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Cancer Chemopreventive Activity Mediated by 4′-Bromoflavone, a Potent Inducer of Phase II Detoxification Enzymes[1]

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ABSTRACT

Induction of phase II enzymes is an important mechanism of chemoprevention. In our search for novel cancer chemopreventive agents, 4′-bromoflavone (4′BF) was found to significantly induce quinone reductase (QR) activity in cultured murine hepatoma 1c1c7 cells (concentration to double activity: 10 nM) and effectively induce the α- and μ-isoforms of glutathione *S*-transferase in cultured H4IIE rat hepatoma cells with no observed toxicity. In short-term dietary studies, 4′BF was also shown to increase QR activity and glutathione levels in rat liver, mammary gland, colon, stomach, and lung in a dose-dependent manner. Induction mediated by 4′BF was bifunctional (induction of both phase I and phase II enzymes) and regulated at the transcriptional level, as revealed by transient transfection studies with plasmid constructs (pDTD-1097CAT, XRE-CAT, and ARE-CAT) and reverse transcription-PCR-based analysis of QR mRNA. In studies conducted with female Sprague Dawley rats, the effects of 4′BF on the relative induction levels of phase I and phase II enzyme activities were investigated in liver and mammary gland. Treatment with 4′BF and 7,12-dimethylbenz[*a*]anthracene (DMBA) or 4′BF alone did not significantly alter DMBA-induced cytochrome P4501A1 activity (phase I enzyme), but it significantly increased QR activity (phase II enzyme), compared with the DMBA treatment group. In addition, 4′BF was found to be a potent inhibitor of cytochrome P4501A1-mediated ethoxyresorufin-*O*-deethylase activity, with an IC_{50} of 0.86 μM. Furthermore, in studies conducted with cultured HepG2 or MCF-7 cells, 4′BF significantly reduced the covalent binding of metabolically activated benzo[*a*]pyrene to cellular DNA. On the basis of these results, a full-term cancer chemoprevention study was conducted with DMBA-treated female Sprague Dawley rats. Dietary administration of 4′BF (2000 and 4000 mg per kg of diet, from 1 week before to 1 week after DMBA) significantly inhibited the incidence and multiplicity of mammary tumors and greatly increased tumor latency. In summary, 4′BF can be viewed as a relatively simple, readily available, inexpensive compound that is a highly effective cancer chemopreventive agent. The full mechanism of action remains to be defined, but enhancement of detoxification pathways appears to be important.

INTRODUCTION

Cancer chemoprevention involves prevention, delay, or reversal of the process of carcinogenesis through ingestion of dietary or pharmaceutical agents (1, 2). A large number of potential chemopreventive agents are known (3), some of which have proven effective in clinical trials (2). These agents may function by a variety of mechanisms, directed at all major stages of carcinogenesis (4). One mechanism of particular note involves the induction of phase II detoxification enzymes, such as QR[4] [NADP(H):quinone oxidoreductase] and GST (5). This type of response is associated with the metabolic detoxification of carcinogens (4, 5), but certain events associated with later stages of the carcinogenic process may also be inhibited (6). In laboratory animals and cell culture systems, several chemopreventive agents have been identified solely on the basis of their ability to induce phase II enzymes (7–9). Similarly, in a program directed toward the discovery of novel chemopreventive agents, we have used cultured Hepa 1c1c7 cells to evaluate the potential of test agents to enhance the activity of QR. This has led to the identification of various active agents, including brassinin (10), withanolides (11), and sulforamate (12). In addition, as exemplified by work conducted with *Tephrosia purpurea*, some novel flavonoids have been found to serve as potent phase II enzyme inducers (13). Flavonoids are a diverse group of compounds that are widely distributed in the plant kingdom. These agents and related synthetic analogues mediate a broad spectrum of biological responses, such as antiallergic (14), anti-inflammatory (15), antioxidant (16), gastroprotective (17), antiviral (18), topoisomerase II-inhibitory (19), protein kinase C-inhibitory (20), antimutagenic (21), and anticarcinogenic (22) activities. Previous work has suggested modulation of enzyme activities associated with carcinogen activation and detoxification may be responsible for cancer chemoprotective activity (23).

On the basis of this information and the observation that various naturally occurring flavonoids induced QR activity with Hepa 1c1c7 cells, a systematic investigation was undertaken to identify agents in this structural class capable of mediating potent stimulatory activity. Over 80 natural or synthetic flavonoids were evaluated, and it was found that a simple derivative of flavone itself, 4′BF, induced QR activity with great potency. Here, we report the synthesis of this compound, possible mechanisms of enzyme induction, and chemopreventive potential with *in vitro* and *in vivo* model systems.

MATERIALS AND METHODS

Chemicals and Cell Cultures. All chemicals, unless specified otherwise, were purchased from Sigma Chemical Co. or Aldrich. Cell culture media and supplements were obtained from Life Technologies, Inc. (Grand Island, NY). Hepa 1c1c7 murine hepatoma cells and its mutants (supplied by Dr. J. P. Whitlock, Jr., Stanford University, Stanford, CA) and HepG2 human hepatoma cells (provided by Dr. D. H. Barch, Northwestern University, Chicago, IL) were cultured as described previously (12). H4IIE rat hepatoma cells were cultured in MEM without ribonucleosides or deoxyribonucleosides, supplemented with 100 units/ml penicillin G, 100 μg/ml streptomycin sulfate, 250 ng/ml amphotericin B, and 5% FCS (37°C, 5% CO_2). MCF-7 human breast carcinoma cells (American Type Culture Collection) were maintained with Earle's MEM supplemented with 10 mg/liter insulin, antibiotic-antimycotics

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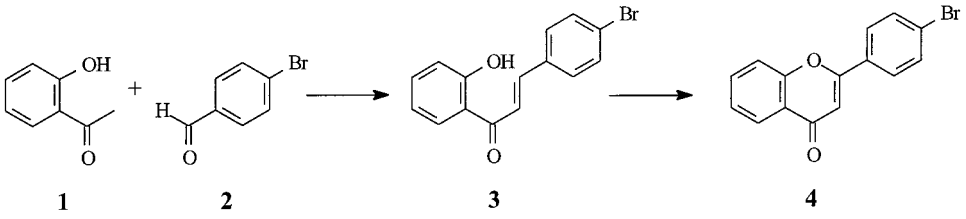
[4] The abbreviations used are: QR, quinone reductase; GST, glutathione *S*-transferase; 4′BF, 4′-bromoflavone; *A*h, arylhydrocarbon; CD, concentration required to double the specific activity of QR; CAT, chloramphenicol acetyltransferase; RT-PCR, reverse transcription-PCR; DMBA, 7,12-dimethylbenz[*a*]anthracene; i.g., intragastric; EROD, ethoxyresorufin-*O*-deethylase; B[*a*]P, benzo[*a*]pyrene; GSH, glutathione; βNF, β-naphthoflavone; XRE, xenobiotic response element; ARE, antioxidant response element.

Fig. 1. Synthesis of 4′BF.



(1×), nonessential amino acids (1×), sodium pyruvate (1×), and 10% fetal bovine serum (37°C, 5% CO_2).

Synthesis of 4′BF. 4′BF (**4**) was synthesized as outlined in Fig. 1. 2′-Hydroxyacetophenone (**1**, 79.48 g, 72.0 ml, 584 mmol) and 4-bromobenzaldehyde (**2**, 100.00 g, 540 mmol) were dissolved in methanol (1.0 liter) with stirring. Potassium hydroxide (89.60 g, 1.60 mol) was added in portions to give a blood-red solution that was stirred for 6 h, during which 4-bromo-2′-hydroxychalcone (**3**) precipitated as the potassium salt. The solution/suspension was poured into cold 1 N HCl (500 ml), and further concentrated HCl was added until the solution was acidic. The resulting yellow precipitate was filtered, washed with water (1 liter), and recrystallized from methanol to give 138.53 g (85%) as yellow crystals: mp 145–147°C [lit. 146–148°C] (24); ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.93 (ddd, J = 8.1, 7.1, 0.9 Hz, 1H, H-5′), 7.01 (dd, J = 8.3, 1.0 Hz, 1H, H-3′), 7.46–7.52 (m, 5H, H-2, -3, -5, -6, -4′), 7.60 (d, J = 15.4 Hz, 1H, H-α), 7.80 (d, J = 15.5 Hz, 1H, H-β), 7.87 (dd, J = 8.1, 1.6 Hz, 1H, H-6′), 10.26 (s, 1H, 2′-OH); ^{13}C NMR (100 MHz, $CDCl_3$): δ 118.59, 118.85, 119.81, 120.50 (C-α), 125.19, 129.54, 129.89, 132.20, 133.37, 136.49, 143.88 (C-β), 163.53 (C-2′), 193.31 (C═O).

This material (**3**) was converted to 4′BF (**4**) through adaptation of the procedure of Doshi *et al.* (25). 4-Bromo-2′-hydroxychalcone (**3**, 138.53 g, 457 mmol) was dissolved in DMSO (1 liter) to which a catalytic amount of iodine (11.60 g, 45.7 mmol) was added (25). The solution was refluxed for 4 h with monitoring by TLC. The solution was cooled to room temperature and poured into saturated aqueous sodium thiosulfate (1 liter), and the resulting precipitate was filtered, washed with cold water, and recrystallized twice from dichloromethane-methanol to give 114.27 g (70% from **2**) of colorless crystals: mp 177–179°C [lit. 177–178°C] (26); ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.80 (s, 1H, H-3), 7.43 (ddd, J = 8.0, 6.9, 0.8 Hz, 1H, H-6), 7.56 (br d, J = 8.5 Hz, 1H, H-8), 7.66 (app d, J = 8.6 Hz, 2H, H-2′, -6′), 7.71 (ddd, J = 8.7, 7.2, 1.7 Hz, 1H, H-7), 7.79 (app d, J = 8.6 Hz, 2H, H-3′, -5′), 8.22 (dd, J = 8.0, 1.5 Hz, 1H, H-5); ^{13}C NMR (100 MHz, $CDCl_3$): δ 107.70, 118.02, 123.91, 125.36, 125.73, 126.28, 127.67, 130.70, 132.33, 133.89, 162.26 (C-8a), 178.21 (C-4).

Determination of QR Activity in Cell Culture. QR activity was assessed in 96-well plates with Hepa 1c1c7 murine hepatoma cells as described previously (12). Specific activity is defined as nmol of 3-(4, 5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide blue formazan formed per mg protein per min (27). Hepa 1c1c7 cells retain many characteristics of normal tissues, particularly the capacity for carcinogen activation and metabolism. They are amenable to precise control of environmental, nutritional, and hormonal factors. Two mutant cell lines derived from wild-type Hepa 1c1c7 cells, TAOcB-P^{r}Cl and BP^{r}Cl, which are either defective in a functional *A*h receptor or unable to translocate the receptor-ligand complex to the nucleus, respectively (28), were used in a similar fashion for the definition of mono- and bifunctional inducers. Induction of QR activity was calculated by comparing the QR specific activity of agent-treated cells with that of solvent-treated cells, and a plot of ratio was generated. CD represents the concentration required to double QR induction. Chemopreventive index is an indexing value generated by comparing CD with IC_{50} (concentration for 50% inhibition of cell viability) values.

Transient Transfection Gene Expression Assays. Cultured HepG2 human hepatoma cells were transfected with plasmid constructs (pDTD-1697CAT, XRE-CAT, and ARE-CAT provided by Dr. C. B. Pickett, Schering-Plough Research Institute, Kenilworth, NJ) containing various portions of the 5′-region of the *QR* gene fused to the *CAT* gene using the calcium phosphate precipitation method (29). After transfection (4 h), the cells were treated with 10% glycerol (3 min), rinsed with PBS, and incubated in MEM containing 100 units/ml penicillin G, 100 units/ml streptomycin sulfate, nonessential amino acids, 1 mM sodium pyruvate (Life Technologies, Inc.), and 10% fetal bovine serum (12–16 h). Treatments with test agents were then performed (48 h), cells were harvested with lysis buffer (Boehringer Mannheim Biochemical), and CAT protein expression was assessed using a CAT ELISA kit (Boehringer Mannheim Biochemical). CAT enzyme expression was normalized for protein content, and data were expressed as mean ± SD, and analyzed using Student's t test (n = 4).

RT-PCR Analysis of QR mRNA Expression. Primers were designed based on the rat *QR* gene (30) and custom synthesized by Ana-Gen Technology, Inc. (Palo Alto, CA). Total RNA was isolated from cultured Hepa 1c1c7 cells with TRIzol reagent (Life Technologies, Inc.) and quantified by UV absorbance. The reverse transcription of RNA was performed in a final volume of 20 μl containing 5× First Strain Synthesis Buffer (Life Technologies, Inc.), 5 mM $MgCl_2$, 1 mM each dNTP, 1 unit of RNase inhibitor (Life Technologies, Inc.), 2.5 units of SuperScript II reverse transcriptase (Life Technologies, Inc.), 2.5 μM oligo$(dT)_{16}$, 0.2 μg of total RNA, and DEPC-treated water. After incubation at 47°C for 30 min, the RT reaction was terminated by heating to 70°C for 15 min. To the newly synthesized cDNA (1 μl), a PCR mixture (Advantage KlenTaq polymerase mix; Clontech) containing 3 mM $MgCl_2$, 2.5 units of Taq polymerase, 6 pmol of primers for *QR* gene (5′-CGAATCTGACCTCTATGCTA-3′; 5′-CACTCTCTCAAACCAGCCTT-3′), and 3 pmol of primers for β-actin (5′-GAGCAAGAGAGGTATCCTGA-3′; 5′-CAGCTCATAGCTCTTCTCCA-3′) as an internal control were added to bring the final volume to 50 μl. The PCR was heated to 94°C for 1 min and immediately cycled 27 times through a 30-s denaturing step at 94°C, a 45-s annealing step at 55°C, and a 60-s elongation step at 72°C, with a Perkin-Elmer 2400 thermocycler. Following the final cycle, a 5-min elongation step at 72°C was performed. Aliquots of the PCR product were electrophoresed on 2.5% agarose gels (Bio-Rad), and PCR fragments were visualized with ethidium bromide staining.

Effect of 4′BF on DMBA-induced QR Activity and Cytochrome P4501A1 Activity in Rat Tissues. At 43 days of age, Sprague Dawley rats were placed on various experimental diets (Table 1) for 1 week prior to administration of a single (i.g.) dose of DMBA (15 mg). Animals were then weighed and sacrificed by CO_2 asphyxiation. Tissues (livers and mammary glands) were collected, rinsed with saline, frozen in liquid nitrogen, and stored at −80°C until analysis. Individual tissues were homogenized in 0.05 M Tris-HCl buffer (pH 7.5) containing 0.25 M sucrose. Cytosolic and microsomal fractions were obtained through differential centrifugation (31). Dicumarol-sensitive QR activity was measured as described above using 50 μl of suitably diluted cytosol supernatants (in duplicate). Cytochrome P4501A1-mediated EROD activities were determined as described previously (32, 33) with slight modifications using suitably diluted microsomal protein. Enzyme activities were expressed as nmol/mg protein/min. Protein content was determined using Bradford reagent with BSA as standard, and data were presented as mean ± SE and analyzed using Student's t test (n = 5).

Table 1 *Protocol for assessing the effect of 4′BF on DMBA-induced phase I and phase II enzyme activities*[a]

Codes	Treatments	DMBA (15 mg, i.g.)
C	Basal oil diet	−
DMBA	Basal oil diet	+
G-1	312.5 mg 4′BF/kg diet	+
G-2	625 mg 4′BF/kg diet	+
G-3	1250 mg 4′BF/kg diet	+
G-4	2500 mg 4′BF/kg diet	+
G-5	5000 mg 4′BF/kg diet	+
G-6	312.5 mg 4′BF/kg diet	−
G-7	1250 mg 4′BF/kg diet	−
G-8	5000 mg 4′BF/kg diet	−

[a] For each group, n = 5.

Inhibition of DMBA-induced Cytochrome P4501A1 Activity. Female Sprague Dawley rats (43 days of age) were treated with DMBA (i.g.), and liver microsomes were isolated through differential centrifugation. Cytochrome P4501A1 activity was monitored by assessing EROD activity. Briefly, 200 μl of microsomal protein (2 mg/ml) were added to a reaction mixture containing 640 μl of 0.05 M Tris-HCl buffer (pH 7.5), 100 μl of BSA (10 mg/ml in Tris buffer), 20 μl of 0.25 M $MgCl_2$, 40 μl of cofactor solution (0.3282 g of $NADP^+$ and 1.417 g of glucose 6-phosphate in 40 ml of 1.15% KCl), 2.5 units of glucose 6-phosphate dehydrogenase, 10 μl of substrate (1 mg of ethoxyresorufin in 10 ml of methanol) and 10 μl of various concentrations of 4′BF in DMSO (α-naphthoflavone as positive control; DMSO as negative control). After incubating at 37°C for 4 min, the reactions were terminated by the addition of 2 ml of methanol. Samples were then centrifuged for 20 min at 2,000 × *g*, and 200 μl of the supernatant fractions were transferred to 96-well plates and read on a spectrofluorimeter (550 nm excitation and 585 nm emission). Assays were conducted in triplicate and the percent of inhibition was calculated as: [1 − (sample *A* − blank)/(DMSO *A* − blank)] × 100.

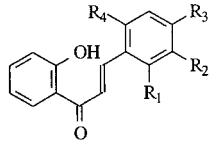
Effect of 4′BF on the Binding of Metabolically Activated [^{3}H]B[*a*]P to DNA of Cultured Cells. HepG2 cells or MCF-7 cells were plated at a density of 1×10^6 cells/dish in 6-cm Petri dishes and allowed to incubate for 18 h. Fresh media containing test samples were added to the cells 2 h prior to the addition of 1 μM [^{3}H]B[*a*]P (10 Ci/mmol). The test compound and tritiated carcinogen were then incubated for 6 h. Following incubation, genomic DNA was extracted by the method described by Sharma *et al.* (34), with minor modifications. Briefly, the cells were washed three times with PBS and harvested by incubation with 0.5 ml of proteinase K (100 μg/ml) in 0.2 M Tris-0.1 M EDTA (pH 8.5) for 10 min at 37°C. The cells were gently scraped from the dishes, transferred to 50 μl of 10% SDS solution, and incubated for 3 h at 55°C. Following the addition of 75 μl of 5 M potassium acetate solution, the cells were placed on ice for 30 min, and centrifuged for 15 min at 13,000 × *g*. The supernatant was transferred to a fresh tube, and 2 volumes of cold ethanol were added to precipitate the DNA overnight at −20°C. The DNA was harvested by centrifugation for 20 min at 13,000 × *g* in an Eppendorf microfuge, and the supernatant was removed. The DNA pellet was washed with 70% ethanol and finally resuspended in 500 μl of 10 mM Tris-1 mM EDTA (pH 8.0) buffer. RNA was removed by treating with 5 μl of RNase A (10 mg/ml) and 5 μl of RNase T1 (5 units/μl) at 37°C for 1 h. An aliquot of harvested DNA was used for measuring the relative absorbancies at 260 and 280 nm ($A_{260\ nm}/A_{280\ nm}$ = 1.7–1.9), and the DNA content was determined by absorbance at 260 nm (1 $A_{260\ nm}$ unit = 50 μg). The remaining sample was used for the determination of radioactivity. All experiments were performed in triplicate; percentage inhibition of carcinogen-DNA binding mediated by test samples was calculated as follows: % inhibition = [1 − (dpm per μg DNA test cells/dpm per μg DNA control cells)] × 100.

Western Blot Analysis of GST Expression. For the investigation of GST induction, Western blots were used with cultured H4IIE rat hepatoma cells. These cells were shown previously to express GST α and GST μ (35), two major detoxification isoforms in liver. Briefly, cells were treated with various concentrations of test agents for varying periods of time. Cells were harvested using SDS lysis buffer [20 mM Tris-HCl (pH 6.8) containing 0.4% SDS (w/v), 4% glycerol (v/v), 0.24 M β-mercaptoethanol, and 0.5% bromphenol], and lysates were boiled for 5 min and stored at −20°C. Aliquots of lysates were used for protein determinations (bicinchoninic acid protein assay kit with BSA as standard) and equivalent amounts of protein were electrophoresed by SDS-PAGE with precast 12.5% Tris-glycine acrylamide gels (Novex, San Diego, CA). Following transfer to polyvinylidene difluoride membranes, non-specific binding sites were blocked with 10% nonfat dry milk, and blotting was performed with goat polyclonal antirat GSTYa (α class) IgG or antirat GSTYb (μ class) IgG and analyzed by alkaline phosphatase-conjugated antigoat IgG secondary antibody (Oxford Biomed Research, Inc.) and quantified using an AP 20 Alkaline Phosphatase Kit detection system (Oxford Biomed Research, Inc.).

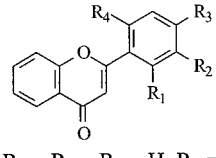
Analysis of QR Activity and GSH Level in Rat Tissues. At 43 days of age, Sprague Dawley rats (n = 10) were placed on various experimental diets (312.5, 625, 1250, 2500, and 5000 mg/kg diet of 4′BF) for 3 weeks. Animals were then weighed and sacrificed by CO_2 asphyxiation. Tissues (liver, mammary gland, colon, stomach, and lung) were collected, rinsed with saline, frozen in liquid nitrogen, and stored at −80°C until analyzed. Individual tissues (except mammary glands) were homogenized in 0.25 M sucrose and centrifuged at 15,000 × *g* for 30 min at 4°C. The supernatant fractions were collected, and 0.1 M $CaCl_2$ in 0.25 M sucrose was added, 20% by volume. After incubation at 0°C for 30 min and further centrifugation at 15,000 × *g* for 30 min at 4°C, a clear cytosolic fraction was used for enzyme assays. Mammary glands of each animal were pooled, homogenized in 1 ml of ice-cold 0.1 M phosphate buffer (pH 6.5) and centrifuged (15,000 × *g*, 30 min at 4°C) to yield clear supernatant fractions suitable for enzyme assays. Dicumarol-sensitive QR activity was measured as described above using 50 μl of suitably diluted tissue supernatants (in duplicate). GSH levels were quantified in 96-well plates as described previously (12). A GSH standard curve was constructed, and GSH levels were expressed as nmol/mg protein.

Inhibition of DMBA-induced Mammary Carcinogenesis in Rats. On the basis of the 3-week feeding study described above, maximum tolerated doses were evaluated (36), and suitable doses were selected for the study of inhibition of DMBA-induced mammary carcinogenesis in rats. Virgin female Sprague Dawley rats were received from Harlan/Sprague Dawley at 35 days of age and placed on a diet of Teklad 4% rat/mouse chow. After 1 week, at 42 days of age, animals were randomized by weight into six groups and placed on experimental diets containing 2000 and 4000 mg 4′BF/kg diet. At 50 days of age, the animals received a single i.g. dose of DMBA (15 mg) in sesame oil (or vehicle only) following an overnight fast. The rats were maintained on the 4′BF diets for 1 additional week, and then returned to the basal diet without test agent at 57 days of age. During the experimental period, all animals were weighed weekly. Palpation for mammary tumors began 3 weeks after animals received DMBA and continued until termination of the study (120 days

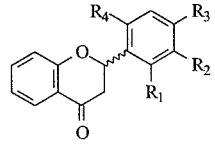
Table 2 *Induction of QR activity in Hepa 1c1c7 cells*



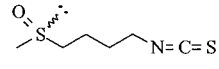
5: $R_1 = R_4 = OCH_3$, $R_2 = R_3 = H$
7: $R_1 = NO_2$, $R_2 = R_3 = R_4 = H$
8: $R_1 = R_3 = R_4 = H$, $R_2 = CF_3$
9: $R_1 = CH_3$, $R_2 = R_3 = R_4 = H$
10: $R_1 = OCH_3$, $R_2 = R_3 = R_4 = H$
17: $R_1 = R_2 = R_4 = H$, $R_3 = CF_3$



4: $R_1 = R_2 = R_4 = H$, $R_3 = Br$
6: $R_1 = R_3 = R_4 = H$, $R_2 = Br$
12: $R_1 = R_3 = R_4 = H$, $R_2 = F$
14: $R_1 = R_3 = R_4 = H$, $R_2 = Cl$
15: $R_1 = F$, $R_2 = R_3 = R_4 = H$
18: $R_1 = R_2 = R_4 = H$, $R_3 = CF_3$
19: $R_1 = R_2 = R_4 = H$, $R_3 = Cl$



13: $R_1 = CH_3$, $R_2 = R_3 = R_4 = H$
16: $R_1 = OCH_3$, $R_2 = R_3 = R_4 = H$



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	Compound tested	CD (μM)[a]	IC_{50} (μM)[b]	CI[c]
5	2′-Hydroxy-2,6-dimethoxychalcone	0.54	14	26
6	3′-Bromoflavone	0.10	3.2	32
7	2′-Hydroxy-2-nitrochalcone	0.30	11	37
8	3-Trifluoromethyl-2′-hydroxychalcone	0.30	12.8	43
9	2′-Hydroxy-2-methylchalcone	0.67	36	54
10	2′-Hydroxy-2-methoxychalcone	0.31	17	55
12	3′-Fluoroflavone	0.67	42	63
13	2′-Methylflavanone	1.00	81	81
14	3′-Chloroflavone	0.27	23	85
15	2′-Fluoroflavone	0.80	>83	>104
16	2′-Methoxyflavanone	0.63	>79	>125
17	4-Trifluoromethyl-2′-hydroxychalcone	0.10	55	550
18	4′-Trifluoromethylflavone	0.03	90	3,000
19	4′-Chloroflavone	0.02	>78	>5,000
4	4′-Bromoflavone	0.01	>166	>17,000
20	Sulforaphane	0.43	11	26

[a] Concentration required to double QR activity.
[b] Concentration required to inhibit cell growth by 50%. The upper limit of test concentration was based on solubility.
[c] CI, chemoprevention index = IC_{50}/CD.

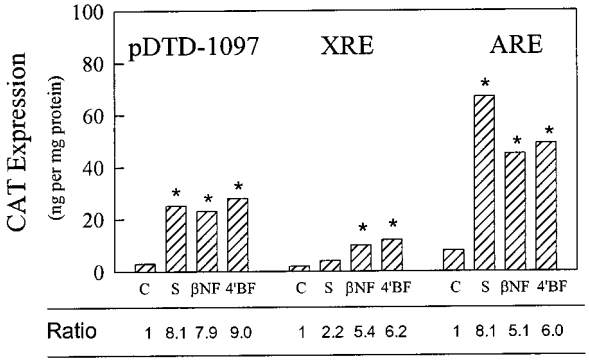

Fig. 2. Quantitation of CAT expression in HepG2 cells transfected with QR-CAT constructs. Transfected cells were treated with 10 μM sulforaphane (*S*), 10 μM βNF, 10 μM 4′BF, or DMSO (0.5% final concentration) as control (*C*). Cells were transfected with pDTD-1097CAT, QR XRE-CAT, or QR ARE-CAT. CAT expression (ng CAT/mg protein) was determined using a CAT ELISA, and the ratio of CAT expression between treated and control cells was calculated (*Ratio* = treated/control). *Columns*, means. Experimental details were as described in "Materials and Methods." *, treatment groups were significantly different from the control group ($P < 0.0001$) using Student's *t* test, with $n = 4$.

post-DMBA). The date of appearance and location of all tumors were recorded. Animals were observed twice daily to assess their general health. Moribund animals were sacrificed by CO_2 asphyxiation. Moribund animals or animals found dead were necropsied immediately. Incidence was evaluated with Fisher's exact test and the log-rank test was used for tumor incidence rate. Tumor multiplicity was calculated using Armitage's test for trends in proportions; latency was evaluated by unpaired *t* test; survival was analyzed by log-rank analysis.

RESULTS AND DISCUSSION

A number of previous studies have suggested that induction of phase II detoxification enzymes is a relevant mechanism for cancer chemoprevention. Various compounds, such as dithiolethione, sulforaphane, indole-3-carbinol, ethoxyquin, and βNF, have been reported to exert broad-based anticarcinogenic activity against a variety of chemical carcinogens at multiple target sites in animal models via induction of phase II detoxification enzymes (9, 37–41), such as QR and GST. As a group, these inducible enzymes facilitate the metabolic detoxification of xenobiotics in mammals and can achieve chemopreventive activity by modification of carcinogen metabolism through increased carcinogen excretion and decreased carcinogen-DNA interactions.

In our continuing effort of searching for novel chemopreventive agents, the Hepa 1c1c7 QR assay was used to identify potent detoxification enzyme inducers because the specific activity of QR rises concomitantly with other phase II detoxification enzymes in many animal tissues in response to various chemopreventive agents (37, 42). In this study, ~80 natural or synthetic B-ring substituted flavones, as well as their biosynthetic precursor chalcones and flavanones, were evaluated for potential to induce QR activity in Hepa 1c1c7 cells and two mutants.[5] Modification of the B-ring of chalcones, flavanones, and flavones was hypothesized to induce torsion of the B-ring and simulate out-of-plane distortions characteristic of polycyclic aromatic hydrocarbons such as DMBA (43). It was found that 15 synthetic flavonoids significantly induced QR activity with CD values in the range of 0.01–1.0 μM (Table 2), and 4′BF demonstrated remarkable induction in terms of the inducing potency and low toxicity. Compared to sulforaphane, 4′BF was ~40 times more active but almost 15 times less toxic. As summarized by the synthetic pathway illustrated in Fig. 1, (*E*)-4-bromo-2′-hydroxychalcone (**3**) was prepared from2′-hydroxyacetophenone (**1**) and 4-bromobenzaldehyde (**2**) via a base-catalyzed Claisen-Schmidt condensation. 4′BF was subsequently formed by oxidative cyclization of **3** with catalytic iodine in DMSO. In this manner, 4′BF can be produced on a 250–500-g scale (overall yield of 70%) at a cost corresponding to less than $2/g.

Compounds functioning in a similar capacity have been classified either as bifunctional inducers, which elevate both phase I and phase II enzymes, or as monofunctional inducers, which selectively elevate phase II enzymes. Induction of phase I enzymes, such as cytochrome P450 isozymes, is required for metabolic disposal of xenobiotics (44) but is also considered a risk factor due to the potential of activating procarcinogens (45). On the basis of data obtained with mutant Hepa 1c1c7 cell lines (data not shown), 4′BF was considered a bifunctional inducer. This was further investigated in transient transfection experiments wherein human hepatoma cells were transfected with DNA constructs containing various portions of the 5′ upstream promoter region of the *QR* gene linked to the *CAT* reporter gene by using the calcium phosphate precipitation method. Construct pDTD-1097CAT contains elements of the rat *QR* gene connected to the *CAT* construct gene. QR XRE-CAT consists of the XRE of the rat *QR* gene containing an *A*h receptor recognition site connected to a heterologous promoter fused to the *CAT* gene, whereas QR ARE-CAT contains the ARE of the rat *QR* gene connected to the heterologous promoter fused to the *CAT* gene (46). As shown in Fig. 2, treatment with 4′BF (10 μM) or βNF (10 μM; a well-known bifunctional inducer) significantly ($P < 0.0001$) induced CAT expression via interaction with both the ARE and the XRE, whereas the monofunctional inducer, sulforaphane (10 μM), selectively interacted with the ARE. These data suggest that 4′BF, as least in part, binds to the *A*h receptor, where its ligand-complex is translocated to the nucleus, interacts with multiple copies of XRE, and transcriptionally activates the phase I cytochrome P4501A1 isozyme gene as well as phase II enzyme genes (28).

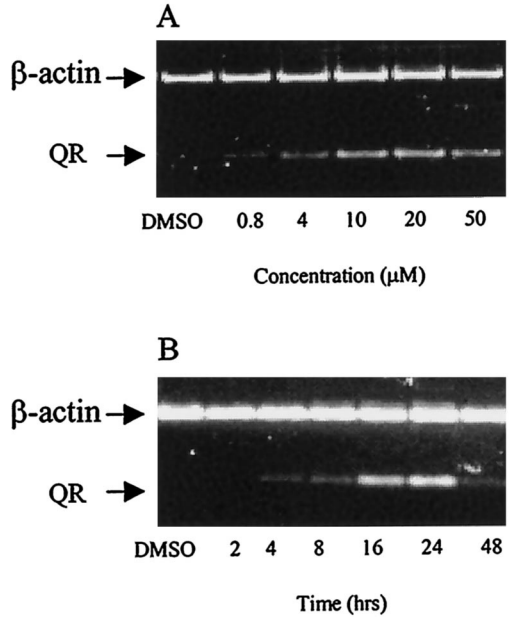

Fig. 3. RT-PCR analysis of QR mRNA levels in Hepa 1c1c7 cells after treatment with 4′BF. *A*, Hepa 1c1c7 cells were treated with the indicated concentrations of 4′BF for 24 h. *B*, Hepa 1c1c7 cells were treated with 10 μM 4′BF for the indicated periods of time. Reverse transcription was carried out by SuperScript II reverse transcriptase (Life Technologies, Inc.) and oligo$(dT)_{16}$. The cDNA was amplified with Advantage Klen*Taq* polymerase (Clontech) by using QR primers and β-actin (internal control), aliquots of the PCR product were eletrophoresed on 2.5% agarose gels (Bio-Rad), and PCR fragments were visualized with ethidium bromide staining (see "Materials and Methods").

[5] J. W. Kosmeder II, L. L. Song, C. Gerhäuser, R. M. Moriarty, and J. M. Pezzuto, Synthesis and evaluation of flavonoids as cancer chemopreventive agents by induction of phase II enzymes, manuscript in preparation.

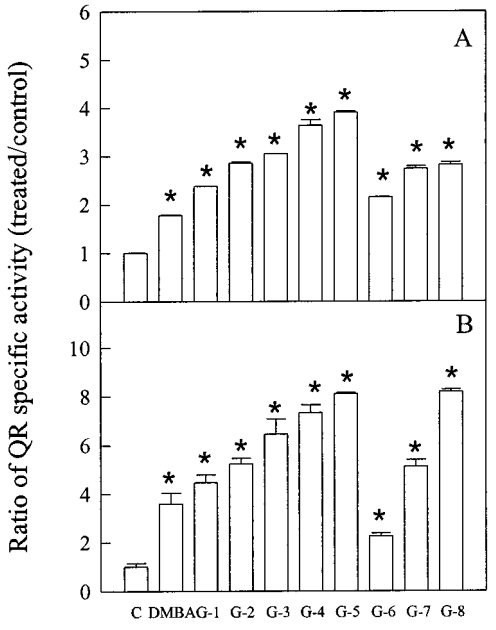

Fig. 4. Effect of 4′BF on DMBA-induced QR activity in rat mammary gland (*A*) or liver (*B*). Induction was calculated by comparing the treatment groups with the control group (basal diet). ∗, treatment groups were significantly different from the basal diet control group ($P < 0.0001$) using Student's *t* test with $n = 5$. See Table 1 for treatment group codes.

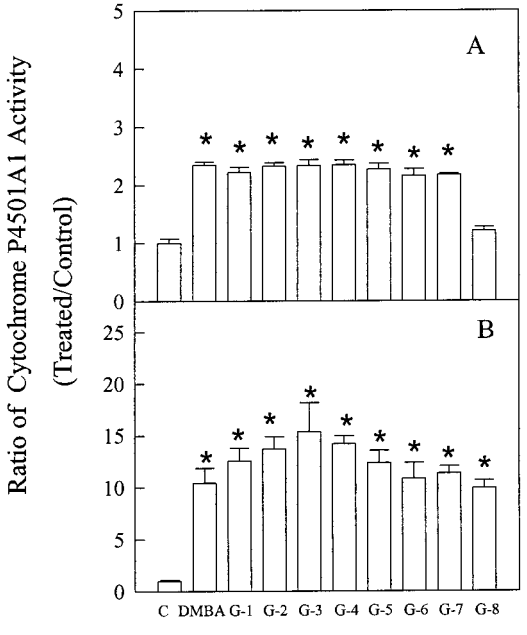

Fig. 5. Effect of 4′BF on DMBA-induced cytochrome P4501A1 activity in rat mammary gland (*A*) or liver (*B*). Induction was calculated by comparing the treatment groups with the control group (basal diet). ∗, treatment groups were significantly different from the basal diet control group ($P < 0.0001$) using Student's *t* test with $n = 5$. See Table 1 for treatment group codes.

Transcriptional regulation of QR activity, as a monitor of phase II enzyme induction, was further demonstrated using RT-PCR. Hepa 1c1c7 cells were treated with 4′BF (10 μM) or DMSO (solvent control) for 2, 4, 8, 16, 24, or 48 h. As illustrated in Fig. 3, QR mRNA levels were increased after an 8-h incubation period, reaching a maximum at 24 h, and showing a decrease at 48 h. Dose-dependent effects of 4′BF were analyzed at the time of maximum induction (24 h), and QR mRNA expression was induced in the concentration range of 0.8–50 μM.

Phase I enzyme induction is considered a potential cancer risk factor due to the activation of procarcinogens to their ultimate reactive forms. Therefore, the relative induction of phase I and phase II enzyme activities by 4′BF might be of critical importance. In the case of βNF, a flavonoid bifunctional inducer with activity similar to 4′BF, it has been shown that induction of cytochrome P450 isozymes can enhance (47) or reduce (48) tumorigenesis. To explore this issue, we investigated the effect of 4′BF on DMBA-induced QR (phase II enzyme) and cytochrome P4501A1 (phase I enzyme) activities in rat liver and mammary gland. DMBA is a potent carcinogen (49) that is selectively active in sites such as the mammary gland and skin and has been widely used as a prototype carcinogen in experimental animal models. To exert its carcinogenic effect, DMBA undergoes bioactivation which involves cytochrome P4501A1 and epoxide hydrolase to form reactive metabolites, notably, DMBA-3,4-diol-1,2-epoxide (50–53). DMBA itself induces phase I and phase II enzyme activities, as well.

Here, animals were placed on various experimental diets for one week (Table 1) prior to a single i.g dose of DMBA (15 mg). One day after DMBA was given, animals were sacrificed, and tissues were collected. QR activity and cytochrome P4501A1 activity were then assessed in the liver and mammary gland. As indicated in Figs. 4 and 5, in liver and mammary gland, both QR and cytochrome P4501A1 activities were significantly induced in comparison with the control groups (basal diet) by each treatment regimen, except cytochrome P4501A1 activity in mammary gland with treatment by 4′BF (5000 mg/kg diet) alone. However, treatment with 4′BF in addition to DMBA did not significantly alter DMBA-induced cytochrome

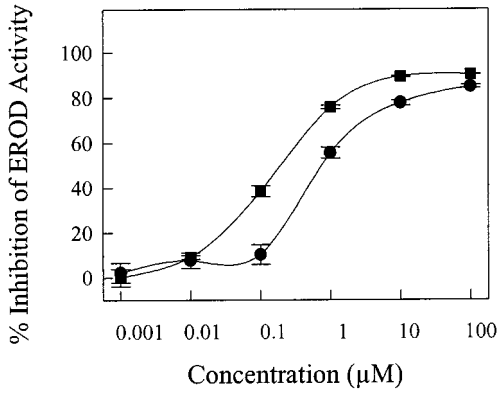

Fig. 6. Inhibition of cytochrome P4501A1-mediated EROD activity using liver microsomes derived from DMBA-treated rats. EROD activity was assessed with the indicated concentrations of 4′BF (●) or α-naphthoflavone (■) as described in "Materials and Methods."

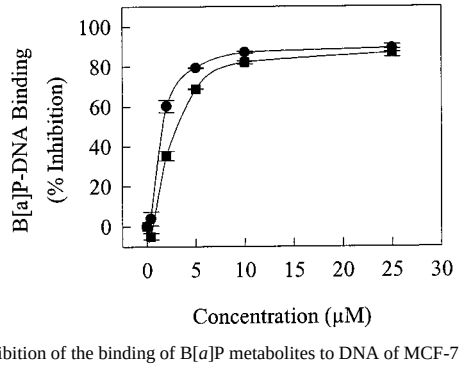

Fig. 7. Inhibition of the binding of B[*a*]P metabolites to DNA of MCF-7 (●) or HepG2 (■) cells in culture. B[*a*]P-DNA binding was determined in the presence of the indicated concentrations of 4′BF as described in "Materials and Methods."

P4501A1 activities in either liver or mammary gland (Fig. 5). On the other hand, significant dose-dependent induction of QR activity was observed on treatment with either DMBA and 4′BF or 4′BF alone, compared to treatment by DMBA alone, in liver and mammary gland. These results suggest that as the dose of 4′BF increased, induction of phase II enzyme expression in rat liver and mammary gland was more favorable, as compared with phase I enzyme induction.

Furthermore, as reported in a previous study by Lu *et al.* (54), 4′BF can significantly inhibit tetrachlorodibenzo-*p*-dioxin-induced EROD activity (phase I cytochrome P4501A1). We now report a similar inhibitory effect of 4′BF on cytochrome P4501A1-mediated EROD activity using microsomes derived from the liver of DMBA-treated rats. As shown in Fig. 6, 4′BF mediated an inhibitory response with an IC_{50} of 0.86 μM. A comparable profile was observed with α-naphthoflavone (IC_{50} of 0.09 μM), a well-known cytochrome P4501A1 inhibitor. Consistent with this activity, we have recently found that 4′BF strongly inhibits DMBA-induced mutagenesis with *Salmonella typhimurium* strain TM677 (data not shown), most likely due to direct inhibition of cytochrome P4501A1 (55). Therefore, it seems logical to speculate that 4′BF binds to the *A*h receptor, interacts with the XRE, and transcriptionally activates the cytochrome P4501A1 gene. In addition, however, 4′BF may also function as a competitive inhibitor that binds to and blocks the activity of cytochrome P4501A1.

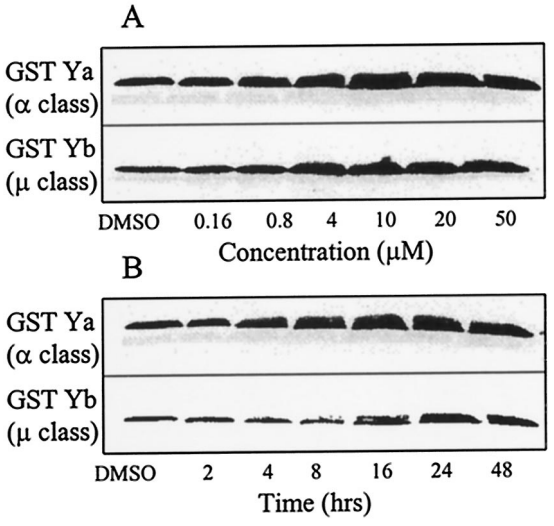

Fig. 8. Expression of the α- and μ-isoforms of GST was determined with cultured H4IIE cells as described in "Materials and Methods." *A*, expression of α- and μ-isoforms of GST in H4IIE cells after treatment with the indicated concentrations of 4′BF for 24 h. *B*, expression of α- and μ-isoforms of GST in H4IIE cells after treatment with 10 μM 4′BF for the indicated periods of time.

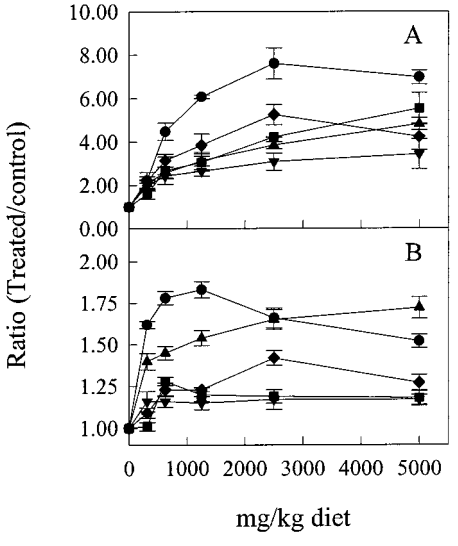

Fig. 9. Induction of QR (*A*) and GSH (*B*) mediated by 4′BF in various rat organs. Sprague Dawley rats (n = 4) at 43 days of age were placed on experimental diets (312.5, 625, 1250, 2500, or 5000 mg 4′BF/kg diet) for 3 weeks. Changes in QR activities and GSH levels were assessed in liver (●), mammary gland (■), colon (▲), stomach (▼), and lung (◆) as described in "Materials and Methods." Induction ratios were calculated by comparing the treatment groups with the control group (basal diet).

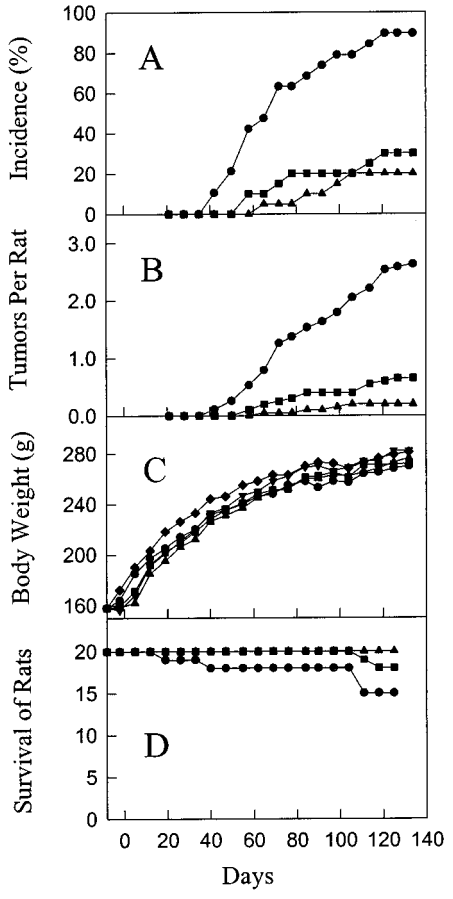

Fig. 10. Effect of dietary 4′BF on percentage incidence of observable mammary tumors (*A*), number of observable mammary tumors per rat (*B*), body weight (*C*), and survival rate of female Sprague Dawley rats given a single i.g. dose of DMBA (*D*). As described in "Materials and Methods," the rat treatment groups were: ●, DMBA in sesame oil; ■, DMBA and 2000 mg/kg diet of 4′BF; ▲, DMBA and 4000 mg/kg diet of 4′BF; ▼, 4000 mg/kg diet of 4′BF; ◆, sesame oil.

To assess the functional significance of the net modulation of phase I and phase II enzyme activities mediated by 4′BF, we determined the effect of this compound on the binding of metabolically activated B[*a*]P to the genomic DNA of cultured MCF-7 or HepG2 cells. It was assumed that a favorable effect on the expression of phase II enzymes would likely enhance metabolic detoxification, decrease the availability of carcinogen reactive metabolites capable of interacting with DNA, and thereby provide a chemoprotective effect. As illustrated in Fig. 7, 4′BF inhibited the formation of B[*a*]P-DNA adducts with IC_{50}s of ~1.7 and 3.1 μM with cultured MCF-7 cells and HepG2 cells, respectively.

Another important activity of 4′BF involves enhanced expression of two major detoxification isoforms of GSTs (Ya: α class; Yb: μ class). To date, the superfamily of mammalian cytosolic GST isozymes has been defined genetically to consist of four classes, α, μ, π, and θ (56, 57), which differ in tissue-specific expression and distribution, as well as substrate specificity. The major detoxification

isoforms of liver, α and μ, were induced by 4′BF in a time- and dose-dependent fashion in H4IIE rat hepatoma cells (Fig. 8).

On the basis of these *in vitro* activities, 4′BF was further investigated in dietary studies performed with female Sprague Dawley rats. Animals were treated with various concentrations of 4′BF (312.5–5000 mg/kg diet) for 3 weeks, and changes in QR activities and GSH levels were assessed in liver, mammary gland, colon, stomach, and lung. As shown in Fig. 9, induction was found in all tissues examined. The maximum induction of QR was observed with doses in the range of 2500–5000 mg/kg diet, whereas the maximum induction of GSH was observed in the range of 1250–2500 mg/kg diet. Induction of GSH began to decline in some of the tissues with 4′BF concentrations higher than 1250 mg/kg diet. Similar effects were observed with βNF,[6] probably as a result of rapid GSH conjugation stimulated by a higher concentration of test agents. However, in addition to the liver, enzymatic and inductive events occurring in the mammary gland may be of critical importance in the genesis of breast cancer. In studies investigating the chemopreventive potential of βNF, dithiolethione, ethoxyquin, and butylated hydroxyanisole with the DMBA-induced rat mammary carcinogenesis model, induction of phase II detoxification enzymes at the mammary level appear to be an important factor for chemopreventive efficacy,[6] and similar factors may apply in the case of 4′BF.

Finally, 4′BF was evaluated and found to be a highly effective chemopreventive agent in the DMBA-induced rat mammary tumorigenesis model. As shown in Fig. 10, *A* and *B*, administration of 2000 or 4000 mg 4′BF/kg diet significantly decreased tumor incidence from 89.5 to 30 and 20% and multiplicity from 2.63 to 0.65 and 0.20 tumors/rat, respectively. Tumor latency was also greatly enhanced by administration of either dose of 4′BF. There were no significant differences in body weight (Fig. 10*C*), and dietary supplementation improved survival (Fig. 10*D*).

To conclude, this is the first description of 4′BF as a highly effective and novel cancer chemoprevention agent. The compound significantly induces phase II detoxification enzymes and exhibits low toxicity with *in vitro* and *in vivo* models. Because it is well known that DMBA requires bioactivation by cytochrome P4501A1 to exert its carcinogenic effect (58), it may be speculated that the chemopreventive activity of 4′BF could be achieved by modification of carcinogen metabolism (inhibition of cytochrome P4501A1), increases in carcinogen excretion (induction of phase II detoxification enzymes), and decreases in carcinogen-DNA interactions. In addition to protection against DMBA-induced mammary tumorigenesis, the current data suggest 4′BF may be an effective inhibitor in other organ sites wherein phase II detoxification enzymes are induced. In summary, 4′BF is a highly active chemopreventive agent that can be economically synthesized in mass quantities using readily available starting materials. These factors provide encouragement for future development.

ACKNOWLEDGMENTS

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