Arch Pharm Res Vol 27, No 9, 919-922, 2004

Archives of Pharmacal Research
http://apr.psk.or.kr

Induction of the Anticarcinogenic Marker Enzyme, Quinone Reductase, by Dalbergiae Lignum

Hu-Quan Yin, Bang-Wool Lee, Youn-Chul Kim, Dong-Hwan Sohn, and Byung-Hoon Lee
College of Pharmacy and MeRRI, Wonkwang University, Iksan 570-749 Korea

(Received June 7, 2004)

The effect of an extract of Dalbergiae Lignum and four components that were isolated from the extract on the anticarcinogenic phase II marker enzyme, quinone reductase (QR), was investigated. Of the solvent extracts of Dalbergiae Lignum, the CH_2Cl_2 fraction was the most potent in inducing QR activity, with a CD value (the concentration required to double the QR activity) of 29.5 μg/mL. The CH_2Cl_2 extract was further separated into six compounds, four of which were identified as 4-methoxydalbergione, latifolin, 4',6-dihydroxy-7-methoxyflavanone, and obtusafuran. Obtusafuran [CD = 1.1 μM; chemopreventive index (CI) = 101.9] and latifolin (CD = 1.7 μM; CI = 154.6) displayed potent QR inducing activity and high chemopreventive indices. Latifolin and 4-methoxydalbergione were identified as strong DPPH-scavengers with half-maximal free radical scavenging concentrations of 15.9 and 17.2 μM, respectively.

Key words: Dalbergiae lignum, Obtusafuran, Latifolin, Quinone reductase, DPPH, Chemoprevention

INTRODUCTION

NAD(P)H:quinone oxidoreductase (quinone reductase; QR; EC1.6.99.2), a cytosolic FAD-containing flavoprotein, forms one of the important components of the phase II drug-metabolizing enzyme systems. It is found in all tested mammalian species and is expressed in many organs, including the liver. QR catalyzes the two-electron reduction of quinones to hydroquinones, thereby suppressing the formation of the superoxide anion radical. Hydroquinones are relatively stable and are subject to detoxification through the glucuronide conjugation reaction (Prochaska and Talalay, 1991). The role of QR is clearly demonstrated in targeted gene-disruption experiments. Radjendirane *et al.* (1998) produced DT-diaphorase (NQO1)-null mice and found that the knock-out animals were considerably more sensitive to the toxicity of menadione.

A wide variety of agents that protect against chemical carcinogenesis are also inducers of phase II enzymes in many animal cells and tissues, and there is convincing evidence that the induction of phase II enzymes is an important mechanism underlying cancer chemoprevention. Many classes of naturally occurring and synthetic compounds, such as β-naphthoflavone, tert-butylhydroxyanisole, and sulforafane, have been identified as potential chemopreventive agents based on their QR-inducing properties (De Long *et al.*, 1986; Prochaska and Talalay, 1988; Zhang *et al.*, 1994). Interestingly, some inducers of QR can also act as inducers of glutathione S-transferase, which detoxifies various types of xenobiotics by conjugating them with glutathione (Prochaska and Talalay, 1988; Talalay *et al.*, 1988; Spencer *et al.*, 1990). An antioxidant response element is usually found in the 5' flanking regions of the genes that code for phase II enzymes, which may be recognized by a similar series of transcription factors (Primiano *et al.*, 1997). Therefore, the induction of QR not only protects against quinone-mediated cytotoxicity, but also acts as a potential mechanism in the prevention of chemical carcinogenesis (Mehta *et al.*, 2002).

Dalbergiae Lignum is a Chinese medicinal plant, which has been reported to produce vasorelaxant, anti-inflammatory, and antiallergenic effects (Yu *et al.*, 1995; Yu and Kuo, 1995; Chan *et al.*, 1998). Although some reports have been published regarding the antioxidant effects of butein, one of the active components of this medicinal plant, little is known of its effects on drug metabolizing enzymes. The present investigation was undertaken to assess the QR-inducing activity of the Dalbergiae Lignum extract and to identify the active principles of the plant.

Correspondence to: Byung-Hoon Lee, College of Pharmacy, Wonkwang University, 344-2 Sinyong-dong, Iksan, Jeonbuk 570-749, Korea
Tel: 82-63-850-6806, Fax: 82-63-856-5606
E-mail: bhlee@wonkwang.ac.kr

MATERIALS AND METHODS

Materials and cell culture

β-Naphthoflavone, menadione, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO). Hepa1c1c1 cells were maintained in the logarithmic phase of growth in DMEM medium (GIBCO BRL, Grand Island, NY), which was supplemented with heat inactivated 10% fetal bovine serum (GIBCO BRL) and 2 mM L-glutamine (Sigma Chemical Co), at 37°C in a 5% CO_2-95% air humidified incubator.

Preparation of the plant extracts and isolation of active compounds

Dalbergiae Lignum (1.2 kg) was extracted with 2 L of hot methanol (MeOH) for 2 h. Dried residue of the extract (173 g) was dissolved in 60% aqueous MeOH (1 L) and partitioned with *n*-hexane and CH_2Cl_2, successively. The MeOH fraction was evaporated, dissolved in distilled water, and partitioned with *n*-butanol (BuOH). The active substances were isolated from the CH_2Cl_2 soluble fraction through the use of silica gel column chromatography.

DPPH free radical scavenging activity

Reduction of 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH) to diphenylpicryl hydrazine by ENN was measured spectrophotometrically at 517 nm using the methods of Ratty *et al.* (1998). L-Ascorbic acid was used as a reference compound, and data were expressed as the percent decrease in the absorbance compared to the control.

Enzyme assay for QR

For the evaluation of QR activity, cultured mouse hepa1c1c7 cells were used as described previously (Gerhäuser *et al.*, 1997; Prochaska and Santamaria, 1998). Briefly, the cells were plated at 5,000 cells/96-well plate and were cultured for 24 h. They were treated with each sample for 48 h, and the medium was removed. Next, the cells were lysed with 0.8% (w/v) digitonin in 2 mM EDTA (pH 7.6), and then, the protein content was determined. QR activity was measured through the NADPH-dependent menadione-mediated reduction of MTT to a blue formazan at 620 nm after incubation at 30°C for 5 min. The induction of QR activity was calculated from the ratio of enzyme activities of treated cells in comparison with a solvent control. Positive controls using β-naphthoflavone (3 μM) were run each time the assay was performed.

RESULTS AND DISCUSSION

Epidemiological studies have demonstrated that the consumption of selective diets protects against many kinds of epithelial tumors (Graham, 1983; Le Marchand *et al.*, 1989; You *et al.*, 1989). It is widely recognized that these diets induce phase II enzymes *in vivo*, protect rodents from toxins and carcinogens, and contain antimutagens or anticarcinogens. In the course of a continuing search for anticancer agents from natural products, Dalbergiae Lignum was evaluated for cancer chemopreventive activity and was identified as an inducer of phase II detoxification enzymes. Extracts of Dalbergiae Lignum prepared with MeOH, BuOH, CH_2Cl_2, or *n*-hexane were tested for QR activity in hepa1c1c7 cells. Each solvent extract produced a dose-dependent induction of QR activity, as shown in Fig. 2. The order of effectiveness of the fraction was CH_2Cl_2 fraction > *n*-hexane fraction > MeOH fraction > BuOH fraction with CD values of 29.5, 37.2, 48.9, and 61.6 μg/mL, respectively. Significant QR-inducing activity was observed at concentrations higher than 5 μg/mL. The CH_2Cl_2 extract, which showed the lowest CD value, was further separated into six compounds by silica-gel column chromatography. The structures of four of these compounds were identified, as shown in Fig. 1.

We next evaluated the QR-inducing effects and DPPH-free-radical-scavenging effects of the following four compounds: 4-methoxydalbergione, latifolin, 4',6-dihydroxy-7-methoxyflavanone, and obtusafuran. They induced QR-specific activity in a dose-dependent manner (Fig. 3). Obtusafuran was identified as the most potent inducer, with a CD value of 1.1 μM and an IC_{50} for the inhibition of cell growth of 108.9 μM (Table I). The ratio between the IC_{50} and CD values, which was previously defined as the chemopreventive index (CI) (Gerhäuser *et al.*, 1997), was calculated to be 101.9, i.e., the margin between activity and toxicity was relatively broad. Because of its lower cytotoxicity, the chemopreventive potential of latifolin was higher than that of obtusafuran despite its low QR-

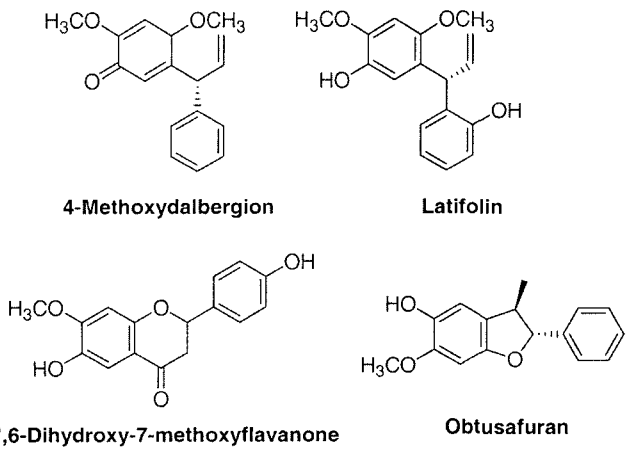

Fig. 1. Structures of compounds that were isolated from the extract of Dalbergiae Lignum.

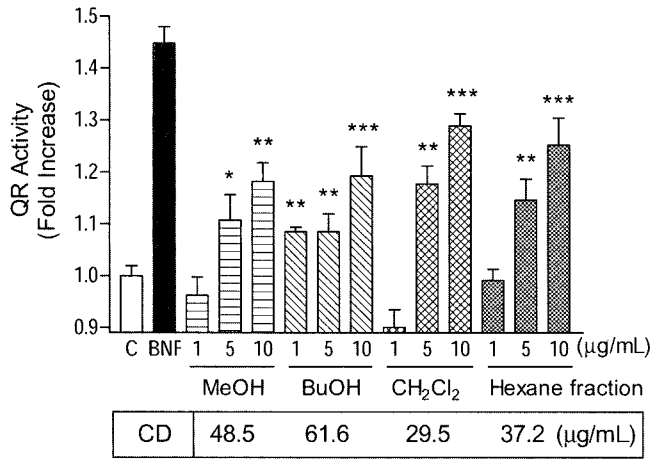

Fig. 2. Effect of the solvent extract of Dalbergiae Lignum on the QR activity in hepa1c1c7 cells. The cells were treated with the extracts for 48 h, and the QR activity was measured as described in Materials and Methods. Each bar represents the mean ± SD for the results from 3 separate experiments. Significantly different from control (C) (*p<0.05; **p<0.01; ***p<0.001). β-Naphtoflavone (BNF) was used for the positive control (3 μM).

inducing activity (CI = 154.6) (Table I).

The activation of carcinogens can lead to the generation of radicals, which have the potential to interact with and damage DNA. Their radical-scavenging potential was determined by their reaction with DPPH free radicals, and their scavenging potential was compared with a solvent control and vitamin C. In addition, the half-maximal scavenging concentration (SC_{50}) was calculated. Latifolin and 4-methoxydalbergione were identified as strong DPPH-scavengers with SC_{50} values of 15.9 and 17.2 μM, respectively, indicating an activity that is about three-folds higher than that of vitamin C, which was used as a positive control (Table I).

Table I. Effects of compounds isolated from CH_2Cl_2 fraction of dalbergiae lignum on quinone reductase activity and DPPH free radical scavenging

Compound	QR Activity			DPPH
	IC_{50} (μM)[a]	CD (μM)[b]	CI[c]	SC_{50} (μM)[d]
4-Methoxydalbergione	129.2	2.2	59.7	17.2
Obtusafuran	108.9	1.1	101.9	168.4
Latifolin	261.7	1.7	154.6	15.9
4,6-Dihydroxy-7-methoxyflavanone	158.5	1.3	124.3	35.9

[a] IC_{50} (half-maximal inhibitory concentration): The concentration at which growth of hepa1c1c7 cells is inhibited by 50% is determined by the MTT assay and calculated by GraphPad Prism™ software.
[b] CD: concentration required to double the activity of QR
[c] CI: ratio between IC_{50}s and CDs.
[d] SC_{50}: half-maximal free radical scavenging concentration. SC_{50} for vitamin C as positive control was 50.5 μM.

A large number of potential chemopreventive agents have been discovered from natural products. A macromolecular targeting strategy that encompasses a variety of pathways and is capable of detecting the major classes of chemopreventive agents was used for the screening of chemopreventive effects. Some molecular targets have also been identified with which the molecular mechanisms of action can be evaluated (Mehta and Pezzuto, 2002). The potential of plant extracts inducing QR activity in hepa1c1c7 cells is a useful marker of their potential to detoxify chemical carcinogens because the specific activity

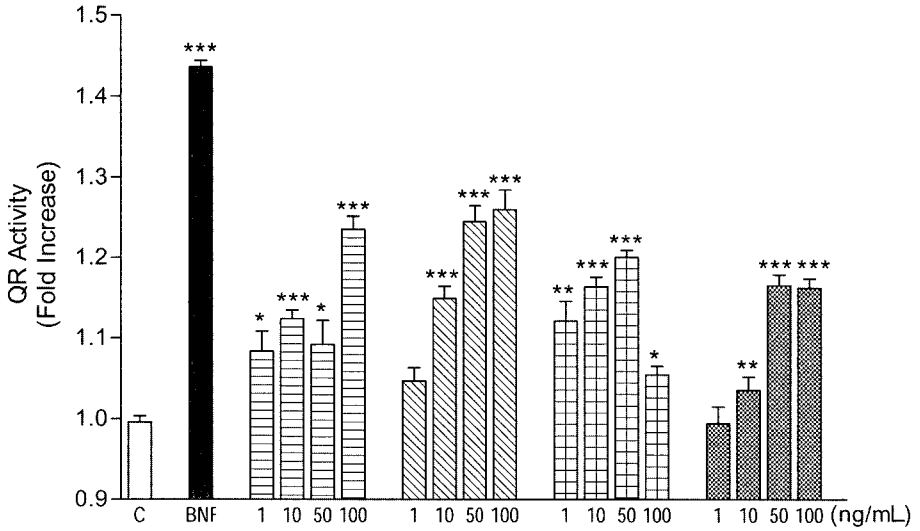

Fig. 3. Induction of QR activity by 4 compounds isolated from the CH_2Cl_2 fraction of Dalbergia Lignum. The cells were treated with 4-methoxydalbergione (▤), obtusafuran (▧), latifolin (▦), and 4,6-dihydroxy-7-methoxyflavanone (▩) for 48 h, and the QR activity was measured as described in Materials and Methods. Each bar represents the mean ± SD for the results from 3 separate experiments. Significantly different from control (C) (*p<0.05; ** p<0.01; ***p<0.001). β-Naphtoflavone (BNF) was used for the positive control (3 μM).

of QR is induced simultaneously with other phase II detoxification enzymes in many animal tissues in response to various chemopreventive agents (De Long *et al.*, 1985; Talalay *et al.*, 1988; Spencer *et al.*, 1990).

In summary, obtusafuran, latifolin, and 4',6-dihydroxy-7-methoxyflavanone were shown to be potent inducers of QR, which is a carcinogen-detoxifying enzyme. Moreover, latifolin was identified as a strong radical scavenger through a DPPH bioassay. These results suggest that the extract of Dalbergiae Lignum or some active compounds therein could be used as chemopreventive agents. Further studies focusing on the direct chemopreventive action of these extracts and compounds and further analysis of the nature of their chemopreventive activity could lead to the development of new cancer preventative agents.

ACKNOWLEDGEMENT

This research was supported by a grant (PF002105-01) from Plant Diversity Research Center of 21st Century Frontier Research Program funded by Ministry of Science and Technology of Korean government.

REFERENCES

Chan, S. C., Chang, Y. S., Wang, J. P., Chen, S. C., and Kuo, S. C., Three new flavonoids and antiallergic, anti-inflammatory constituents from the heartwood of Dalbergia odorifera. *Planta Med.*, 64, 153-158 (1998).

De Long, M. J., Prochaska, H. J., and Talalay, P., Induction of NAD(P)H:quinone reductase in murine hepatoma cells by phenolic antioxidants, azo dyes, and other chemoprotectors: a model system for the study of anticarcinogens. *Proc. Natl. Acad. Sci. U.S.A.*, 83, 787-791 (1986).

De Long, M. J., Prochaska, H. J., and Talalay, P., Tissue-specific induction patterns of cancer-protective enzymes in mice by *tert*-butyl-4-hydroxyanisole and related substituted phenols. *Cancer Res.*, 45, 546-551 (1995).

Gerhäuser, C., You, M., Liu, J., Moriarty, R. M., Hawthorne, M., Metha, R. G., Moon, R. C., and Pezzuto, J. M., Cancer chemopreventive potential of sulforamate, a novel analogue of sulforaphane that induces phase 2 drug-metabolizing enzymes. *Cancer Res.*, 57, 272-278 (1997).

Graham, S., Results of case-control studies of diet and cancer in Buffalo, New York. *Cancer Res.*, 43, 2409s-2413s (1983).

Le Marchand, L., Yoshizawa, C. N., Kolonel, L. N., Hankin, J. H., and Goodman, M. T., Vegetable consumption and lung cancer risk: a population-based case-control study in Hawaii. *J. Natl. Caner Inst.*, 81, 1158-1164 (1989).

Mehta, R. G. and Pezzuto, J. M., Discovery of cancer preventive agents from natural products: from plants to prevention. *Curr. Oncol. Rep.*, 4, 478-486 (2002).

Primiano, T., Sutter, T. R., and Kensler, T. W., Redox regulation of genes that protect against carcinogens. *Comp. Biochem. Physiol.*, 118, 487-497 (1997).

Prochaska, H. J. and Santamaria, A. B., Direct measurement of NAD(P)H:quinone reductase from cells cultured in microtiter wells: a screening assay for anticarcinogenic enzyme inducers. *Anal. Biochem.*, 169, 328-336 (1988).

Prochaska, H. J. and Talalay, P., Regulatory mechanisms of monofunctional and bifunctional anticarcinogenic enzyme inducers in murine liver. *Cancer Res.*, 48, 4776-4782 (1988).

Prochaska, H. J. and Talalay, P., The role of NAD(P)H:quinone reductase in protection against the toxicity of quinones and related agents. In Sies, H. (Eds), Oxidative Stress: Oxidants and Antioxidants, Academic Press, London, pp. 195-211, (1991).

Radjendirane, V., Joseph, P., Lee, Y. H., Kimura, S., Klein-Szanto, A. J., Gonzalez, F. J., and Jaiswal, A. K., Disruption of the DT diaphorase (NQO1) gene in mice leads to increased menadione toxicity. *J. Biol. Chem.*, 273, 7382-7389 (1998).

Ratty, A. K., Sunamoto, J., and Das, N. P., Interaction of flavonoids with 1,1-diphenyl-2-picrylhydrazyl free radical liposomal membranes and soyabean lipoxygenase 1. *Biochem. Pharmacol.*, 37, 989-995 (1998).

Spencer, S. R., Wilczak, C. A., and Talalay, P., Induction of glutathione transferases and NAD(P)H:quinone reductase by fumaric acid derivatives in rodent cells and tissues. *Cancer Res.*, 50, 7871-7875 (1990)

Talalay, P., De Long, M. J., and Prochaska, H. J., Identification of a common chemical signal regulating the induction of enzymes that protect against chemical carcinogenesis. *Proc. Natl. Acad. Sci. U.S.A.*, 85, 8261-8265 (1988).

You, W. C., Blot, W. J., Chang, Y. S., Ershow, A., Yang, Z. T., An, Q., Henderson, B. E., Fraumeni Jr., J. F., and Wang, T. G., Allium vegetables and reduced risk of stomach cancer. *J. Natl. Cancer Inst.*, 81, 162-164 (1989).

Yu, S. M., Cheng, Z. J., and Kuo, S. C., Endothelium-dependent relaxation of rat aorta by butein, a novel cyclic AMP-specific phosphodiesterase inhibitor. *Eur. J. Pharmacol.*, 280, 69-77 (1995).

Yu, S. M. and Kuo, S. C, Vasorelaxant effect of isoliquiritigenin, a novel soluble guanylate cyclase activator, in rat aorta. *Br. J. Pharmacol.*, 114, 1587-1594 (1995).

Zhang, Y., Kensler, T. W., Cho, C. G., Posner, G. H., and Talalay, P., Anticarcinogenic activities of sulforaphane and structurally related synthetic norbornyl isothiocyanates. *Proc. Natl. Acad. Sci. U.S.A.*, 91, 3147-3150 (1994).