL-6) by γ-oryzanol in peritoneal macrophages in response to priming with rIFN-γ and LPS (Table 3). ELISA analysis showed that production of the cytokines increased about twofold in cells from 1% γ-oryzanol-treated tumor-bearing mice compared to the vehicle-treated control. Based on the observed cytokine production, it seems that 1% γ-oryzanol restored macrophage activity to about 72–82% of that in normal mice. These results imply that restoration of macrophage activity by γ-oryzanol might be an important factor for tumoricidal or tumoristatic action of γ-oryzanol or γ-oryzanol-containing rice bran in vivo.

3.6 Inhibition of angiogenesis

Antitumor activity of γ-oryzanol could in part also be attributed to the inhibition of the formation of new blood vessels inside the tumor tissue, resulting in tumor cell death through hypoxia [42]. To demonstrate this possibility, we examined the effect of γ-oryzanol on angiogenesis in the induced tumors by counting the number of blood vessels under the microscope in the control and experimental mice groups. Figure 7 shows that compared to the vehicle-treated control, blood-vessel formation was significantly suppressed in the induced tumors treated with 0.2, 0.5, and 1% γ-oryzanol by about 33, 47, and 61%, respectively. It appears that γ-oryzanol blocked new microblood-vessel formation needed for tumor growth inside the tumor.

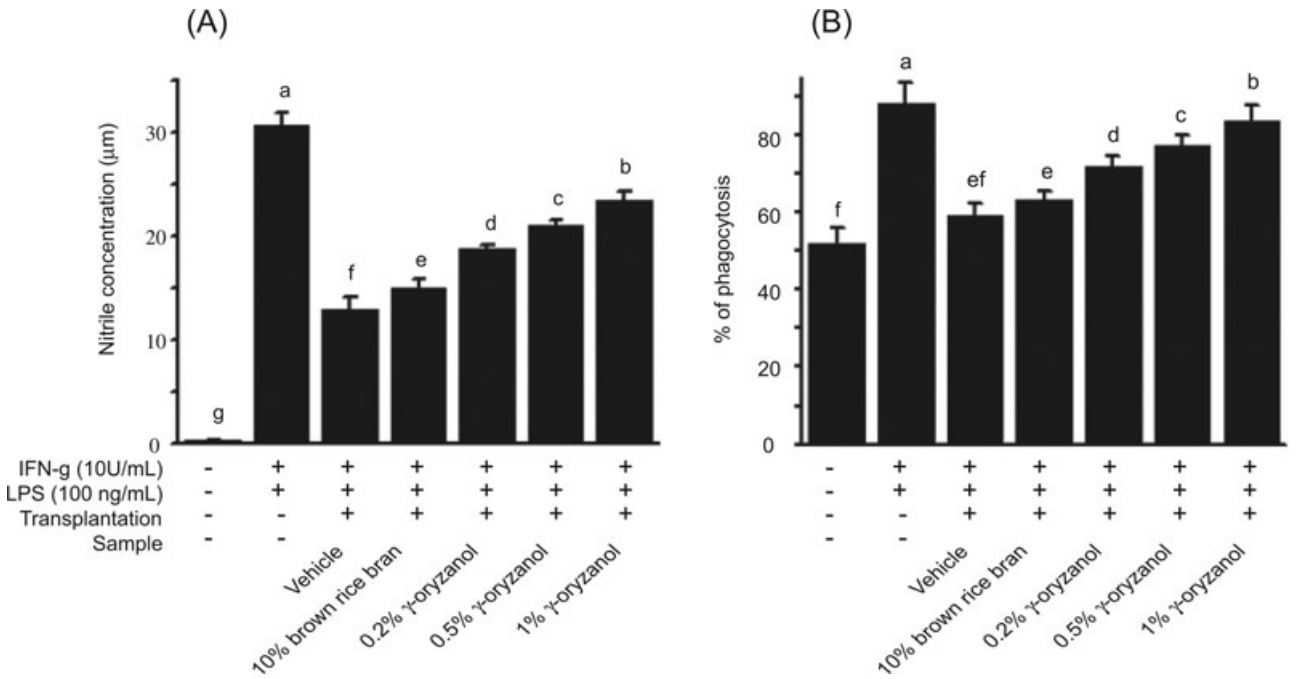

Figure 6. Upregulation of NO production and phagocytosis activities of peritoneal macrophages from tumor-induced mice by rice bran and γ-oryzanol. (A) Macrophage cells from tumor-induced mice subjected to oral administration with 0.2, 0.5, and 1% γ-oryzanol-supplemented diets (w/w) were primed with rIFN-γ and then stimulated with LPS for 48 h. (B) To induce phagocytosis, macrophage cells were stimulated with rIFN-γ and LPS for 48 h, and then incubated with Dextran-FITC (1 mg/mL) for 1 h. Fluorescence intensity was then determined by flow cytometry. First two bars show groups of macrophages from control animals with no tumors. Results are expressed as mean ± SD ($n = 10$). Bars with the same letters are not significantly different between groups at $p < 0.05$.

Table 3. Stimulation of pro-inflammatory cytokines by rice bran and γ-oryzanol in peritoneal macrophages from tumor-induced mice[a)]

Sample	Cytokines (pg/mL)		
	TNF-α	IL-1β	IL-6
Control (−)[b)]	47 ± 4^{1}	21 ± 2^{1}	23 ± 1^{1}
Control (+)[c)]	5211 ± 241^{2}	308 ± 17^{2}	423 ± 15^{2}
Vehicle[d)]	2025 ± 121^{3}	$131 \pm 10^{3,4}$	144 ± 6^{3}
10% rice bran	2315 ± 103^{4}	143 ± 9^{4}	170 ± 10^{4}
0.2% γ-oryzanol	3114 ± 227^{5}	183 ± 10^{5}	225 ± 13^{5}
0.5% γ-oryzanol	3497 ± 179^{6}	227 ± 14^{6}	267 ± 12^{6}
1% γ-oryzanol	3931 ± 226^{7}	251 ± 11^{7}	304 ± 21^{7}

a) Values expressed as mean ± SD ($n = 10$) in each column with the same superscript are not significantly different at $p < 0.05$.
b) Normal mice macrophages not stimulated with rIFN-γ and LPS.
c) Normal mice macrophages stimulated with rIFN-γ (10 U/mL) and LPS (100 ng/mL).
d) Tumor-induced mice macrophages subjected to oral administration with PBS-supplemented diet and then stimulated with rIFN-γ and LPS.

3.7 Regulation of pro-angiogenic factors

Two possible mechanisms may govern the suppression of new blood-vessel formation in the induced tumors. The first is a direct cytotoxic or antiangiogenic effect of γ-oryzanol. The second acts indirectly, involving inhibition of specific growth-related signals of vascular endothelial cell proliferation. We could not examine the former possibility using an in vitro cell culture system because of extremely low solubility of the test substances. We did, however, investigate whether elevated angiogenesis-related gene expression levels in the induced tumors were downregulated by the treatments. Translation levels of VEGF, COX-2, 5-LOX, and iNOS determined by Western blot analysis show that 0.2, 0.5, and 1% γ-oryzanol inhibited the known angiogenic factor VEGF by 37, 56, and 63%, respectively (Fig. 8A).

Similar inverse relationships between dose transcription levels of these genes were also observed by RT-PCR (Fig. 8B). PGE2 and LTB4, the respective arachidonic acid metabolites produced by COX-2 and 5-LOX, measured by ELISA (Table 4) confirmed the inhibitory actions of γ-oryzanol on COX-2 and 5-LOX activities; 0.2, 0.5, and 1% γ-oryzanol inhibited PGE2 by 17, 25, and 31%, respectively; the same doses of γ-oryzanol inhibited LTB4 by 21, 25, and 31%, respectively. The fact that the upregulation of VEGF determined with target genes was induced by PGE2 and 5-LOX (Fig. 8B and Table 4) supports our finding that downregulation of COX-2 expression is associated with the blockade of blood-vessel formation inside the tumors [43, 44].

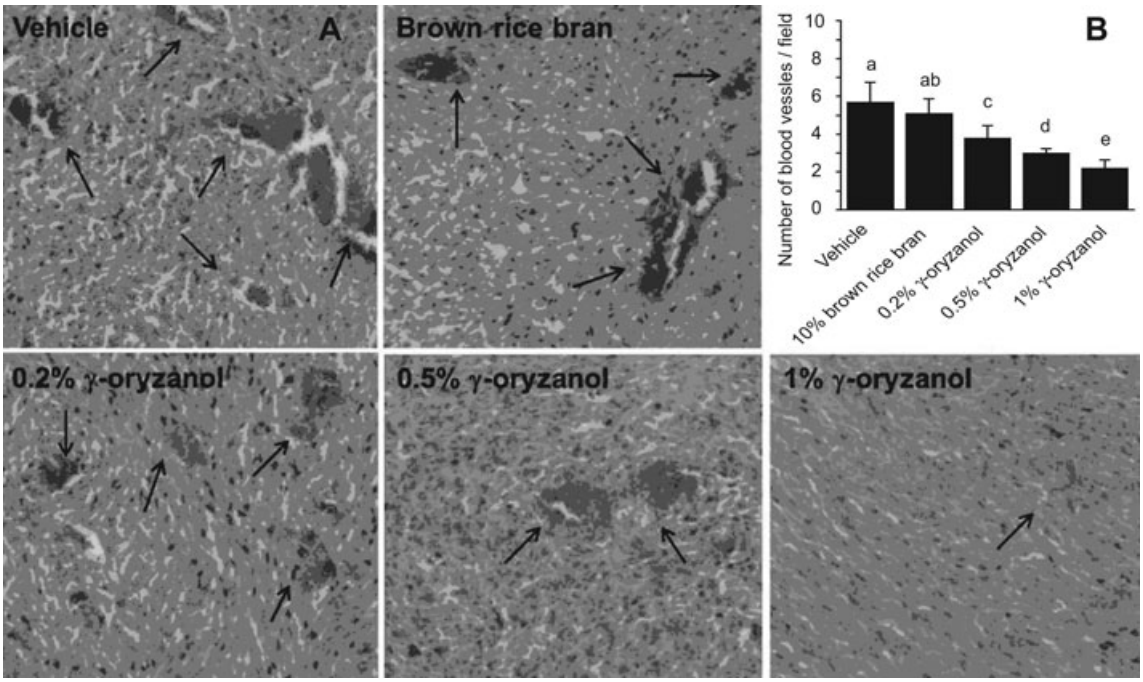

Figure 7. Antiangiogenic effect of rice bran and γ-oryzanol. (A) To observe blood-vessel formation, paraformaldehyde-fixed and paraffin-embedded tumor sections were stained with hematoxylin and eosin Y (H&E). The images were photographed by microscope at 200× magnification. Arrows indicate blood vessels inside the tumor. (B) To record vessel density, blood microvessels were counted under a microscope in six randomly chosen fields. Results are expressed as mean ± SD ($n = 10$). Bars with the same letters are not significantly different between groups at $p < 0.05$.

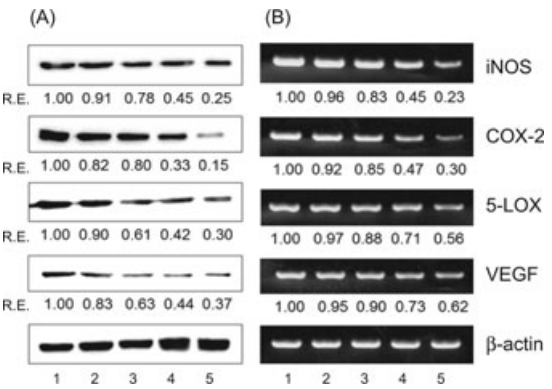

Figure 8. Modulation of iNOS, COX-2, 5-LOX, and VEGF expression in induced tumors of mice fed rice bran- and γ-oryzanol-supplemented diet. (A) Western blot analysis of iNOS, COX-2, 5-LOX, and VEGF protein expressions. (B) Semiquantitative RT-PCR analysis of iNOS, COX-2, 5-LOX, and VEGF mRNA synthesis. The relative ratio is expressed as a relative expression (R.E.) value calculated from target gene/β-actin gene expression. β-Actin was used as a control for constitutively expressed gene. Oral administration with diet supplemented as follows: lane 1, control group, normal diet; lane 2, 10% rice bran (w/w) supplementation; lane 3, 0.2% γ-oryzanol (w/w) supplementation; lane 4, 0.5% γ-oryzanol (w/w) supplementation; lane 5, 1% γ-oryzanol (w/w) supplementation. Figures represent results from at least three individual experiments.

4 Discussion

We found that compared to mice treated with the vehicle, oral administration for 2 weeks of γ-oryzanol to mice with induced tumors significantly reduced tumor size in a dose-dependent manner by up to 44%. Further mechanistic studies showed that the inhibition of tumor growth was accompanied with changes in several ex vivo and in vivo biomarkers (NK, NO, phagocytosis, tumor vascularity, histopathology, cellular RNA, and proteins; VEGF, COX-2, PGE2, 5-LOX, LTB4, TNF-α, IL-1β, and IL-6) associated with normal and tumor cells.

The observed results indicate that reduction of tumor size is accompanied by modulation of gene expressions associated with tumor growth and the consequent effects on levels of the pro-inflammatory mediators and angiogenic factors. Although we do not know the mechanism of how γ-oryzanol modulates expression of the COX-2 and 5-LOX, it is likely that downregulation of COX-2 and 5-LOX might be the cause of repressed VEGF expression and subsequent tumor cell death due to a lack of diffusion of nutrients and oxygen to the tumor cells. Earlier studies also indicated that VEGF is the downstream mediator of 5-LOX in the induction of the angiogenic process and subsequent tumorigenesis [45, 46].

We do not know the specific molecular cellular targets of γ-oryzanol or whether it is concentrated or metabolized in

Table 4. Inhibitory effects of rice bran and γ-oryzanol on release of eicosanoids in induced tumors[a)]

Sample	Eicosanoids (pg/mL)	
	PGE2	LTB4
Vehicle[b)]	914 ± 10^1	2305 ± 77^1
10% rice bran	823 ± 35^2	2156 ± 100^2
0.2% γ-oryzanol	724 ± 23^3	1912 ± 69^3
0.5% γ-oryzanol	683 ± 7^4	1733 ± 82^4
1% γ-oryzanol	635 ± 17^5	1603 ± 34^5

a) Values expressed as mean ± SD ($n = 10$) in each column with the same superscripts are not significantly different at $p < 0.05$.
b) Tumor-induced mice macrophages subjected to oral administration with PBS-supplemented diet and then stimulated with rIFN-γ (10 U/mL) and LPS (100 ng/mL).

tumor tissues. Because γ-oryzanol is known to exhibit strong antioxidative activities [47], it may interfere with intracellular signaling pathways through reactive oxygen radicals. It is still unclear whether the anticarcinogenic effects of rice bran phytosterols are related to antioxidant and/or inhibition of NF-κB activity [17]. Results from this study and toxicity studies by other investigators [17] indicate that γ-oryzanol may be almost completely free of harmful effects. These considerations suggest that γ-oryzanol may have therapeutic potential against human cancers. Whether the γ-oryzanol content of white and pigmented brans from different rice varieties correlates with anticarcinogenic activity also merits study.

The authors have declared no conflict of interest.

5 References

[1] Parrado, J., Miramontes, E., Jover, M., Márquez, J. C. et al., Prevention of brain protein and lipid oxidation elicited by water-soluble oryzanol enzymatic extract derived from rice bran. *Eur. J. Nutr.* 2003, *42*, 307–314.

[2] Zhang, M.-W., Guo, B.-J., Zhang, R.-F., Chi, J.-W. et al., Separation, purification and identification of antioxidant compositions in black rice. *Agr. Sci. China.* 2006, *5*, 431–440.

[3] Watanabe, S., Imawaka, N., Katsube, T., Yamasaki, Y., Radical scavenging activity and identification of anthocyanin pigments in shikokumai (*Oryza sativa*). *Nippon Shokuhin Kagaku Kogaku Kaishi* 2009, *56*, 419–423.

[4] Kong, S., Lee, J., Antioxidants in milling fractions of black rice cultivars. *Food Chem.* 2010, *120*, 278–281.

[5] Min, B., Chen, M.-H., Green, B. W., Antioxidant activities of purple rice bran extract and its effect on the quality of low-NaCl, phosphate-free patties made from channel catfish (*Ictalurus punctatus*) belly flap meat. *J. Food Sci.* 2009, *74*, C268–C277.

[6] Roschek, B., Fink, R. C., Li, D., McMichael, M. et al., Pro-inflammatory enzymes, cyclooxygenase 1, cyclooxygenase 2, and 5- lipooxygenase, inhibited by stabilized rice bran extracts. *J. Med. Food* 2009, *12*, 615–623.

[7] Muntana, N., Prasong, S., Study on total phenolic contents and their antioxidant activities of Thai white, red and black rice bran extracts. *Pak. J. Biol. Sci.* 2010, *13*, 170–174.

[8] Sukhonthara, S., Theerakulkait, C., Miyazawa, M., Characterization of volatile aroma compounds from red and black rice bran. *J. Oleo Sci.* 2009, *58*, 155–161.

[9] Nam, S. H., Choi, S. P., Kang, M. Y., Koh, H. J. et al., Bran extracts from pigmented rice seeds inhibit tumor promotion in lymphoblastoid B cells by phorbol ester. *Food Chem. Toxicol.* 2005, *43*, 741–745.

[10] Nam, S. H., Choi, S. P., Kang, M. Y., Kozukue, N. et al., Antioxidative, antimutagenic, and anticarcinogenic activities of rice bran extracts in chemical and cell assays. *J. Agric. Food Chem.* 2005, *53*, 816–822.

[11] Nam, S. H., Choi, S. P., Kang, M. Y., Koh, H. J. et al., Antioxidative activities of bran extracts from twenty one pigmented rice cultivars. *Food Chem.* 2006, *94*, 613–620.

[12] Choi, S. P., Kang, M. Y., Koh, H. J., Nam, S. H. et al., Antiallergic activities of pigmented rice bran extracts in cell assays. *J. Food Sci.* 2007, *72*, S719–S726.

[13] Choi, S. P., Nam, S. H., Kang, M. Y., Inhibitory effects of pigmented black rice bran on airway inflammation in mouse asthma model. *J. Clin. Biochem. Nutr.* 2008, *43*, S363–S369.

[14] Kim, S. P., Kang, M. Y., Park, J. C., Nam, S. H. et al., Rice hull smoke extract inactivates *Salmonella* Typhimurium in laboratory media and protects infected mice against mortality. *J. Food Sci.* 2012, *77*, M80–M85.

[15] Kim, S. P., Yang, J. Y., Kang, M. Y., Park, J. C. et al., Composition of liquid rice hull smoke and anti-inflammatory effects in mice. *J. Agric. Food Chem.* 2011, *59*, 4570–4581.

[16] Lerma-García, M. J., Herrero-Martínez, J. M., Simó-Alfonso, E. F., Mendonça, C. R. B. et al., Composition, industrial processing and applications of rice bran γ-oryzanol. *Food Chem.* 2009, *115*, 389–404.

[17] Islam, M. S., Nagasaka, R., Ohara, K., Hosoya, T. et al., Biological abilities of rice bran-derived antioxidant phytochemicals for medical therapy. *Curr. Top. Med. Chem.* 2011, *11*, 1847–1853.

[18] Tunçel, N. B., Yilmaz, N., Gamma-oryzanol content, phenolic acid profiles and antioxidant activity of rice milling fractions. *Eur. Food Res. Technol.* 2011, *233*, 577–585.

[19] Yoshie, A., Kanda, A., Nakamura, T., Igusa, H. et al., Comparison of γ-oryzanol contents in crude rice bran oils from different sources by various determination methods. *J. Oleo Sci.* 2009, *58*, 511–518.

[20] Patel, M., Naik, S. N., Gamma-oryzanol from rice bran oil—a review. *J. Sci. Ind. Res.* 2004, *63*, 569–578.

[21] Kim, D.-J., Oh, S.-K., Yoon, M.-R., Chun, A. et al., The change in biological activities of brown rice and germinated brown rice. *J. Korean Soc. Food Sci. Nutr.* 2011, *40*, 781–789.

[22] Phutthaphadoong, S., Yamada, Y., Hirata, A., Tomita, H. et al., Chemopreventive effect of fermented brown rice and rice

bran (FBRA) on the inflammation-related colorectal carcinogenesis in $Apc^{Min/+}$ mice. *Oncol. Rep.* 2010, *23*, 53–59.

[23] Phutthaphadoong, S., Yamada, Y., Hirata, A., Tomita, H. et al., Chemopreventive effects of fermented brown rice and rice bran against 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced lung tumorigenesis in female A/J mice. *Oncol. Rep.* 2009, *21*, 321–327.

[24] Hudson, E. A., Dinh, P. A., Kokubun, T., Simmonds, M. S. J. et al., Characterization of potentially chemopreventive phenols in extracts of brown rice that inhibit the growth of human breast and colon cancer cells. *Cancer Epidemiol. Biomarkers Prev.* 2000, *9*, 1163–1170.

[25] Nurul-Husna, S., Norhaizan, M. E., Hairuszah, I., Abdah, M. A. et al., Rice bran phytic acid (IP6) induces growth inhibition, cell cycle arrest and apoptosis on human colorectal adenocarcinoma cells. *J. Med. Plant Res.* 2010, *4*, 2283–2289.

[26] Norazalina, S., Norhaizan, M. E., Hairuszah, I., Norashareena, M. S., Anticarcinogenic efficacy of phytic acid extracted from rice bran on azoxymethane-induced colon carcinogenesis in rats. *Exp. Toxicol. Pathol.* 2010, *62*, 259–268.

[27] Miyazawa, T., Shibata, A., Nakagawa, K., Tsuzuki, T., Antiangiogenic function of tocotrienol. *Asia Pac. J. Clin. Nutr.* 2008, *17*, 253–256.

[28] Kannappan, R., Ravindran, J., Prasad, S., Sung, B. et al., γ-Tocotrienol promotes TRAIL-induced apoptosis through reactive oxygen species/extracellular signal-regulated kinase/p53-mediated upregulation of death receptors. *Mol. Cancer Ther.* 2010, *9*, 2196–2207.

[29] Xu, Z., Godber, J. S., Purification and identification of components of γ-oryzanol in rice bran oil. *J. Agric. Food Chem.* 1999, *47*, 2724–2728.

[30] Kanaya, Y., Doi, T., Sasaki, H., Fujita, A. et al., Rice bran extract prevents the elevation of plasma peroxylipid in KKAy diabetic mice. *Diabetes Res. Clin. Pract.* 2004, *66*, S157–S160.

[31] Choi, S. P., Kim, S. P., Kang, M. Y., Nam, S. H. et al., Protective effects of black rice bran against chemically-induced inflammation of mouse skin. *J. Agric. Food Chem.* 2010, *58*, 10007–10015.

[32] Trop, S., Samsonov, D., Gotsman, I., Alper, R. et al., Liver-associated lymphocytes expressing NK1.1 are essential for oral immune tolerance induction in a murine model. *Hepatology* 1999, *29*, 746–755.

[33] Roden, M. M., Lee, K.-H., Panelli, M. C., Marincola, F. M., A novel cytolysis assay using fluorescent labeling and quantitative fluorescent scanning technology. *J. Immunol. Methods* 1999, *226*, 29–41.

[34] Narumi, S., Finke, J. H., Hamilton, T. A., Interferon and interleukin 2 synergize to induce selective monokine expression in murine peritoneal macrophages. *J. Biol. Chem.* 1990, *265*, 7036–7041.

[35] Xie, Q., Cho, H. J., Calaycay, J., Mumford, R. A. et al., Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science* 1992, *256*, 225–228.

[36] Duperrier, K., Eljaafari, A., Dezutter-Dambuyant, C., Bardin, C. et al., Distinct subsets of dendritic cells resembling dermal DCs can be generated *in vitro* from monocytes, in the presence of different serum supplements. *J. Immunol. Methods* 2000, *238*, 119–131.

[37] Chomczynski, P., Sacchi, N., Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 1987, *162*, 156–159.

[38] Shin, E. K., Kim, D. H., Lim, H., Shin, H.-K. et al., The anti-inflammatory effects of a methanolic extract from *Radix isatidis* in murine macrophages and mice. *Inflammation* 2010, *33*, 110–118.

[39] Cicero, A. F., Gaddi, A., Rice bran oil and gamma-oryzanol in the treatment of hyperlipoproteinaemias and other conditions. *Phytother. Res.* 2001, *15*, 277–289.

[40] Mullins, D. W., Burger, C. J., Elgert, K. D., Paclitaxel enhances macrophage IL-12 production in tumor-bearing hosts through nitric oxide. *J. Immunol.* 1999, *162*, 6811–6818.

[41] Tung, Y.-T., Chen, H.-L., Lai, C.-W., Shen, C.-J. et al., Curcumin reduces pulmonary tumorigenesis in vascular endothelial growth factor (VEGF)-overexpressing transgenic mice. *Mol. Nutr. Food Res.* 2011, *55*, 1036–1043.

[42] Bertout, J. A., Patel, S. A., Simon, M. C., The impact of O_2 availability on human cancer. *Nat. Rev. Cancer* 2008, *8*, 967–975.

[43] Leahy, K. M., Ornberg, R. L., Wang, Y., Zweifel, B. S. et al., Cyclooxygenase-2 inhibition by celecoxib reduces proliferation and induces apoptosis in angiogenic endothelial cells *in vivo*. *Cancer Res.* 2002, *62*, 625–631.

[44] Ding, Y. B., Shi, R. H., Tong, J. D., Li, X. Y. et al., PGE2 up-regulates vascular endothelial growth factor expression in MKN28 gastric cancer cells via epidermal growth factor receptor signaling system. *Exp. Oncol.* 2005, *27*, 108–113.

[45] Romano, M., Clária, J., Cyclooxygenase-2 and 5-lipoxygenase converging functions on cell proliferation and tumor angiogenesis: implications for cancer therapy. *FASEB J.* 2003, *17*, 1986–1995.

[46] Ye, Y.-N., Liu, E. S.-L., Shin, V. Y., Wu, W. K.-K. et al., Contributory role of 5-lipoxygenase and its association with angiogenesis in the promotion of inflammation-associated colonic tumorigenesis by cigarette smoking. *Toxicology* 2004, *203*, 179–188.

[47] Xu, Z., Hua, N., Godber, J. S., Antioxidant activity of tocopherols, tocotrienols, and γ-oryzanol components from rice bran against cholesterol oxidation accelerated by 2,2′-azobis(2-methylpropionamidine) dihydrochloride. *J. Agric. Food Chem.* 2001, *49*, 2077–2081.

