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Induction of Phase II Enzymes and Inhibition of Cytochrome P450 Isozymes by Chitosanoligosaccharides

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Abstract The cancer chemopreventive potential of chitosanoligosaccharides was investigated by measuring the induction of quinone reductase and glutathione S-transferase activities and inhibition of cytochrome P450 1A1, 2B1, and 2E1 activities. Chitosanoligosaccharide I (1-kDa<MW< 3-kDa) significantly induced glutathione S-transferase activity with a maximal 1.5-fold increase at 500 μg/ml, while chitosanoligosaccharide II (3-kDa<MW<5-kDa) (500 μg/ml) strongly induced quinone reductase ($p<0.01$) and glutathione S-transferase ($p<0.005$) activities. The *in vitro* incubation of rat liver microsomes with chitosanoligosaccharides I and II (2.5, 5, 50, and 500 μg/ml) showed a dose-dependent inhibiton of cytochrome P450 1A1, 2B1, and 2E1 activities. Chitosanoligosaccharide II was a more potent inhibitor of cytochrome P450 2B1 activity than chitosanoligosaccharide I. Accordingly, these findings suggest that chitosanoligosaccharides are potential chemopreventive agents.

Key words: Chitosanoligosaccharide, cytochrome P450, glutathione S-transferase, quinone reductase

Cancer chemoprevention can be defined as the prevention of neoplastic disease or inhibition of its progression by the administration of chemical or natural substances. A large number of potential chemopreventive agents have already been identified from epidemiological surveys and experimental and preclinical observations, some of which have proven effective in clinical trials [9]. Chemopreventive agents can function by a variety of mechanisms, directed at all major stages of carcinogenesis. One mechanism of particular note involves the induction of phase II detoxification enzymes, such as quinone reductase (QR) or glutathione S-transferase (GST) [26]. The cytochrome P450 (CYP) enzyme is involved in the activation of diverse chemical carcinogens, and modulation of its activity by chemopreventive agents is an important part of their action mechanism. Benzo[a]pyrene requires metabolic activation mediated by CYP 1A1 and/or 1A2 [28], while CYP 2B1 is one of the major isozymes involved in the activation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK, a tobacco-specific carcinogen) [6]. CYP 2E1 is primarily responsible for the initial metabolism of *N*-nitrosodimethylamine (NDMA) and azoxymethane [28].

Chitin and chitosan are both widely used as health foods in several countries. Chitin is a β-(1→4) polymer of *N*-acetyl-D-glucosamine, with a molecular size of more than one million. Meanwhile, chitosan is a β-(1→4) polymer of D-glucosamine and can be readily obtained from chitin by deacetylation with alkali. Chitin and chitosan have numerous pharmacological actions, including immunopotentiating [17], antitumor [27], antihypertensive [12], hypocholesterolemic [19], and antimicrobial actions [20]. However, their high molecular weight and high viscosity retards absorption in the human intestine, which does not possess chitinase and chitosanase activities that degrade the β-glucosidic linkages in chitin and chitosan. Therefore, the preparation of chitosanoligosaccharide by chemical, fermentation, and enzymatic methods has been developed [8, 11]. The yield of oligosaccharides with a high degree of polymerization is the greatest after enzymatic hydrolysis [10].

Previously, the current authors reported on the antimutagenic activities [18], inhibition of oxidative stress resulting from exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [23], and inhibition of the polyamine biosynthesis of *Acanthamoeba castellanii* and 12-*O*-tetradecanoylphorbol-13-acetate-induced ornithine decarboxylase activity by chitosanoligosaccharides [24]. Accordingly, this study prepared chitosanoligosaccharides using a membrane reactor system, then investigated their effect on phase II enzyme activities and CYP isozymes.

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MATERIALS AND METHODS

Preparation of Chitosanoligosaccharide I and Chitosanoligosaccharide II

Water soluble chitosanoligosaccharide I (1-kDa<MW<3-kDa) and chitosanoligosaccharide II (3-kDa<MW<5-kDa) were prepared from 1% (w/v) chitosan using a dual reactor system, as described by Shon and Nam [24].

Determination of Quinone Reductase Activity

The QR specific activity was measured in Hapa1c1c7 murine hapatoma cells grown in 96-well microtiter plates according to the method of Shon *et al.* [25]. The induction of quinone reductase activity was calculated from the ratio of the specific enzyme activity of the sample-treated cells in comparison with a solvent control. Ellagic acid was used as the reference compound in this experiment.

Glutathione S-Transferase Activity

The GST activity was measured using a modification of the procedure developed by Habig *et al.* [7] with 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate. The protein content was measured in a duplicate plate using a bicinchoninic acid protein assay kit (Sigma, St. Louis, MO, U.S.A.) with bovine serum albumin (BSA) as the standard. The GST activity was expressed as the slope/min/mg of protein. The data derived from the sample-treated cells were compared to the values obtained for the solvent control. Ellagic acid was used as the reference compound.

Preparation of Rat Liver Microsome

Male Sprague-Dawley rats (body weight 130–150 g) were obtained from the Dae-Han Laboratory Animal Research Center (Eumsung, Korea) and induced with β-naphthoflavone (three daily intraperitoneal injections of 80 mg/kg body weight), phenobarbital (four daily intraperitoneal injections of 75 mg/kg body weight), or pyrazole (four daily intraperitoneal injections of 200 mg/kg body weight). The animals were killed by cervical dislocation 1 day after the last 3-β-naphthoflavone treatment and on day 5 of the phenobarbital or pyrazole regimen. Hepatic tissue from five rodents per treatment was assayed individually. The hepatic tissue was homogenized in a 0.15 M KCl buffer (pH 7.0) (5.0 ml/g of tissue) and centrifuged at 9,000 ×*g* for 20 min. The microsomal pellets were then obtained by recentrifugation at 27,000 ×*g* for 20 min. All steps were performed at 4°C. The β-naphthoflavone-exposed rat liver microsomes were used to study the effect of chitosanoligosaccharides on the dealkylation of ethoxyresorufin by ethoxyresorufin *O*-deethylase (EROD, CYP 1A1), while the phenobarbital-treated rat liver microsomes were used to study the effect of chitosanoligosaccharides on the dealkylation of pentoxyresorufin by pentoxyresorufin *O*-dealkylase (PROD, CYP 2B1), and the pyrazole-treated rat liver microsomes were used to study the effect of chitosanoligosaccharides on aniline hydroxylase (CYP 2E1) activity. The protein contents of the microsomal fractions were determined using a bicinchoninic protein assay kit with BSA as the standard.

Determination of Activities of Cytochrome P450 1A1, 2B1, and 2E1

The activities of the CYP 1A1 and 2B1 enzymes were determined by monitoring the formation of resorufin from ethoxyresorufin and pentoxyresorufin, respectively, as described by Burke *et al.* [3]. The results are expressed as pmol of resorufin formed/min/mg protein. Meanwhile, the activity of CYP 2E1 was evaluated as the rate of *p*-aminophenol formation from aniline, according to the method of Dicker *et al.* [4].

Statistical Analysis

The data were analyzed for statistical significance using Student's *t*-test. p-Values less than 0.05 were considered to be significant.

RESULTS AND DISCUSSION

Previous studies have suggested that the induction of phase II detoxification enzyme activities is responsible for cancer chemopreventive activity [1, 29]. These inducible enzymes facilitate the metabolic detoxification of xenobiotics in mammalians and can achieve chemopreventive activity by modifying the carcinogen metabolism through increased carcinogen excretion and the decreased availability of carcinogen reactive metabolites capable of interacting with DNA. Prochaska and Fernandes [21] reported that dietary treatment of mice with 2(3)-*tert*-butyl-4-hydroxy-anisole increased the hepatic-specific activities of QR and GST, and phase II enzyme induction by protective foodstuffs played a role in reducing the risk of cancer.

GSTs, a family of phase II detoxification enzymes, play a critical role in cellular protection against a wide variety of xenobiotics by catalyzing the conjugation of carcinogens with glutathione. The reversible conjugation of carcinogens to glutathione leads to reduced toxicity. Highly reactive epoxides of numerous compounds, including benzo[a]pyrene, are substrates for human GSTs. The inhibition of chemically induced organ damage or tumors in animal models by chemopreventive agents such as oltipraz has been accompanied by an increase in GST activity [13]. Furthermore, a daily dietary intake of glucosinolate-containing brussel sprouts leads to an increase of α-class GST levels in human blood plasma [2].

The phase II enzyme (quinone reductase and glutathione S-transferase) profiles induced by the chitosanoligosaccharides

Table 1. Effect of chitosanoligosaccharide I and chitosanoligosaccharide II on quinone reductase (QR) and glutathione S-transferase (GST) activities.

Samples	Concentration (μg/ml)	Ratio (treated/control)	
		QR	GST
Chitosanoligosaccharide I	2.5	1.0±0.1	1.2±0.1
	5	1.0±0.1	1.2±0.1
	50	1.0±0.1	1.4±0.2*
	500	1.2±0.1	1.5±0.1**
Chitosanoligosaccharide II	2.5	1.1±0.1	1.2±0.1
	5	1.2±0.1	1.3±0.1*
	50	1.2±0.2	1.3±0.1*
	500	2.2±0.1***	1.5±0.1**
Ellagic acid	3	1.4±0.1*	1.3±0.1*
	15	1.9±0.2***	1.6±0.1**
	30	2.1±0.2***	1.7±0.1**

Cultured Hepa1c1c7 hepatoma cells were treated with chitosanoligosaccharide I or chitosanoligosaccharide II within a concentration range of 2.5- 500 μg/ml for 48 h. The quinone reductase (QR) and glutathione S-transferase (GST) activities in the sample-treated cells were analyzed and compared with a solvent-control to calculate the ratio of quinone reductase and glutathione S-transferase induction. The induction of quinone reductase and glutathione S-transferase in the solvent control was 143.8±15.7 nmol/min/mg protein and 16.3±1.9 slope/min/mg protein, respectively. Values are mean±SD (standard deviation) of three experiments. *p<0.05, **p< 0.01, ***p<0.005 as compared to the solvent control.

are shown in Table 1. Chitosanoligosaccharide I only moderately induced quinone reductase activity, yet significantly induced glutathione S-transferase activity with a maximal 1.5-fold induction at 500 μg/ml. Meanwhile, chitosanoligosaccharide II induced quinone reductase activity in a dose-dependent manner within a concentration range of 2.5- 500 μg/ml with a maximal 2.2-fold induction at the highest concentration level tested. Chitosanoligosaccharide II also significantly induced glutathione S-transferase enzyme activity in cultured Hepa 1c1c7 cells. Chitosanoligosaccharides I and II were both found to induce drug-metabolizing enzymes in a cell culture, and the chemopreventive activity was mainly attributed to the increased detoxification of xenobiotics and carcinogens.

CYPs are the principal enzymes that catalyze the bioactivation of many procarcinogens and toxins to their ultimate reactive forms. Therefore, specific CYPs have been identified as potential targets for cancer chemoprevention.

Chitosanoligosaccharides I and II dose-dependently inhibited microsomal ethoxyresorufin *O*-deethylase activity (Fig. 1), and were found to be a more potent inhibitor of CYP 1A1 activity than resveratrol [15], a well-known inhibitor of CYP 1A1 (Fig. 1). CYP 1A1 is involved in the activation of the procarcinogens of the polycyclic aromatic hydrocarbons [22] formed by the incomplete combustion of tabacco during smoking as causative agents for lung cancer. Thus,

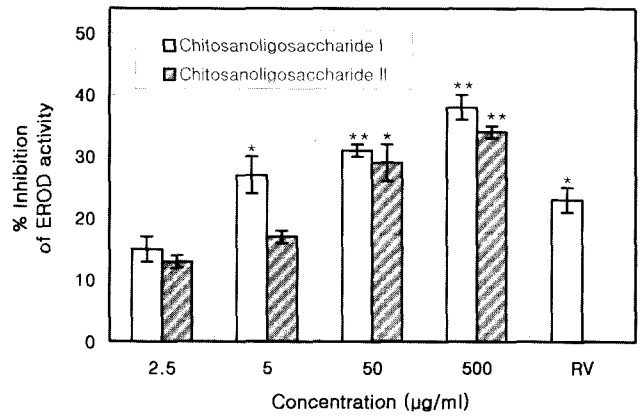

Fig. 1. Inhibition of rat liver microsomal cytochrome P450 1A1-dependent ethoxyresorufin *O*-de-ethylase (EROD) activity by chitosanoligosaccharide I and chitosanoligosaccharide II.
The EROD activity in the control was 307±41 pmol resorufin/min/mg protein. RV, 50 μg/ml resveratrol. Data shown are mean values with bars indicating the SD of the mean (n=3). *p<0.05, **p<0.01 compared with the control.

inhibitors of CYP 1A1 could be considered as potential agents for cancer chemoprevention, particularly lung cancer.

Chitosanoligosaccharide II was a more potent inhibitor of CYP 2B1 activity than chitosanoligosaccharide I (Fig. 2). A comparable effect was observed with ethynylestradiol [14], a known inhibitor of cytochrome P450 2B1 (Fig. 2). Previous studies have provided evidence that aflatoxin B_1 (AFB_1) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are metabolically activated by isozymes of the CYP 2B subfamily [5]. As such, the potent inhibition of CYP 2B1 activity by chitosanoligosaccharides I and II may

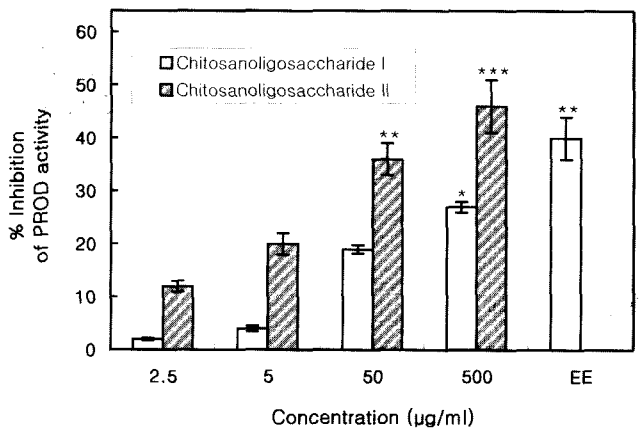

Fig. 2. Inhibition of rat liver microsomal cytochrome P450 2B1-dependent pentoxyresorufin *O*-de-alkylase (PROD) activity by chitosanoligosaccharide I and chitosanoligosaccharide II.
The PROD activity of the control was 139±12 pmol resorufin/min/mg protein. EE, 700 μg/ml ethynylestradiol. Data shown are mean values with bars indicating the SD of the mean (n=3). *p<0.05, **p<0.01, ***p<0.005 compared with the control.

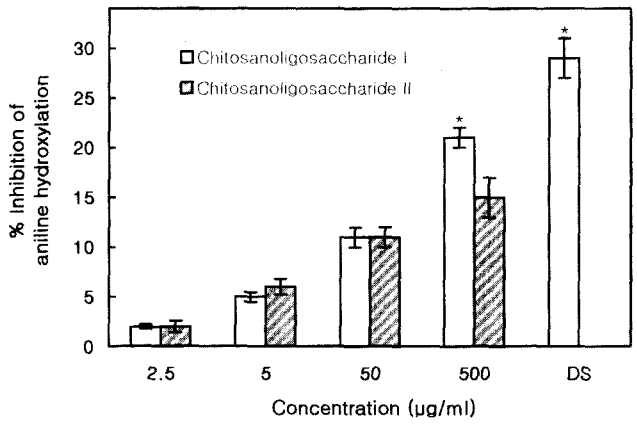

Fig. 3. Inhibition of rat liver microsomal cytochrome P450 2E1 activity by chitosanoligosaccharide I and chitosanoligosaccharide II.

The aniline hydroxylation activity of the control was 36±3 nmol aminophenol/min/mg protein. DS, 60 μg/ml diallyl sulfide. Data shown are mean values with bars indicating the SD of the mean (n=3). *$p<0.05$ compared with the control.

contribute to a protective effect against AFB_1 and NNK carcinogenicities.

Chitosanoligosaccharide I also had an inhibitory effect on aniline hydroxylase, primarily catalyzed by CYP 2E1 in the pyrazole-induced rat liver microsome (Fig. 3). Meanwhile, chitosanoligosaccharide II only moderately inhibited CYP 2E1 activity (Fig. 3). Diallyl sulfide used as the reference compound [16] showed a significant inhibition of CYP 2E1 activity (Fig. 3).

Accordingly, the present results suggest that chitosanoligosaccharides may act as chemopreventive agents due to the induction of phase II enzymes (quinone reductase and glutathione S-transferase) and their specific inhibitory activity toward CYP isozymes (1A1, 2B1, and 2E1). Therefore, the current data may provide useful information for the further development of chitosanoligosaccharides as chemoprevention agents in animal studies and later in human clinical trials.

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References

1. Bandaru, S. R., V. R. Chinthalapally, R. Abraham, and K. Gary. 1993. Chemoprevention of colon carcinogenesis by organosulfur compounds. *Cancer Res.* **53:** 3493-3498.
2. Bogaards, J. J. P., H. Verhagen, M. I. Willems, G. van Poppel, and P. J. van Bladeren. 1994. Consumption of Brussels sprouts results in elevated α-class glutathione S-transferase levels in human blood plasma. *Carcinogenesis* **15:** 1073-1075.
3. Burke, M. D., S. Thompson, C. R. Elcombe, J. Halpert, T. Haaparanta, and R. T. Mayer. 1985. Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: A series of substrates to distinguish between different induced cytochromes P-450. *Biochem. Pharmacol.* **34:** 3337-3345.
4. Dicker, E., Y. McHugh, and A. I. Cederbaum. 1990. Increased oxidation of *p*-nitrophenol and aniline by intact hepatocytes isolated from pyrazole-treated rats. *Biochim. Biophys. Acta* **1035:** 249-256.
5. Guengerich, F. P. 1988. Roles of cytochrome P450 enzymes in chemical carcinogenesis and cancer chemotherapy. *Cancer Res.* **48:** 2946-2954.
6. Guo, Z., T. J. Smith, E. Wang, K. I. Eklind, F. L. Chung, and C. S. Yang. 1993. Structure activity relationships of arylalkyl isothiocyanates for the inhibition of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone metabolism and the modulation of xenobiotic-metabolising enzymes in rats and mice. *Carcinogenesis* **14:** 1167-1173.
7. Habig, W. H., M. J. Pabst, and W. B. Jakoby. 1974. Glutathione S-transferases: The first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* **249:** 7130-7139.
8. Hong, I. P., K. J. Hong, Y. L. Shin, and G. C. Shin. 2003. Cloning and characterization of a bifunctional cellulase-chitosanase gene from *Bacillus licheniformis* NBL420. *J. Microbiol. Biotechnol.* **13:** 35-42.
9. Hong, W. K. and M. B. Sporn. 1997. Recent advances in chemoprevention of cancer. *Science* **278:** 1073-1077.
10. Izume, M. and A. Ohtakara. 1987. Preparation of D-glucosamine oligosaccharides by the enzymatic hydrolysis of chitosan. *Agric. Biol. Chem.* **51:** 1189-1191.
11. Jo, Y. Y., K. J. Jo, Y. L. Jin, W. J. Jung, J. H. Kuk, K. Y. Kim, T. H. Kim, and R. D. Park. 2003. Characterization of endochitosanases-producing *Bacillus cereus* P16. *J. Microbiol. Biotechnol.* **13:** 960-968.
12. Kato, H., T. Taguchi, H. Okuda, M. Kondo, and M. Takara. 1994. Antihypertensive effect of chitosan in rats and humans. *J. Tradit. Chin. Med.* **11:** 198-205.
13. Kensler, T. W., P. A. Egner, P. M. Dolan, J. D. Groopman, and B. D. Roebuck. 1987. Mechanism of protection against aflatoxin tumorigenicity in rats fed 5-(2-pyrazinyl)-4-methyl-1,2-dithiol-3-thione (oltipraz) and related 1,2-dithiol-3-thiones and 1,2-dithiol-3-thones. *Cancer Res.* **47:** 4271-4277.
14. Kent, U. M., D. E. Mills, R. V. Rajnarayanan, W. L. Alworth, and P. F. Hollenberg. 2002. Effect of 17-alpha-ethynylestradiol on activities of cytochrome P450 2B (P450 2B) enzymes: Characterization of inactivation of P450s 2B1 and 2B6 and identification of metabolites. *J. Pharmacol. Exp. Ther.* **300:** 549-558.
15. Lee, J. E. and S. Safe. 2001. Involvement of a post-transcriptional mechanism in the inhibition of CYP1A1 expression by resveratrol in breast cancer cells. *Biochem. Pharmacol.* **62:** 1113-1124.

16. Loizou, G. D. and J. Cocker. 2001. The effects of alcohol and diallyl sulphide on CYP2E1 activity in humans: A phenotyping study using chlorzoxazone. *Hum. Exp. Toxicol.* **20:** 321-327.
17. Marcinkicwicz, J., A. Polewska, and J. Knapczyk. 1991. Immunoadjuvant properties of chitosan. *Arch. Immunol. Ther. Exp. (Warsz)* **39:** 127-132.
18. Nam, K. S., Y. R. Choi, and Y. H. Shon. 2001. Evaluation of the antimutagenic potential of chitosan oligosaccharide: Rec, Ames and Umu assays. *Biotechnol. Lett.* **23:** 971-975.
19. Ormrod, D. J., C. C. Holmes, and T. E. Miller. 1998. Dietary chitosan inhibits hypercholesterolemia and atherogenesis in the apolipoprotein E-deficient mouse model of atherosclerosis. *Atherosclerosis* **138:** 329-334.
20. Park, P. J., H. K. Lee, and S. K. Kim. 2004. Preparation of hetero-chitooligosaccharides and their antimicrobial activity on *Vibrio parahaemolyticus*. *J. Microbiol. Biotechnol.* **14:** 41-47.
21. Prochaska, H. J. and C. L. Fernandes. 1993. Elevation of serum phase II enzymes by anticarcinogenic enzyme inducers: Markers for a chemoprotected state? *Carcinogenesis* **14:** 2441-2445.
22. Schweikl, H., J. A. Taylor, S. Kitareewan, P. Linko, D. Nagorney, and J. A. Goldstein. 1993. Expression of CYP 1A1 and CYP 1A2 genes in human liver. *Pharmacogenetics* **3:** 239-249.
23. Shon, Y. H., I. K. Park, I. S. Moon, H. W. Chang, I. K. Park, and K. S. Nam. 2002. Effect of chitosan oligosaccharide on 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced oxidative stress in mice. *Biol. Pharm. Bull.* **25:** 1161-1164.
24. Shon, Y. H. and K. S. Nam. 2003. Inhibition of polyamine biosynthesis of *Acanthamoeba castellanii* and 12-*O*-tetradecanoylphorbol-13-acetate-induced ornithine decarboxylase activity by chitosanoligosaccharide. *Biotechnol. Lett.* **25:** 701-704.
25. Shon, Y. H., K. S. Nam, and M. K. Kim. 2004. Cancer chemopreventive potential of *Scenedesmus* spp. cultured in medium containing bioreacted swine urine. *J. Microbiol. Biotechnol.* **14:** 158-161.
26. Talalay, P., J. W. Fahey, W. D. Holtzclaw, T. Prestera, and Y. Zhang. 1995. Chemoprotection against cancer by phase II enzyme induction. *Toxicol. Lett. (Amst.)* **82:** 173-179.
27. Tsukada, K., T. Matsumoto, K. Aizawa, A. Tokoro, R. Naruse, S. Suzuki, and M. Suzuki. 1990. Antimetastatic and growth-inhibitory effects of N-acetylchitohexaose in mice bearing Lewis lung carcinoma. *Jpn. J. Cancer Res.* **81:** 259-265.
28. Yang, C. S., T. J. Smith, and J. Y. Hong. 1994. Cytochrome P450 enzymes as targets for chemoprevention against chemical carcinogenesis and toxicity: Opportunities and limitations. *Cancer Res.* **54(Suppl.):** 1982s-1986s.
29. Zhang, Y., T. W. Kensler, C. G. Cho, G. H. Posner, and P. Talalay. 1994. Anticarcinogenic activities of sulforaphane and structurally related synthetic norbornyl isothiocyanates. *Proc. Natl. Acad. Sci. USA* **91:** 3147-3150.