RESEARCH COMMUNICATION

Quinone Reductase Inducers in *Azadirachta indica* A. Juss Flowers, and their Mechanisms of Action

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Abstract

We have previously shown that the flowers of neem tree (*Azadirachta indica* A. Juss, family Meliaceae), Thai variety, strongly induced the activity of glutathione *S*-transferase (GST) while resulting in a significant reduction in the activities of some cytochrome P_{450}-dependent monooxygenases in rat liver, and possess cancer chemopreventive potential against chemically-induced mammary gland and liver carcinogenesis in rats. In the present study, 2 chemicals possessing strong QR inducing activity were fractionated from neem flowers using a bioassay based on the induction of QR activity in mouse hepatoma Hepa 1c1c7 cultured cells. Spectroscopic characteristics revealed that these compounds were nimbolide and chlorophylls, having CD (concentration required to double QR specific activity) values of 0.16 and 3.8 μg/ml, respectively. Nimbolide is a known constituent of neem leaves, but was found for the first time here in the flowers. Both nimbolide and chlorophylls strongly enhanced the level of QR mRNA in Hepa 1c1c7 cells, as monitored by northern blot hybridization, indicating that the mechanism by which these constituents of neem flowers induced QR activity is the induction of QR gene expression. These findings may have implication on cancer chemopreventive potential of neem flowers in experimental rats previously reported.

Key Words: *Azadirachta indica* A. Juss - Neem flowers - Quinone reductase -Nimbolide - Chlorophylls - Hepa 1c1c7 cells

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Introduction

Cancer chemoprevention is a new promising strategy for cancer prevention. It means the use of pharmacologic or natural agents that inhibit the development of invasive cancer either by blocking the DNA damage or by arresting or reversing the progression of premalignant cells in which such damage has already occurred (Hong and Sporn, 1997). Chemopreventive agents may function by a variety of mechanisms, and induction of phase II detoxification enzymes, such as glutathione *S*-transferase (GST) and NAD(P)H:quinone oxidoreductase [quinone reductase (QR)], is one of the major mechanism of protection against initiation of chemically induced carcinogenesis (Talalay et al., 1995). At present, a large number of cancer chemopreventive agents have been isolated from vegetables and fruits. These include, for examples, phenethyl isothiocyanates (Fenwick et al., 1983; Kelloff et al., 1996a; Hecht, 2000), sulforaphane (Zhang et al., 1992, 1994) and indole-3-carbinol (Grubbs et al., 1995; Kelloff et al., 1996b) from cruciferous vegetables, organosulfur compounds from garlic (Wargovich et al., 1992; Hong et al., 1992; Haber-Mignard et al., 1996), epigallocathechin gallate from green tea (Yang and Wang, 1993; Kelloff et al., 1996c), curcumin from curcuma longa (Kelloff et al., 1996d; Chuang et al., 2000) and lycopene from tomato (Giovannucci, 1999; Kelloff et al., 2000). Many of these compounds have the ability to induce phase II detoxification enzymes, either GST in animals or QR in murine hepatoma cell line (Delong et al., 1986; Sparnin et al., 1988; van Lieshout et al., 1996), and some of these compounds have been identified solely on the basis of their ability to induce phase II enzymes, especially QR (Lam et al., 1982; Zhang et al., 1992, 1994).

Neem tree (*Azadirachta indica* A. Juss, family Meliaceae), Thai variety, is the evergreen tree growing throughout Thailand. Neem tree is the magic tree, almost every part of the tree has been used for a long time in agriculture and traditional medicine (van der Nat et al.,

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1991). Flowers and young leaves are also among the common vegetables eaten in Thailand especially in the winter. We have previously reported that the extracts of neem flowers and young leaves exhibited antimutagenicity against indirect mutagens/carcinogens towards *Salmonella typhimurium* (Rojanapo and Tepsuwan, 1992; Kusamran et al., 1998b). In addition, dietary neem flowers could inhibit the formation of micronucleated erythrocytes in mice induced by 9,10-dimethyl-1,2-benzanthracene (DMBA) (Kupradinun et al., 1997). It has also been clearly demonstrated in our laboratory that dietary neem flowers caused a marked increase in the activity of GST in rat liver, while resulting in a significant reduction in the activities of some cytochrome P_{450}-dependent monooxygenases, namely, aminopyrine demethylase, aniline hydroxylase, as well as the capacity to metabolically activate the mutagenicities of aflatoxin B_1 (AFB_1) and benzo(a)pyrene [B(a)P] towards *Salmonella typhimurium* (Kusamran et al., 1998a). Interestingly, neem flowers exhibited strong chemopreventive potential against AFB_1-induced liver and DMBA-induced mammary gland carcinogenesis in rats (Tepsuwan et al., 2002). These results strongly indicate that neem flowers contain cancer chemopreventive agents that probably possess the ability to induce phase II enzymes. It is, therefore, of much interest to isolate and characterize such novel compounds, and to study their possible mechanism of phase II enzyme induction as well as their anticarcinogenic activity.

QR induction assay in Hepa 1c1c7 cells is a convenient method for rapid screening the compounds having QR inducing capacity (Prochaska et al., 1992) and anticarcinogenic phase II enzyme inducers (Prochaska and Santamaria, 1988). It is also now widely used as the bioassay method to guide the isolation of cancer chemopreventive agents from plants (Zhang et al., 1992; Mehta and Pezzuto, 2002; Jang et al., 2003). In the present study we, therefore, used this bioassay as a guided method to isolate and purify such novel compounds from neem flowers. In this communication we also report the effect of these compounds on the level of QR mRNA in Hepa 1c1c7 cells.

Materials and Methods

Chemicals

α-Minimal essential medium (α-MEM without both ribonucleosides and deoxyribonucleosides), fetal calf serum and TRIzol reagent were purchased from Gibco BRL, life technologies (Grand island, NY), β–NADP from Yeast Oriental Co. Ltd. (Osaka, Japan), and dimethyl sulfoxide (DMSO), spectrophotometric grade, from Merck (Darmstadt, Germany). 3-(4- Morpholino) propanesulfonic acid, 100 base-pair ladder and RNA ladder marker were obtained from Amersham Pharmacia International (UK), *Pst* I restriction endonucleus from Biolabs Inc., and Dig High Prime Labeling, Detection Starter kit I and digitonin from Boehringer Mannheim (USA). Seakem LE agarose was obtained from FMC Bio Products (USA), and CDP-Star™ substrate and polyATtract system III, IV from Promega Life Science (USA). Antibiotics and chemicals used for the determination of QR activity were from Sigma-Aldrich Co. (MO, USA), and all other chemicals were of analytical grade and obtained locally.

Plant Material

Neem tree, Thai variety, was formerly named as *Azadirachta indica* A. Juss var. *siamensis* Valeton. But until recently, taxonomists decided to place the plant as *Azadirachta indica* A. Juss. This plant belongs to the family Meliaceae.

Bunches of neem flowers were purchased from local markets in Bangkok, Thailand. The specimen was compared with that at the herbaria of the Ministry of Agriculture, Thailand (SN035710). Flowers were removed from the stems, successively washed with tap water, distilled water, and finally freeze-dried. Freeze-dried materials were blended to powder and stored at -20°C until extraction.

Hepa 1c1c7 Cells and QR cDNA Probe

Murine hepatoma Hepa 1c1c7 cell line was kindly provided by Drs. Nebert Frank and Clarissa Gerhauser (German Cancer Research Center, Heidelberg, Germany). The plasmid pDTD55 that was used as the source of QR cDNA probe was a gift from Professor Cecil B. Pickett and Dr. Truyen Nguyen, Schering-Plough Research Institute, USA.

Isolation and Characterization of QR Inducers from Neem Flowers

Lyophilized neem flower powder was successively extracted with petroleum ether, chloroform, ethyl acetate and methanol using a vacuum soxhlet apparatus at 40°C. All extracts were evaporated using a rotary evaporator at 40°C, dissolved in DMSO and determined for their QR inducing capacity in Hepa 1c1c7 mouse hepatoma cells. Chloroform and petroleum ether extracts that exhibited strongest QR inducing activity were further fractionated by silica gel column chromatography using petroleum ether, petroleum ether and chloroform, chloroform, chloroform and ethyl acetate, ethyl acetate and methanol as eluents. The major fractions were then assayed for QR inducing capacity. Compound A was crystallized after evaporation of a fraction showing strongest CD value. After re-crystallization in chloroform and methanol, pure compound was obtained. Another fraction showing strong QR inducing activity and single spot on thin layer chromatography (TLC) plate was submitted to purification using preparative TLC and Sephadex LH-20 column chromatography, another pure compound (compound B) was obtained. The identification of pure compounds was performed either by spectroscopic techniques, such as infrared spectrometry (IR), mass spectrometry (MS) and ^{1}H-nuclear magnetic resonance (^{1}H-NMR) (compound A), or by comparing both TLC characteristic and absorption spectrum with known compound (compound B).

Determination of QR Activity in Hepa 1c1c7 Cells

QR activity was measured in mouse hepatoma Hepa 1c1c7 cells essentially as previously described by Prochaska and Santamaria (Prochaska and Santamaria, 1988) and Gerhauser et al. (Gerhauser et al., 1997). Briefly, cells were grown in 96-well plates in α-MEM medium (200 µl of $1x10^4$ cells/ml) containing 100 units/ml penicillin G sodium, 100 µg/ml streptomycin sulfate, 250 ng/ml amphotericin B and 10% heat-treated fetal calf serum at 37° C in a 5% CO_2 atmosphere. After 24 hr of preincubation, the medium was changed, and 10 µl of test compounds dissolved in 10% DMSO (final concentration of DMSO was 0.5%) was added and the plates were subsequently incubated for an additional 48 hr. QR activity in one set of test plates was assayed by measuring the NADPH-dependent menadiol-mediated reduction of 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide to a blue formazan, while protein was determined by crystal violet staining of an identical set of plates. QR induction was calculated from the ratio of enzyme specific activities of compound-treated cells in comparison with those of control cells. CD value, representing the concentration required to double QR specific activity, and CI (chemopreventive index) value, representing the ratio of the IC_{50} (concentration required to inhibit cell growth of by 50%) and the CD, were thereafter generated.

Preparation of Labeled QR cDNA Probe

QR cDNA probe (~ 0.9 kb) was prepared as a *Pst* I fragment of the 1.9 kb cDNA fragment of the plasmid pDTD55. Briefly, the plasmid pDTD55 was propagated in *E. coli* competent cells (K12 JM109) and then purified using QIAGEN-tip 100. QR cDNA probe was prepared from purified pDTD55 following *Pst* I digestion, agarose gel electrophoresis and QIA quick spin-column chromatography. After that, QR cDNA probe (~ 0.9 kb) was labeled with digoxigenin (Dig High Prime labeling kit) and used for hybridization.

Analysis of QR mRNA Expression in Hepa 1c1c7 Mouse Hepatoma Cells

Hepa 1c1c7 mouse hepatoma cells ($1 x 10^5$ cells/ml) were cultured in 75 cm^2 tissue culture flasks containing 10 ml of α-MEM medium as described in the previous section. After preincubation for 24 hr, cells were treated with test compounds in DMSO for an additional 24 hr. After that, cells were lyzed, total RNA was extracted by using TRIzol reagent and mRNA was isolated using PolyATtract system III and IV according to the manufacturer's protocol (Promega Life Science, USA) and then size fractionated by 1.2% agarose/0.36 M formaldehyde gel electrophoresis. The poly A-mRNA in formaldehyde gel was transferred to a Hybond™ -N+ nylon transfer membrane, hybridized with the digoxigenin-labeled 0.9-kb QR cDNA and then incubated with anti-digoxigenin-AP (Boehringer Mannheim Corp., USA). After equilibrating in CDP-Star™ substrate, the membrane was exposed to the chemiluminescent films (Amersham International plc). The blot was stripped and reprobed with the coding region of the β-actin gene that was used as an internal control.

Statistical Analyses

The data were analyzed for statistical significance using Student's *t*-test. *P* values less than 0.05 were considered significant.

Results

Isolation and Characterization of QR Inducers from Neem Flowers

Neem flower powder was successively extracted with petroleum ether, chloroform, ethyl acetate and methanol, and determined for the capacity to induce QR activity in Hepa 1c1c7 cells. The chloroform extract showed strongest induction capacity and followed by petroleum ether, ethyl acetate and methanol extracts with CD values being 2.0, 6.7, 13.0 and 23.3 µg/ml, respectively (Table 1). On the other hand, chloroform extract was most toxic to the cells among the 4 extracts, showing IC_{50} value of 8.3 µg/ml. However, CI value of chloroform extract was highest comparing to those of petroleum ether and ethyl acetate extracts, being 4.15, 3.04 and 2.50 µg/ml, respectively. The yield of chloroform and petroleum ether extracts was high (Table 1), these 2 extracts were therefore subjected to further fractionation.

Fractionation of chloroform extract using silica gel column chromatography yielded 14 fractions. Eleven fractions were active in inducing QR activity with CD values being less than 10 µg/ml. Compound A, a major constituent in a fraction with CD value of 1.9 µg/ml, was isolated by crystallization and identified by spectroscopic techniques such as IR, MS and ^{1}H-NMR. This compound was a white crystal having m.p. 231.5-232.5°C. IR (KBr) spectrum showed v_{max} at 1678, 1736 and 1782 cm^{-1} indicating the presence of -C = C-, - O-C = O, -C=O, respectively. MS (EI) spectrum of compound A showed M^+ ion at m/z 466 as the base peak and the other abundant ions at m/z 435, 422, 335 and 259. ^{1}H-NMR ($CDCl_3$) spectrum showed δ : 1.23,

Table 1. Yield and QR Inducing Activity of Neem Flower Extracts in Hepa 1c1c7 Cells

Extract	Yield (% dry wt.)	CD (µg/ml)	IC_{50} (µg/ml)	CI
Petroleum ether	2.1	6.7	20.3	3.03
Chloroform	3.4	2.0	8.3	4.15
Ethyl acetate	0.2	13.0	32.5	2.50
Methanol	2.1	23.3	>50	>2.15

Results are means of 2 separate assays
Ground freeze-dried neem flowers were extracted sequentially by petroleum ether, chloroform, ethyl acetate and methanol, and each extract was assayed for the capacity to induce QR in Hepa 1c1c7 cells as described in Materials and Methods.
CD, concentration required to double QR specific activity
IC_{50}, concentration required to inhibit cell growth by 50%
CI, chemopreventive index

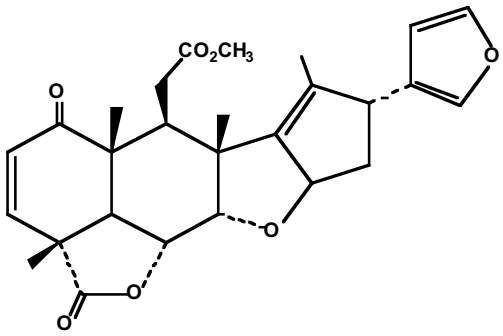

Figure 1. Structure of Nimbolide (compound A)

3H (S, -CH_3); 1.37, 3H (S, -CH_3); 1.48, 3H (S, -CH_3); 1.71, 3H (S, -CH_3); 2.08, 1H (t, *J* = 8 Hz, -CH,); 2.14, 1H (t, *J* = 8 Hz, -CH,); 2.38, 1H (dd, *J* = 5.5, 16 Hz, -CH,); 2.74, 1H (t, *J* = 5.5 Hz, -CH-); 3.18, 1H (d, *J* = 12 Hz, -CH-); 3.26, 1H (dd, *J* = 5.5 Hz, -CH-); 3.56, 3H (S, -OCH_3); 3.67, 1H (d, *J* = 8 Hz, -CH-); 4.27, 1H (d, *J* = 3.5 Hz, -CH-); 4.63, 1H (dd, *J* = 3.5,12 Hz, -CH-); 5.52. 1H (tt, *J* = 1.5,8 Hz, -CH-); 5.92, 1H (d, *J* = 10 Hz, -CH=C); 6.26, 1H (dd, *J* = 1.2 Hz, AR-H); 7.22, 1H (dd, *J* = 1 Hz, AR-H); 7.28 1H (d, *J* = 10 Hz, C=CH-); 7.32, 1H (t, *J* = 1.5 Hz, AR-H). These characteristics allowed us to conclude compound A as nimbolide. Its structure, shown in Figure 1, was also confirmed by comparing the ^{1}H-NMR spectrum with that of authentic nimbolide. This compound exhibited very strong QR inducing activity (Figure 2A) with a CD value of 0.16 μg/ml or 0.35 μM (Table 2), however, it was quite toxic to Hepa 1c1c7 cells (Figure 2A), showing IC_{50} of 0.42 mg/ml or 0.89 μM (Table 2). CI value of nimbolide was then calculated and found to be 2.62 (Table 2).

Compound B was isolated from a fraction, which exhibited a CD value of 2.1 μg/ml and showed a single green spot on TLC plate, by preparative TLC and Sephadex LH-20 column chromatography. The UV spectrum of this compound showed maximum absorption at 413 and 668 nm which is the characteristic of chlorophylls (Gross, 1991). Moreover, its TLC characteristic was also identical to chlorophylls of other sources. Thus, it was then concluded that this compound might be chlorophyll mixture. However, an attempt to separate this mixture failed to obtain chlorophyll a and chlorophyll b due to decomposition. This chlorophyll mixture had moderate QR inducing activity (Figure 2B) with the CD value of 3.8 μg/ml (Table 2). However, it did not exhibit any toxicity to the cell culture although it was tested up to 25 μg/ml (Figure 2B). The CI value was therefore found to be greater than 6.6 (Table 2).

Table 2. QR Inducing Activity, Toxicity and Chemopreventive Index of Nimbolide and Chlorophyllsk

Compound	CD (μg/ml)	IC_{50} (μg/ml)	CI
Nimbolide	0.16 ± 0.02 (0.35 ± 0.04 mM)	0.42 ± 0.02 (0.91 ± 0.05 mM)	2.62 ± 0.15
Chlorophylls	3.80 ± 0.20	> 25	> 6.60 ± 0.35

Results are means ± SD of 3 separate assays
CD, concentration required to double QR specific activity
IC_{50}, concentration required to inhibit cell growth by 50%
CI, chemopreventive index

Petroleum ether extract was also fractionated using silica gel column chromatography to afford 27 fractions. Nine fractions were active, showing CD values being less than 10 μg/ml. Among these, two fractions that exhibited CD values of 1.3 and 1.8 μg/ml were submitted to TLC analysis. The major constituent in these 2 fractions showed the same characteristic as compound A, thus they were not submitted to further purification. Another 2 fractions that also showed low CD values (1.1 and 1.5 μg/ml) were submitted to further purification, but failed to obtain pure compound with low CD values.

Effects of Nimbolide and Chlorophylls on QR mRNA Level in Hepa 1c1c7 Cells

mRNAs were extracted from Hepa 1c1c7 cells treated separately with DMSO (0.5%, negative control), chlorophylls (25 μg/ml), nimbolide (0.52 μg/ml or 1.12 μM) and β–naphthoflavone (β–NF, 1 μM, positive control) using the TRIzole reagent and polyATtract systems, size-fractionated and visualized by hybridization with digoxigenin-labeled 0.9-kb QR cDNA probe as described in Materials and Methods. Results in Fig. 3A revealed that the levels of QR mRNA (1.4 kb) in cells treated with chlorophylls (lane 2), nimbolide (lane 3), and β–NF (lane 4) were much higher than that in DMSO treated cells (lane 1). The level of QR mRNA in cells treated with chlorophylls was comparable to that in nimbolide treated cells. The level of β-actin mRNA, which was used as an internal control of total mRNA extraction, was comparable in most cases (Figure 3B).

Discussion

Isolation and Characterization of QR Inducers from Neem Flowers

Biological-guided fractionation of neem flowers, which has recently been shown in our laboratory to exhibit cancer

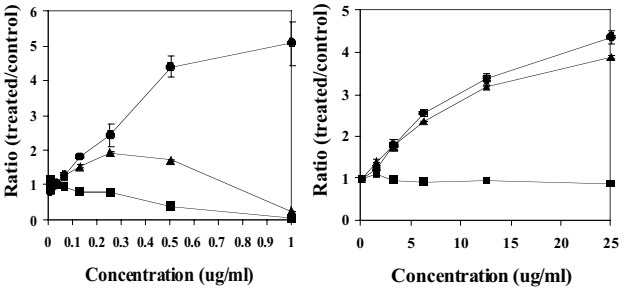

Figure 2. Induction of QR Activity, QR Specific Activity and Protein Level by Nimbolide (A) and Chlorophylls (B) in Hepa 1c1c7 Cells. Cells were grown for 24 h and then exposed to nimbolide and chlorophylls in a concentration range of 0.0625-1 and 1.5625-25 μg/ml, respectively, for 48 h. QR activity (▲) and protein level (■) in compound-treated cells were measured and compared with those of solvent-treated cells (0.5% DMSO final concentration) to calculate their ratio as well as QR specific activity (●).

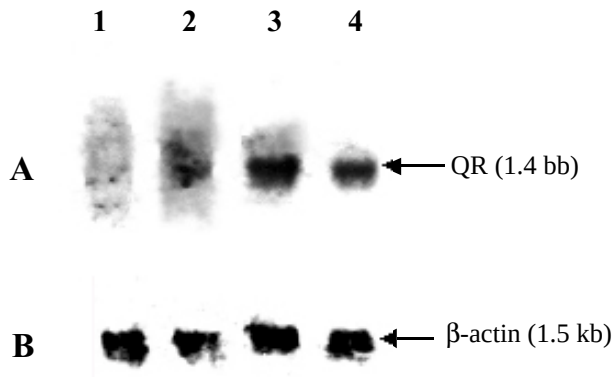

Figure 3. Northern Blot Hybridization Analysis of QR mRNA Levels in Hepa 1c1c7 Cells after Treatment with Nimbolide and Chlorophylls. Eight μg of total mRNA was loaded per lane. A, hybridization with a QR cDNA probe. B, hybridization with a *β*-actin cDNA probe. Lane 1, treatment with DMSO (0.5% final concentration, solvent control); Lane 2, treatment with 25 μg/ml chlorophylls; Lane 3, treatment with 0.52 μg/ml or 1.12 μM nimbolide; Lane 4, treatment with 1 μM *β*-NF (positive control).

chemopreventive potential against chemically-induced liver and mammary gland carcinogenesis, based on the induction of QR in Hepa 1c1c7 mouse hepatoma cells led to the isolation of 2 compounds, namely, nimbolide and chlorophylls. There were other fractions possessing QR induction capacity, however, they were not further separated due to small amount obtained. Nimbolide has been found abundantly in neem leaves (Rochanakij et al., 1985; Kigodi et al., 1989; van der Nat et al., 1991), but has never been reported in the flowers. In addition, it has never been previously shown to induce either QR or other phase II enzymes. On the contrary, chlorophylls are common constituents of all green plants, thus their presence in neem flowers observed here is not unexpected, since most of the flowers that we used were not yet bloomed and were still green. They have also never been previously reported to induce either QR or other phase II enzymes. However, chlorophyllin, their water-soluble derivative, has been reported to induce GST activity in mice (Singh et al., 1996a, 1996b) and to exhibit cancer chemopreventive potential in various systems (Dashwood et al., 1998; Xu and Dashwood, 1999).

Both nimbolide and chlorophylls were isolated from chloroform extract that showed strongest QR inducing capacity among 4 extracts of neem flowers. Nimbolide exhibited extremely strong QR inducing activity with CD value of 0.16 μg/ml or 0.35 μM, comparable with that of sulforaphane (Zhang et al., 1992; Gerhauser et al., 1997), a well-known chemopreventive agent in broccoli. On the other hand, however, chlorophylls showed moderate inducing capacity with CD value of 3.8 μg/ml. It has been mentioned that compounds showing CD values less than 10 μg/ml were considered active (Chang et al., 1997). According to the major mechanism for the induction of phase II enzymes of cancer chemopreventive agents, it may be suggested that nimbolide and chlorophylls may have chemopreventive potentials. Considering the properties of neem flowers that include phase II enzyme induction (Kusamran et al., 1998a) and cancer chemopreventive potential in experimental animals (Tepsuwan et al., 2002), the results here also suggest that these 2 compounds might be major active constituents responsible for the induction of phase II enzymes and might also involve, at least partly, in the chemopreventive potentials of neem flowers.

Nimbolide has never been determined for its chemopreventive potential, however, it is one of the limonoids, the compounds possessing cancer chemopreventive potential. Some limonoids, for example, nomilin and limonin from citrus fruits, have been found to induce increased activity of GST, and the increased enzyme activity was correlated with the ability of these compounds to inhibit B(a)P-induced forestomach tumor in mice (Lam and Hasegawa, 1989). In addition, limonin has also been shown to increase GST activity as well as to reduce azoxymethane-induced rat colon carcinogenesis (Tanaka et al., 2000). Thus, this is another information to support that nimbolide might have cancer chemopreventive property in which we are currently investigating in our laboratory.

On the other hand, nimbolide was quite toxic to Hepa 1c1c7 cells, showing IC_{50} value of 0.42 μg/ml or 0.89 μM while chlorophylls was not toxic although it was tested up to 25 μg/ml. Nimbolide has been reported to exhibit cytotoxicity to various human and mouse cell lines (Cohen et al., 1996; Cui et al., 1998). The IC_{50} value of nimbolide in this study was much less than that previously reported in some cell lines. Hence, it is also of much interest to further pursue the anticancer activity of this compound.

Effects of Nimbolide and Chlorophylls on QR mRNA Level in Hepa 1c1c7 Cells

Regarding to the effect of nimbolide and chlorophylls on the induction of QR, both of them strongly increased the level of QR mRNA in Hepa 1c1c7 cells. The amount of induced QR mRNA seemed to correlate with the specific activity of the enzyme. These results suggest that these 2 compounds induced QR activity by inducing QR gene expression, thereby resulting in the increased synthesis of QR mRNA and protein. This mechanism of induction is also reported for other inducers of phase II detoxification enzymes, for examples, benzyl isothiocyanate, *tert*-butylhydroquinone, 1,2-dithiole-3-thione, *β* -NF and sulforaphane (Prestera, et al., 1993).

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