

Characterization of Potentially Chemopreventive Phenols in Extracts of Brown Rice That Inhibit the Growth of Human Breast and Colon Cancer Cells¹

E. Ann Hudson,² P. Anh Dinh, Tetsuo Kokubun, Monique S. J. Simmonds, and Andreas Gescher

Medical Research Council Toxicology Unit, University of Leicester, Leicester LE1 9HN, United Kingdom [E. A. H., P. A. D., A. G.], and Jodrell Laboratory, Royal Botanic Gardens Kew, Richmond, Surrey TW9 3AB, United Kingdom [T. K., M. S. J. S.]

Abstract

Rice is a staple diet in Asia, where the incidence of breast and colon cancer is markedly below that in the Western world. We investigated potential colon and breast tumor-suppressive properties of rice, testing the hypothesis that rice contains phenols that interfere with the proliferation or colony-forming ability of breast or colon cells. Brown rice, its white milled counterpart, and bran from brown rice were boiled and extracted with ethyl acetate. The extracts were analyzed by high pressure liquid chromatography-mass spectrometry. Eight phenols, protocatechuic acid, *p*-coumaric acid, caffeic acid, ferulic acid, sinapic acid, vanillic acid, methoxycinnamic acid, and triclin, were identified in the extracts of bran and intact brown rice. These extracts were separated into nine fractions by column chromatography. The effect of bran extract and its fractions at 100 $\mu\text{g/ml}$ on cell viability and colony-forming ability of human-derived breast and colon cell lines was assessed. Bran extract decreased numbers of viable MDA MB 468 and HBL 100 breast cells and colon-derived SW 480 and human colonic epithelial cells as judged by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. It also reduced colony formation of SW 480 colon and MDA MB 468 breast cells. Of the eight phenols identified in the brown rice bran, when applied at 50 μM , caffeic acid decreased numbers of all cell types except HBL 100. Triclin, ferulic acid, and methoxycinnamic acid interfered with cell viability in one or more cell lines. Triclin (50 μM) and the other phenols (200 μM) inhibited colony formation of SW 480 cells. Clonogenicity of MDA MB 468 cells was inhibited by caffeic acid, ferulic acid, and triclin (50 μM). Triclin was the most potent anticlonogenic of the compounds with

IC₅₀s of 16 μM in the SW 480 colon cells and 0.6 μM in the MDA MB 468 breast cells. The results suggest that: (a) brown rice and bran contain compounds with putative cancer chemopreventive properties; (b) certain phenols contained in brown rice bran, e.g., triclin, may be associated with this activity; and (c) these phenols are present at much lower levels in white than in brown rice. Thus, the consumption of rice bran or brown rice instead of milled white rice may be advantageous with respect to cancer prevention.

Introduction

Considerable research efforts are currently focused on the identification and elucidation of the mechanism of action of compounds from dietary sources that might prevent or postpone the onset of cancer. Notable examples of plant-derived substances with such properties in *in vitro* and *in vivo* experiments are genistein from soybeans (1), indole-3-carbinol from cruciferous vegetables such as Brussels sprouts and broccoli (2), curcumin from the root of *Curcuma*, resveratrol from red wine (3), and epigallocatechin gallate from tea (4). In contrast, the staple components of the human diet have received less attention as sources of cancer chemopreventive substances. This notion is exemplified by rice, *Oryza sativa*, the staple food of over half the world's population. Rice possesses special dietary importance in Asia, where the incidence of breast and colon cancer is markedly below that in the Western world (5), although the incidence of colon cancer has increased over recent years in industrialized parts of Asia such as Japan, where the diet is becoming more Westernized. Intriguingly, consumption of brown rice and other whole grain foods was inversely associated with incidence of adenomatous polyps after suitable adjustment for differences with reference to, e.g., race, body mass index, physical activity, smoking, and fat consumption (6). Witte *et al.* (6) suggest that the potential protective effects of grains on polyps might be related to the presence of dietary constituents other than fiber or antioxidants in these foods. Some investigations of potential beneficial effects of specific rice constituents in terms of prevention or amelioration of malignant disease have been published. They include reports suggesting that rice constituents counteract chemical-induced mutagenicity (7–9), tumor promotion (10), carcinogenicity (11–13), and established neoplastic growth in rodents (14–16). However, relatively little is known about which specific molecules may be responsible for these activities. Rice is mostly consumed in its milled white form, which is obtained by milling and polishing brown rice, causing the removal of the bran from the grain. Yet some of the evidence concerning the chemopreventive and antitumor properties of rice suggests that it is predominantly the bran portion of the grain that contains biologically active substances (8–15).

Received 12/28/99; revised 8/9/00; accepted 8/20/00.

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¹ This study was supported by a generous grant from the World Cancer Research Fund.

² To whom requests for reprints should be addressed, at MRC Toxicology Unit, University of Leicester, PO Box 138, Leicester LE1 9HN, United Kingdom. Phone: 44 (0) 116 2525541; Fax: 44 (0) 116 2525616; E-mail: eah5@le.ac.uk.

Clinically, chemopreventive agents that suppress tumor development are of primary interest, because they might counteract cancers even in tissues in which carcinogenic initiation has already occurred. A number of mechanisms have been proposed by which such agents suppress tumors, but they all culminate in interference with proliferation and survival of initiated cells. In the light of these considerations, we decided to study the potential colon- and breast tumor-suppressive properties of rice further. Specifically, we wanted to test the hypotheses that: (a) rice contains substances that interfere with the proliferation or colony-forming ability of breast or colon cells; and (b) there is a difference between white and brown rice in terms of antiproliferative or anticlonogenic properties. Because the extracts of brown rice bran did indeed inhibit cell growth, they were chemically analyzed and found to contain phenolic constituents. We investigated the potential role of these phenols in the mediation of the growth inhibition exerted by brown rice bran extract. Overall, the study was designed to contribute to the knowledge base that may eventually lead to the dispensation of rational advice concerning both the potential usefulness of particular diets and the prudence of recommending consumption of specific nutraceuticals isolated from the diet.

Materials and Methods

Cell Lines. Immortalized HCECs³ and human-derived colon carcinoma cell lines SW 480 and HT 29 were kindly provided by Dr. A. Pfeifer (Nestec, Ltd., Lausanne, Switzerland) and Prof. C. Paraskeva (Bristol University, Bristol, United Kingdom), respectively. These cells were routinely cultured in DMEM (containing 4.5 g/l glucose and 2 mM Glutamax) supplemented with 10% FCS. For HCEC cell culture, flasks and dishes were precoated with medium containing 10 μ l/ml Vitrogen 100, 2.5 μ g/ml human fibronectin, and 50 μ g/ml BSA prior to plating, as described previously (17). Immortalized human breast cells HBL 100 and human-derived tumor cell lines MCF 7 and MDA MB 468 were kindly provided by Prof. R. Walker (Department of Pathology, University of Leicester, Leicester, United Kingdom). HBL 100 cells were cultured routinely in DMEM (containing 1 g/liter glucose, 2 mM Glutamax, and 110 mg/l sodium pyruvate), supplemented with 10% FCS; MDA MB 468 and MCF 7 cells were cultured in RPMI 1640 supplemented with 10% FCS and 2 mM Glutamax. All cell lines tested negative for *Mycoplasma* infection and were cultured without antibiotics.

Chemicals. Cell culture medium and reagents were obtained from Life Technologies, Inc. (Paisley, Scotland), except Vitrogen 100, which was purchased from Collagen Corp. (Palo Alto, CA), and human fibronectin, which was bought from Sigma Chemical Co. (Poole, United Kingdom). To test whether the phenols that we identified in the brown rice fractions (see below) have cell growth-modulatory properties themselves and may therefore contribute to the activity of the fractions investigated, we purchased pure caffeic acid, ferulic acid, *p*-coumaric acid, methoxycinnamic acid, sinapic acid, vanillic acid, and protocatechuic acid to be able to use them in amounts sufficient for assays in cells *in vitro*. These phenols, genistein and two reagents used in the MTS assay, MTS and phenazine

methosulfate, were obtained from Sigma. Brown and white rice varieties were purchased from a local supermarket.

Preparation of Rice Extract. Brown or white rice (1.5 kg) was cooked in water (2.7 liter) for 30 min. The rice was cooled, mixed with water, and successively and exhaustively extracted with diethyl ether, ethyl acetate, and methanol (twice at 1.5 liter each). Rice bran was prepared by milling brown rice with a food processor prior to cooking. Bran powder was separated from the endosperm with a colander (mesh size, \sim 1 mm). The bran was boiled in water and extracted as described above. Extracts were concentrated *in vacuo*, and the residues were analyzed by HPLC using a Waters system 600 (with 717 autosampler) linked to a LiChrosphere RP-18 C₁₈ column (250 \times 4.6 mm; 5 μ m) and a Waters 996 photodiode array detector. For detection, wavelengths were scanned between 200 and 400 nm. The mobile phase consisted of 2% acetic acid in water (eluant A), and methanol:acetic acid:water (18:1:1; eluant B). A linear gradient was run from 40 to 100% B over 20 min (flow rate, 1 ml/min), followed by 100% B for 5 min, before the column was reconditioned prior to injection of the next sample.

Chromatographic Separation and Analysis of Fractions.

The ethyl acetate extracts of brown rice or brown rice bran were chromatographed on an Amberlite XAD-2 styrene-divinylbenzene polymer column (diameter 5 \times 32 cm) and eluted with increasing amounts of methanol (0–100%) in water. Ten fractions (250 ml each) were collected and analyzed by HPLC using the eluant as described above. The first fraction did not contain any detectable peaks and was not tested further. The subsequent nine fractions afforded HPLC peaks. They will be referred to as “fractions 1–9.” Fractions were eluted with 10% methanol:water (fraction 1) to 100% methanol (fraction 9). Weights of fractions 1–9 were 1.42, 0.11, 0.14, 0.88, 0.39, 0.40, 0.39, 0.08, and 0.06 g, respectively. Tentative assignment of chromatographic peaks in the fractions to compounds was performed on the basis of chromatographic retention times, UV absorption spectra (using data in a library of flavonoids at the Jodrell Laboratory providing retention times and UV absorption maxima), and by cochromatography with authentic reference compounds. Unambiguous identification was achieved by quadrupole ion trap mass spectrometry (Finnigan LCQ instrument) in negative atmospheric pressure chemical ionization mode, linked to the HPLC-UV detector (set at 260 nm). The HPLC eluant used was 25–100% methanol in water for 20 min; the flow rate was 1 ml/min. The [M-H]⁻ ions at the apposite retention times were subjected to tandem mass spectrometric analysis in which the appropriate fragments corresponding to [M-H-CH₃]⁻, [M-H-OCH₃]⁻, and/or [M-H-CO₂]⁻ were observed. It has to be pointed out that the HPLC retention times (as shown in Table 1) of individual phenols do not reflect accurately the order in which the phenols eluted from the XAD-2 column as constituents of the fractions, reflecting chemical differences between the columns used for fractionation and for HPLC analysis.

Isolation of Tricin. Tricin was found in fractions 8 and 9 of the ethyl acetate extract of brown rice. Because triclin is not readily available commercially, we had to isolate it in quantities sufficient for evaluation in cell assays. Tricin occurs at high concentrations in the leaves of Gramineae (18); therefore, it was isolated from powdered dried leaves of *Festuca* spp. in the following way. Leaves were extracted with 80% aqueous methanol, and the extract was concentrated *in vacuo* at 50°C. The concentrate was dissolved in a small amount of methanol and washed with hexane; the methanol layer was then reconcentrated and extracted with chloroform. The chloroform extract

³ The abbreviations used are: HCEC, human colon epithelial cell; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance.

Table 1 Phenols identified in rice bran fractions and their abundance in the fractions

	Approximate retention time (min) ^a	Fraction	% ^b
Protocatechuic acid	2.7–3.3	1	0.07
Caffeic acid	7.5	1	0.03
<i>p</i> -Coumaric acid	12.0	1	0.41
		2	0.02
Vanillic acid	10.2	2	0.01
Methoxycinnamic acid	17.5	3	<0.01
		4	<0.01
Ferulic acid	12.5	4	0.31
		5	0.59
		6	<0.01
Sinapic acid	13.8	6	<0.01
		7	0.38
Tricin	19.7	8	0.25
		9	0.2

^a Retention times (for peak apex) are consistent with those shown in Fig. 1.

^b Approximate percentage values with respect to the weight of the fractions obtained by integration of HPLC peak areas after baseline correction and comparison with authentic standards. Values are the mean of duplicate determinations from one experiment representative of two.

was chromatographed on a silica gel column (diameter, 4 × 17 cm) packed in chloroform and eluted with a chloroform:ethyl acetate mixture with increasing proportions of ethyl acetate. Fractions containing triclin (detected by polyamide TLC, Rf 0.24, developed in methanol:acetic acid:water 18:1:1) were pooled and rechromatographed on a polyamide column (diameter, 4 × 18 cm) that was eluted with methanol:acetic acid (19:1). The eluate was subjected to chromatography on a Sephadex LH-20 column (diameter, 2 × 31 cm) with elution by methanol, yielding 14.9 mg of pure triclin from ~450 g of dried plant material. Tricin was recrystallized from methanol, and its structure was confirmed by means of HPLC retention time, UV absorption (λ_{\max} in methanol 352, 266, and, 243 nm), ¹H NMR, and mass spectra. In the ¹H NMR spectrum, triclin (in DMSO-*d*₆, 500 MHz) afforded the following diagnostic resonances (δ in ppm): 3.88 (6H, H-3' and -5'), 6.21 (1H, H-6), 6.56 (1H, H-8), 6.96 (1H, H-3), 7.32 (2H, H-2' and -6'), 9.24 (1H, 4'-OH), 10.73 (1H, 7-OH), and 12.94 (1H, 5-OH). It yielded the following diagnostic mass units (*m/z* with abundance expressed as % in parentheses): 329 (100), 119 (23), 141 (8), 223 (4), 299 (4), and 157 (3).

Assessment of Cell Proliferation and Clonogenicity. Stock solutions of ethyl acetate extract of brown rice, its chromatographic fractions or of phenols detected in the fractions were taken up in DMSO and diluted in HEPES buffer (1 M; pH 7.4), such that the final concentrations of DMSO and HEPES in the cell incubations did not exceed 0.02% and 10 mM, respectively, concentrations that did not alter cell growth or clonogenicity. Effects of the fractions of the ethyl acetate extract and of the phenols on the proliferation and clonogenicity of breast and colon cells were investigated both in high density cultures using the MTS assay and under low cell density conditions by clonogenic assay. Extracts were applied at a concentration of 100 μ g/ml.

In the MTS assay, six cell types were used, three human breast-derived lines, immortalized HBL 100, tumorigenic MCF 7 and MDA MB 468 cells, and three human colon-derived cell types, immortalized HCEC and tumorigenic SW 480 and HT 29 cells. Cells (750 per well in 0.2 ml of medium) were seeded in 96-well plates and cultured for 4 h prior to treatment. Extract

and fractions were added to cellular incubates at 100 μ g/ml. Solvent control incubates contained the appropriate mixture of buffer and DMSO. Cells were exposed to test substances for 7 days, after which mitochondrial function of the cells was determined by the MTS assay as described by Malich *et al.* (19) using 2 h as the time period of incubation with MTS and an automated plate reader set at a wavelength of 490 nm (Lab-systems iEMS Reader MF). In this assay, the number of viable cells is reflected by extent of reduction of MTS by intact mitochondria to generate a dye that absorbs at 490 nm.

The effect of test substances on clonogenicity was determined in SW 480 cells (300 per well in 2 ml) and MDA MB 468 cells (200 per well in 2 ml) in six-well plates. Cells were allowed to attach for 4 h prior to exposure to test substances. MDA MB 468 cell cultures contained 50% conditioned medium, as described previously (20). Phenols were applied either at 50 μ M (MDA MB 468 cells) or 200 μ M (SW 480 cells), except triclin, which was applied at 50 μ M throughout. After an addition of test substance, cells were cultured for 10 days before the colony number was assessed. Cells were washed with PBS, fixed in 100% ethanol, and stained with 25% Giemsa blue solution. Colonies consisting of >10 cells were counted. Colony-forming efficiency of SW 480 and MDA MB 468 cells was 59 ± 16.9% and 58 ± 9.5%, respectively. Genistein (30 μ M) was included as a positive control in both assay types.

IC₅₀s (mean ± SD; see Table 4) were determined for individual phenols from at least three independent clonogenic assay experiments in which the following concentrations were used: caffeic acid at 1, 5, 10, 25, and 50 μ M; protocatechuic acid at 50, 100, 200, and 300 μ M in the MDA MB 468 cells and 10, 20, 40, and 50 μ M in the SW 480 cells; triclin at 0.05, 0.1, 0.5, 1.0, and 10 μ M in the MDA MB 468 and 10, 20, 25, 40, and 50 μ M in the SW 480 cells. IC₅₀s for each data set were derived from graphs in which colony number, expressed as percentage of control incubate, was plotted against phenol concentration.

Statistical Analysis. Results were analyzed by the ANOVA General Linear Model (21) using Minitab software (Minitab Inc.), followed by Fisher's least significant difference posthoc test (22). In Figs. 3 and 4, cell colony numbers are presented as predicted means as determined by the ANOVA model; the SD of the set is shown on the control bar only. For Tables 2 and 3, absorbance values obtained in the MTS assay were analyzed as above, but results are presented as percentages of control cell cultures (omitting extracts or phenols).

Results

Analysis of Rice Extracts. The ether extract of either rice variety consisted of colorless and waxy material, which solidified upon cooling to 4°C. The ethyl acetate extract of brown rice contained brown-colored material, which we assumed to contain various phenols because HPLC analysis with photodiode array detection separated compounds with absorption maxima between 250 and 360 nm (Fig. 1B). White rice afforded an ethyl acetate extract of similar weight to that of the brown variety, but it contained only traces of phenols (Fig. 1A). Further extraction of either rice variety with methanol and subsequent concentration yielded gummy materials, characteristic of oligo- and polysaccharides. In the case of brown rice, the methanol extract contained also trace amounts of phenols.

Because our interest was focused on phenolic rice components, HPLC profiles of the ethyl acetate extracts prepared from whole intact brown rice, *i.e.*, with bran, were compared with those from bran obtained by milling brown rice. The profiles were very similar (Fig. 1, B and C), which suggests that

Table 2 Effect of ethyl acetate extract of rice bran and chromatographic fractions 1–9 on proliferation of breast- and colon-derived cells as assessed by the MTS assay

	Breast cell lines			Colon cell lines		
	MDA MB468	MCF7	HBL100	HT29	SW480	HCEC
Extract	27.3 ^{a,b}	90.4	22.0 ^b	76.8	37.6 ^b	36.4 ^b
Fraction 1	39.4 ^b	34.6 ^b	81.7 ^b	101.4	80.0 ^b	85.2
Fraction 2	90.9	93.3	88.1	114.4	125.9 ^b	120.5 ^b
Fraction 3	101.5	95.2	85.3 ^b	123.8 ^b	122.3 ^b	105.7
Fraction 4	112.1	109.6	90.8	134.8 ^b	118.8 ^b	129.5 ^b
Fraction 5	110.6	104.8	107.3	123.2	138.8 ^b	125.0 ^b
Fraction 6	107.6	99.0	96.3	117.4	144.7 ^b	129.5 ^b
Fraction 7	98.5	94.2	94.5	143.5 ^b	128.2 ^b	121.6 ^b
Fraction 8	59.1 ^b	27.9 ^b	57.8 ^b	71.0 ^b	56.5 ^b	62.5 ^b
Fraction 9	48.5 ^b	32.7 ^b	51.4 ^b	71.0 ^b	58.8 ^b	56.8 ^b
Genistein	47.0 ^b	60.6 ^b	69.7 ^b	66.7 ^b	65.9 ^b	72.7 ^b

^a Results (mean of three to five observations) are presented as percentages of viable cell numbers in control incubations (omitting extracts or fractions) as reflected by the respective absorbance units (490 nm). Extract and fractions were applied at 100 µg/ml; 30 µM genistein served as a positive control. The mean and SD of the absorbance units of each set relating to a particular cell line were calculated by the ANOVA General Linear Model. Mean absorbances in control incubations were 0.66, 1.04, 1.09, 0.69, 0.85, and 0.88 in MDA MB468, MCF7, HBL 100, HT29, SW480, and HCEC cells, respectively; the SD of each set varied between 0.11 (MDA MB468 cells) and 0.16 (MCF7 cells) absorbance units. For details of cell culture and experimental design, see "Materials and Methods."

^b Absorbance values are significantly different from those for control cells ($P < 0.05$).

Table 3 Effect of eight phenolic constituents of brown rice on proliferation of breast- and colon-derived cells as assessed by the MTS assay

	Breast cell lines			Colon cell lines		
	MDA MB468	MCF7	HBL100	HT29	SW480	HCEC
Protocatechuic acid	97.0 ^a	107.1	131.8 ^b	102.1	110.1	95.9
Caffeic acid	83.6 ^b	72.9 ^b	96.6	71.9 ^b	59.6 ^b	79.4 ^b
<i>p</i> -Coumaric acid	100.0	109.3	100.0	86.5	111.2	86.6
Vanillic acid	94.0	92.1	117.1 ^b	81.3	102.2	85.6
Methoxycinnamic acid	94.0	109.3	93.2	64.6 ^b	113.3	80.4 ^b
Ferulic acid	97.0	89.3	110.2	95.8	105.6	75.3 ^b
Sinapic acid	104.4	102.9	121.6 ^b	106.3	117.3 ^b	88.7
Tricin	65.7 ^b	104.3	77.3 ^b	55.2 ^b	104.5	84.5
Genistein	62.7 ^b	60.0 ^b	72.7 ^b	58.3 ^b	77.5 ^b	80.4 ^b

^a Results (mean of three to five observations) are presented as percentages of viable cell numbers in control incubations (omitting phenols) as reflected by the respective absorbance units (490 nm). Phenols were applied at 50 µM; 30 µM genistein served as a positive control. The mean and SD of the absorbance units of each data set relating to a particular cell line were calculated by the ANOVA General Linear Model. Mean absorbances in control incubations were 0.67, 1.40, 0.88, 0.96, 0.89, and 0.97 in MDA MB468, MCF7, HBL 100, HT29, SW480, and HCEC cells, respectively; the SD of each set varied between 0.08 (MDA MB468 cells) and 0.26 (MCF7 cells) absorbance units. For details of cell culture and experimental design, see "Materials and Methods."

^b Values are significantly different from those for control cells ($P < 0.05$).

the bran layer rather than the rice kernel is the predominant location of phenolic constituents of rice. The ethyl acetate extract of brown rice or bran was subjected to column chromatographic fractionation on Amberlite XAD-2 using methanol:water with increasing concentrations of the former as eluant. This procedure furnished nine phenol-enriched fractions that were further analyzed by HPLC. Characterization of compounds associated with selected peaks in the chromatograms of the fractions was performed, and the structures of the identified compounds are shown in Fig. 2. Precise quantitation of the amounts of each compound in the fractions was not carried out, but approximate percentage values, with respect to the weight of the fractions, were computed by integration of the chromatographic peak areas (Table 1). On the basis of the weights of the fractions collected and the tentative assumption that the recovery of constituents from the chromatography column was 100%, computation of the approximate content of these substances in brown rice yields the following estimated values, expressed as µg per 100 g dry weight of rice: 66 µg of protocatechuic acid, 390 µg of *p*-coumaric acid, 28 µg of caffeic acid, 334 µg of ferulic acid, 105 µg of sinapic acid, and 7 µg of triclin. It is pertinent to point out that because the rice

was boiled before extraction, the compounds thus far identified are probably products of hydrolysis of their glycosidic precursors. The fractions also contained other compounds at low levels, including flavonoids, which we have not yet identified.

Effects of Rice Extract and Fractions on Number of Viable Cells and Clonogenicity. The rice bran extract reduced number of viable cells as judged by the MTS assay, but cells differed in their susceptibility toward the cytostatic/cytotoxic activity of the extract (Table 2). Although it caused a significant decrease in numbers of MDA MB 468, HBL 100, SW 480, and HCECs, proliferation of MCF 7 or HT 29 cells was not affected. Four of the nine fractions of the brown rice extract decreased cell numbers in some but not all cell types (Table 2); fraction 1 reduced numbers of the three breast and SW 480 colon cells, fraction 3 caused a significant decrease in HBL 100 cells, and the most lipophilic fractions, 8 and 9, attenuated consistently the number of viable cells of all six types, thus resembling genistein, which served as positive control. In contrast, fractions 2–7 increased, rather than decreased, the numbers of colon cells to a moderate extent over control cell numbers.

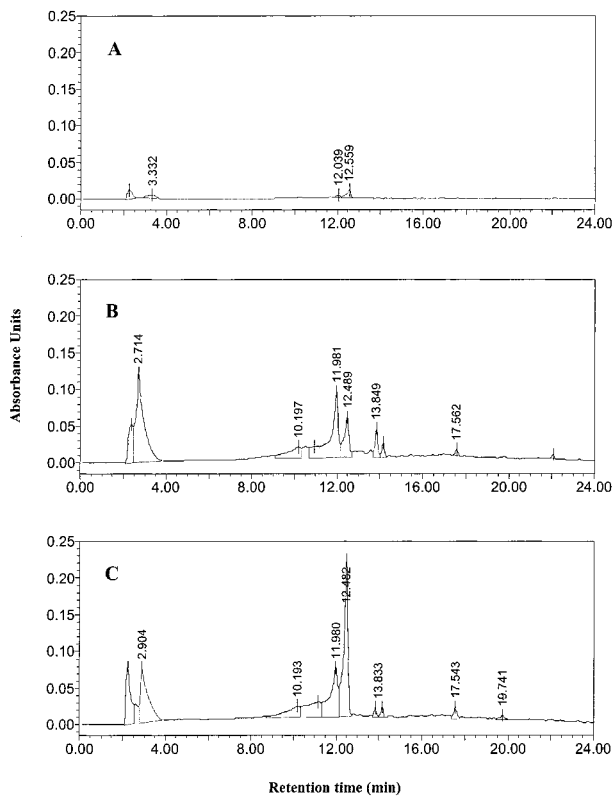


Fig. 1. High-pressure liquid chromatograms of ethyl acetate extracts of white rice (A), brown rice (B), and brown rice bran (C). Extracts were dissolved in methanol:acetic acid:water (36:3:61), and an aliquot equivalent to 100 μg of material was injected. UV detection was at 320 nm. The following peaks were identified by co-chromatography and mass spectrometry after fractionation of extracts (retention times in minutes are given in parentheses): protocatechuic acid (2.7–3.3), vanillic acid (10.2), *p*-coumaric acid (12.0), ferulic acid (12.5), sinapic acid (13.8), methoxycinnamic acid (17.5), and triclin (19.7). Caffeic acid was below the detection limit in the extract, but it was identified in fraction 1. The chromatograms shown are representative of at least three per material source. For details on rice extraction, HPLC conditions and peak identification, see “Materials and Methods.”

In the clonogenic assay, the unfractionated brown rice bran extract decreased colony formation in both cell types. Its effect was particularly potent against MDA MB 468 cells, the colony-forming ability of which was reduced by 90%, comparable with the efficacy of genistein (Fig. 3). The white rice extract did not affect clonogenicity (result not shown). The clonogenicity of SW 480 cells was inhibited by all nine rice fractions, with fractions 4, 5, and 8 being most potent (Fig. 3A). Formation of MDA MB 468 cell colonies was decreased by fractions 4, 5, 8, and 9 of the rice bran extract (Fig. 3B).

Effects of Phenols on Number of Viable Cells and Clonogenicity. The eight phenols that had been identified as constituents of the ethyl acetate extract of brown rice and rice bran (for structures, see Fig. 2) were characterized in terms of their antiproliferative and anticlonogenic potential in comparison with genistein. They were applied at 50 μM (MTS assay) and 50 and 200 μM (clonogenic assay) and compared with genistein (30 μM). Using the MTS assay, caffeic acid caused a significant decrease in numbers of viable cells in all six lines except HBL 100 (Table 3). Tricin was active in MDA MB 468, HBL 100, and HT 29 cells but not in MCF 7, HCEC, and SW 480 cells. Ferulic acid decreased the number of viable HCECs, and me-

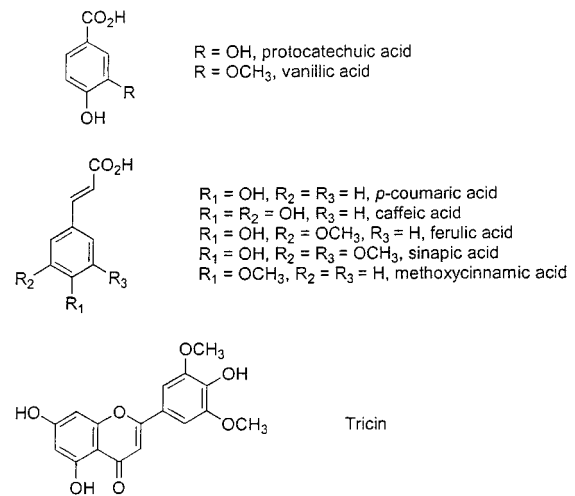


Fig. 2. Chemical structures of phenols investigated in this study.

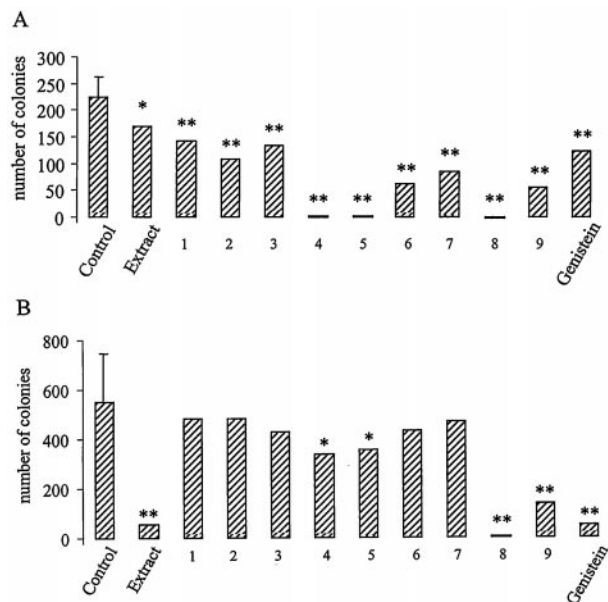


Fig. 3. Effect of ethyl acetate extract (Extract) of rice bran and nine column chromatographic fractions on clonogenicity of SW 480 (A) and MDA MB 468 (B) cells. *Bottom*, fraction numbers. Cells were exposed for 10 days to extract or fractions (100 $\mu\text{g}/\text{ml}$) as described in “Materials and Methods.” Genistein (30 μM) served as positive control. Results are the predicted means (SW480, $n = 7-9$ observations; MDA MB 468, $n = 8-12$ observations) determined by the ANOVA General Linear Model. The SD (bars), which is representative for the whole data set, is only shown for the control cell colony number. Values that are significantly different compared with controls are indicated by * ($P < 0.05$) or ** ($P < 0.001$).

thoxycinnamic acid reduced numbers of both HCEC and HT 29 cells.

In low-density cultures of SW 480 cells, caffeic acid, ferulic acid, *p*-coumaric acid, methoxycinnamic acid, sinapic acid, vanillic acid, protocatechuic acid (all at 200 μM), and triclin (50 μM) interfered with colony formation (Fig. 4A). The clonogenicity of MDA MB 468 cells was inhibited in response to caffeic acid, ferulic acid, and triclin (50 μM ; Fig. 4B). Table 4 shows the IC_{50} s for inhibition of clonogenicity for the three

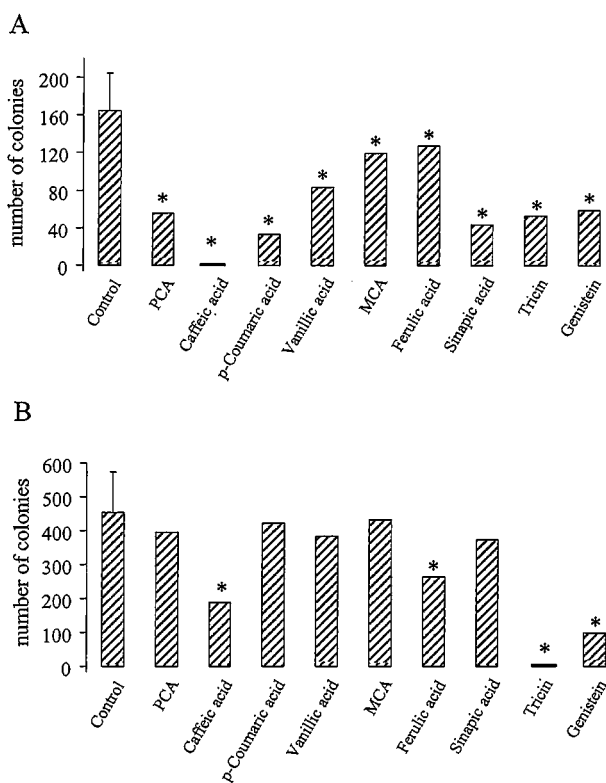


Fig. 4. Effect of phenols identified in rice bran on clonogenicity of SW 480 (A) and MDA MB 468 (B) cells. MCA, methoxycinnamic acid; PCA, protocatechuic acid. Cells were exposed for 10 days to the phenols at 200 μM (A) or 50 μM (B), except triclin, which was used at 50 μM in either, as described in "Materials and Methods." Genistein (30 μM) served as positive control. Results are the predicted means (SW480, $n = 6$ –18 observations; MDA MB 468, $n = 9$ observations) determined by the ANOVA General Linear Model. The SD (bars), which is representative for the whole data set, is only shown for the control cell colony number. *, values are significantly different compared with control cells ($P < 0.05$).

most anticlonogenic compounds, caffeic acid, protocatechuic acid, and triclin. Of these phenols, triclin was the most potent with IC₅₀s of 16 μM in the colon and 0.6 μM in the breast cell line.

Discussion

The results outlined above support the notion that brown rice, in contrast to its white counterpart, contains agents that possess cytostatic/cytotoxic and anticlonogenic properties in human-derived immortalized and tumorigenic breast and colon cancer cells. This investigation focused on phenolic constituents that were extractable by ethyl acetate from brown rice, but present only in trace amounts, or at amounts below the limit of detection in white rice. These compounds appear to be predominantly located in rice bran, which forms a part of staple foodstuff consumed commonly in East Asian countries. It is tantalizing to speculate as to the nature of the active principles that may have caused, or contributed to, the growth modulation exerted by the rice bran extract and its fractions. The characterization of eight of the compounds present in the extract allows some tentative inferences to be made as to potential active constituents involved in the growth effects of individual fractions. It is conceivable that triclin caused, or contributed to,

the decrease in cell numbers and clonogenicity elicited by fractions 8 and 9. This notion is borne out by a calculation based on the finding that triclin constituted $\sim 0.25\%$ of fraction 8 (see Table 1). Therefore, the concentration of triclin as constituent of fraction 8 was $\sim 0.7 \mu\text{M}$ when the fraction was applied at 100 $\mu\text{g/ml}$ to the cellular incubation. This value is indeed close to the IC₅₀ of triclin, which was 0.6 μM when it was added as pure compound to the MDA MB 468 cell incubation mixture in the clonogenic assay (see Table 4). Furthermore, ferulic acid may have contributed to the anticlonogenicity of fractions 4 and 5 in both breast and colon cells, and sinapic acid may have been involved with the inhibiting effect of fraction 7 on the clonogenicity of SW 480 cells. The interpretation of these findings is confounded by the fact that fractions 3–7 elevated, rather than decreased, numbers of viable colon cells (Table 2). This result suggests that the rice bran extract contains both growth-inhibitory and -stimulatory constituents, intimating the possibility that the potentially beneficial effect of some of the bran components may be masked, or counteracted, by the opposite activity of others.

There are some incongruities when the results presented above in the clonogenic assay are compared with those in the MTS assay. For example, fractions 4 and 5 interfered with clonogenicity (Fig. 3) but did not reduce the numbers of viable cells as measured by the MTS assay (Table 2). Likewise, protocatechuic acid at 50 μM was unable to reduce numbers of viable cells of any of the six types (Table 3), but its IC₅₀ with respect to anticlonogenicity in SW480 cells was 21.6 μM (Table 4). These discrepancies are consistent with the notion that the growth behavior of cells cultured at very low density in the clonogenic assay is more susceptible to cytotoxic and cytostatic stimuli than that of more densely seeded cells. An illustrative example of this phenomenon is the susceptibility of lung carcinoma cells toward the growth effects of the marine compounds bistratene A (23) and bryostatin 1 (24). In contrast, it is more difficult to explain how fraction 1 reduced the number of viable MDA MB468 cells (Table 2) without affecting their clonogenicity (Fig. 3). Furthermore, there were inconsistencies concerning the effects of the fractions on the one side and those of their constituent phenols on the other. For example, the potent anticlonogenic activity of fractions 4 and 5 against SW480 cells (Fig. 3) contrasts with only weak efficacy of methoxycinnamic acid and ferulic acid (Fig. 4), two phenols identified as constituents of these fractions. Fractions 8 and 9, which contain triclin, inhibited the viability of SW 480 cells in the MTS assay (Table 2), but triclin (50 μM) on its own failed to affect it (Table 3). This lack of correlation between activities of fractions and constituent phenols suggests that agents other than those characterized above are likely to contribute to the observed effects of the fractions, and it reinforces the conclusion that the bran extract contains phytochemicals with growth-inhibiting and -promoting properties.

One potential role of *in vitro* experiments of the type described here is to furnish conjectures as to the potential tissue specificity of the agents under study. The results outlined above were obtained in three breast and three colon cell types and allow tentative suggestions as to potential differences in sensitivity between these two tissues. The rice bran extract interfered more potently with the colony formation of the breast than of the colon cells (Fig. 3), whereas the individual fractions were overall more potently anticlonogenic in the colon cell lines. Nevertheless, there was no such tissue specificity of extract or fractions in the MTS assay. In the clonogenic assay, all eight phenols investigated exerted activity against the colon cells, whereas only three, caffeic acid, ferulic acid, and triclin, affected the breast cells, albeit at lower concentrations than those used in the SW 480 cell assay. Protocatechuic acid and triclin showed the most striking indication of potential tissue specificity in terms of anticlonogenicity (Table 4). The colon cancer cells were eight times more sensitive to protocatechuic acid than the breast cells, whereas the breast cancer cells were 25 times more sensitive toward triclin than the colon cells. In the MTS assay, ferulic and methoxycinnamic acids reduced the numbers of viable colon cells but not breast cells. Specificity was not observed when effects of rice fractions and constituent phenols on the tumorigenic cell types were analyzed in comparison with those on the immortalized ones.

Of the eight phenols investigated here, the flavone triclin is arguably the most potent anticlonogenic agent in cells of either breast or colon tissue origin. Triclin occurs predominantly as 5-glucoside in the Gramineae (18), the plant family to which wheat, barley, maize and other grain crops apart from rice belong. It was first isolated from wheat straw almost 70 years ago (25). Although a plethora of information exists on biological properties pertinent to anticarcinogenesis for flavonoids and isoflavonoids such as genistein and quercetin, there is to our knowledge only one report with reference to triclin, which suggests that triclin isolated from stems of the plant *Wikstroemia indica* (Thymelaeaceae) possesses antineoplastic activity against the P388 murine leukemia model (26). The potency of triclin documented here renders it an attractive candidate for elucidation of its cancer chemopreventive efficacy *in vivo*, especially with respect to breast cancer, and of its biochemical mechanisms, which are unknown.

The results presented here on cell growth modulation by rice constituents have to be interpreted in the light of the beneficial effects of rice that have been published before. Rice bran constituents possess antimutagenic (7–9), antitumor-promoting (10), and cytotoxic (14–16) properties. Among the bioactive constituents are the triterpene cycloartenol ferulate, which counteracted the tumor-promoting activity of phorbol ester in the skin of 7,12-dimethylbenz[*a*]anthracene-initiated mice (10), (10*E*,12*Z*)-9-hydroxy-10,12-octadecadienoic acid (14), and hydrolyzed anthocyanin (16), which were cytotoxic against murine P388 leukemia and HCT-15 cells, respectively, *in vitro*, and a dextran-like α -glucan, which exhibited antitumor activity against Meth-A fibrosarcoma and Lewis lung carcinoma grown in mice *in vivo* (15). Rice bran polysaccharides inhibited gastrointestinal carcinogenicity induced by *N*-ethyl-*N'*-nitrosoguanidine (13). Arabinoxylan hemicellulose, an indigestible rice bran fiber macromolecule, decreased exposure of rodents to carcinogenic xenobiotics by facilitating their intestinal excretion (11), and hemicellulose inhibited 1,2-dimethylhydrazine-induced intestinal carcinogenesis in rats (12). Among other agents that have been found in rice and may conceivably confer cancer chemopreventive activity on this staple diet are β -sitosterol (27, 28), phytic acid (29), and toco-

triols and tocopherols (30). The results described here add eight phenols to the list of rice constituents with potential cancer chemopreventive activity, but it is important to note that although these agents are discussed here as constituents of rice, they occur ubiquitously in the plant kingdom.

Are there any conclusions of the results presented above that might be exploited for dietary advice? The results support the hypothesis that brown rice contains substances with putative cancer preventive properties, and that these substances are present at much lower levels in the white variety. Furthermore, the phenols contained in rice bran described here may be associated with cancer chemopreventive activity of brown rice. Thus, the consumption of rice bran or brown rice instead of milled white rice may be advantageous with respect to cancer prevention. This conclusion has to be interpreted in the light of the fact that worldwide the consumption of brown rice is probably minuscule as compared with that of the white variety. The conclusion is consistent with the general realization that consumption of whole grain foodstuffs, including whole-grain bread, pasta, and rice is more beneficial to the maintenance of human health than that of their refined products. In an overview of the epidemiological evidence, whole-grain intake was associated with an odds ratio of <1, suggesting protection in 11 of 12 studies, and whole-grain diets have been suspected to be of particular benefit in the prevention of colorectal, gastric, and endometrial cancers and coronary heart disease (31). The conclusion also enforces the dietary recommendations proffered by the World Cancer Research Fund and American Institute for Cancer Research, which advocate diets rich in whole-grain and minimally refined cereals (32).

There are several reasons that underline the tentative nature of the conclusions drawn from our results: (a) it is possible that it is the intact rice matrix containing the sum of nutrient and nonnutrient constituents that is required to confer optimal chemopreventive activity on brown rice, and this may be diminished or lost on investigation of fractions or constituents in isolation; (b) the complete spectrum of growth-inhibitory constituents contained in brown rice was not elucidated by us. It is therefore conceivable that agents other than those identified and discussed here contribute much more potently to the antiproliferative and anticlonogenic activity of the rice extract than the phenols which were characterized here, and furthermore, there was evidence of growth-promoting constituents in rice bran fractions, but we did not attempt their chemical identification; (c) it is important to relate, at least putatively, the findings described here to the *in vivo* situation in which rice is ingested as part of the diet. It is exceedingly difficult to estimate the amount of the phenols that has to be ingested to precipitate growth-arresting effects *in vivo*. On the basis of our gross calculation, it is conceivable that in total ~1 mg of the free phenols investigated here is ingested with 100 g of brown rice. Even assuming satisfactory bioavailability, the amounts of the individual growth-modulating phenols, such as for example 28 μ g of caffeic acid and 7 μ g of triclin, present in the body after a meal containing 100 g of brown rice, are unlikely to be sufficient to furnish systemic levels that elicit effects equivalent to those observed here under highly defined artificial cell culture conditions. These levels are also markedly below those of bioactive rice bran constituents that have been identified previously. These levels are based on a computation taking into account the amounts that have been isolated and the fact that rice bran constitutes ~15% of the whole grain. For example, 100 g of brown rice yielded 2.1 mg cycloartenol ferulate (10) and 1.4 mg of (10*E*,12*Z*)-9-hydroxy-10,12-octadecadienoic acid (14). Nevertheless, it is conceivable that the phenols char-

acterized here, when present together with each other and with other rice constituents, affect biological targets synergistically and thus exert much higher growth-modulating and chemopreventive efficacy than those observed on assessment of individual compounds. This consideration may be especially pertinent under conditions of daily ingestion of these compounds with rice as part of the diet over a long period of time.

Finally, this type of study can give hints as to potentially useful nutraceuticals isolated from foodstuffs that may prevent cancer. Our results suggest that among the phenols contained in brown rice, triclin might be a prime candidate nutraceutical with colon or particularly breast cancer chemopreventive activity.

Acknowledgments

We thank Nigel Veitch for the NMR analysis, Geoffrey Kite for mass spectral data, and Renée Grayer for helpful discussions.

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