PHYTOTHERAPY RESEARCH
Phytother. Res. **22**, 1500–1505 (2008)
Published online 13 August 2008 in Wiley InterScience
(www.interscience.wiley.com) **DOI**: 10.1002/ptr.2521

Nrf2-mediated Induction of Detoxifying Enzymes by Alantolactone Present in *Inula helenium*

Ji Yeon Seo[1], Soon Sung Lim[2], Ju Ryoung Kim[1], Ji-Sun Lim[1], Young Ran Ha[1], In Ae Lee[1], Eun Ji Kim[2], Jung Han Yoon Park[2] and Jong-Sang Kim[1]*

[1]Department of Animal Science and Biotechnology, Kyungpook National University, Deagu 702-701, Republic of Korea
[2]Regional Innovation Center and Department of Food Science and Nutrition, Hallym University, Chuncheon, 200-702, Republic of Korea

Our previous study showed that a methanol extract of *Inula helenium* had the potential to induce detoxifying enzymes such as quinone reductase (QR) and glutathione *S*-transferase (GST) activity. In this study the methanol extract was further fractionated using silica gel chromatography and vacuum liquid chromatography, to yield pure compounds alantolactone and isoalantolactone as QR inducers. Alantolactone caused a dose-dependent induction of antioxidant enzymes including QR, GST, γ-glutamylcysteine synthase, glutathione reductase, and heme oxygenase 1 in hepa1c1c7 mouse hepatoma cells. The compound increased the luciferase activity of HepG2-C8 cells, transfectants carrying antioxidant response element (ARE)-luciferase gene, in a dose-dependent manner, suggesting ARE-mediated transcriptional activation of antioxidant enzymes. Alantolactone also stimulated the nuclear accumulation of Nrf2 that was inhibited by phosphatidylinositol 3-kinase (PI3K) inhibitors. In conclusion, alantolactone appears to induce detoxifying enzymes via activation of PI3K and JNK signaling pathways, leading to translocation of Nrf2, and subsequent interaction between Nrf2 and ARE in the encoding genes. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: Inula helenium; alantolactone; detoxifying enzymes; Nrf2; PI3K.

INTRODUCTION

It is widely recognized that induction of cellular defence genes, such as phase 2 detoxifying and antioxidant enzyme genes, play an important biological role in protection against carcinogenesis and in the attenuation of cancer development (Talalay *et al.*, 2004; Jaiswal, 2000). A wide variety of chemical compounds including dithiolethiones, isothiocyanates, diphenols, quinones, Michael reaction acceptors and heavy metals can inhibit the carcinogenesis process by inducing phase 2 enzymes which detoxify toxic molecules by directly destroying their reactive centres and reactive oxygen species (ROS), or by conjugating them with endogenous ligands, facilitating their excretion from the cells (Begleiter and Fourier, 2004). The induction of phase 2 enzymes was reported to be mediated by Nrf2 (nuclear factor erythroid-related factor 2), which is a leucine zipper-type transcription factor and known to regulate its various genes through a common DNA regulatory element, called antioxidant response element (ARE) (Sporn and Liby, 2005).

* Correspondence to: Dr Jong-Sang Kim, Department of Animal Science and Biotechnology, Kyungpook National University, Daegu 702-701, Korea.
E-mail: vision@knu.ac.kr
Contract/grant sponsor: Korea Research Foundation Grant funded by the Korean Government (MOEHRD); contract/grant number: KRF-2005-F00055.
Contract/grant sponsor: BioGreen 21 Program, Rural Development Administration, Republic of Korea; contract/grant number: 20070301-034-039-007-03-00.

Our previous study showed that a methanol extract of *Inula helenium* contained a significantly high capacity to induce NAD(P)H:(quinone acceptor) oxidoreductase 1 (EC 1.6.99.2) (QR) activity (Im *et al.*, 2007). The herb has been traditionally used for asthma, as an expectorant and insecticide (Chevallier, 2001). The methanol extract of the plant was further fractionated and it was found that the hexane fraction was most active in inducing detoxifying enzymes such as QR and glutathione *S*-transferase (GST). In this study the compounds responsible for induction of phase 2 detoxifying enzymes were isolated by using silica gel chromatography and vacuum liquid chromatography (VLC) and identified by spectrometry. Alantolactone identified as one of the QR inducers was subjected to further study to investigate its action mechanism in cultured cell system.

MATERIALS AND METHODS

Materials. All cell culture reagents and fetal bovine serum were obtained from Gibco BRL (Gaitherburg, MD, USA). Hepa1c1c7 and BPRc1 cells were from the American Type Culture Collection (Rockville, MD, USA). All other chemicals were of reagent grade. The roots of *Inula helenium* L. were purchased from DeaGuang in Chuncheon in 2006. A voucher (No. 325) is deposited at the Hallym University RIC Center in Chuncheon, Republic of Korea.

Cell culture. Hepa1c1c7 and its mutant (BPRc1) cells were plated at a density of 3×10^5 and 5×10^5 cells per

Received 8 August 2007
Revised 29 December 2007
Accepted 4 February 2008

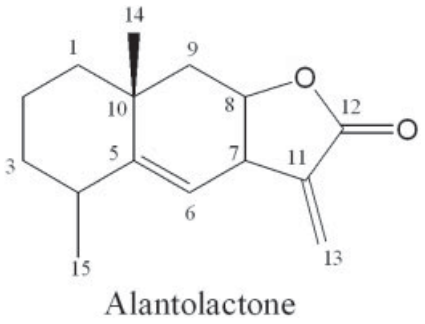

Figure 1. Structure of alantolactone.

100 mm plate (Nunc, Rochester, NY) in 10 mL of α-MEM supplemented with 10% FBS, respectively. The HepG2-C8 cell line established in Dr An Kong's laboratory at Rutgers, The State University of New Jersey, by transfecting human hepatoma HepG2 cells with pARE-TI-luciferase construct was used for the reporter assay (Kim *et al.*, 2003). HepG2-C8 cells were maintained in modified DMEM supplemented with 10% FBS, GlutaMax (Gibco No. 35050-061), 100 units/mL penicillin and 0.5 mg/mL G418. The cells were normally starved overnight in 0.5% FBS-containing medium before treatment. The cells were cultured for 48 h, exposed to various concentrations of sample for another 24 h, followed by biochemical assays.

Extraction and purification of QR inducer from *Inula helenium*. Roots (1.5 kg) of *Inula helenium* were air-dried followed by grinding in a Willey-Mill plant grinder. The ground plant material was soaked in *n*-hexane (8.5 L) for 24 h. The solvent was decanted from the plant residue and evaporated *in vacuo* to yield 40.8 g of crude extract. A portion of *n*-hexane extract (5.2 g) of the herbal root was adsorbed to silica gel and applied to a silica gel chromatography column (40–63 μm, 60 × 300 mm, 60 Å). Elution of the column was performed using increasing polarity mixtures of *n*-hexane/EtOAc in a series of three linear gradient steps. Step 1 consisted of 100/0 to 90/10 using 2 L with step 2 consisting of 90/10 to 75/25 using 1 L. Step 3 consisted of 75/25 to 0/100 using 1 L and the column was washed with 2 L of EtOAc. Column eluate was collected in 30 mL test tubes and, based on TLC similarities, recombined into 13 fractions (1, 1–60, 80 mg; 2, 61–75, 85 mg; 3, 76–89, 1.3 g; 4, 90–105, 620 mg; 5, 106–145, 45 mg; 6, 1–60, 55 mg; 7, 61–75, 86 mg; 8, 76–89, 1.2 g; 9, 90–105, 520 mg; 10, 106–145, 52 mg; 11, 1–60, 65 mg; 12, 61–75, 96 mg; 13, 76–89, 1.1 g). Further purification of bioactive fraction 8 was accomplished using repeated VLC procedures on silica gel, as described previously (Cantrell *et al.*, 1999), to yield pure compound **1** (57 mg). The purity of this compound was 98.8% determined by HPLC peak area percentage.

Identification of alantolactone. The identification of QR inducer was accomplished by comparison of spectr scopic data with those reported in the literature (Marshall and Cohen, 1964; Kauer and Kalsi, 1985). ^{1}H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ on a Bruker Avance 400 MHz spectrometer (Billerica, Massachusetts, USA). All ^{13}C multiplicities were deduced from 90° and 135° DEPT experiments. IR spectra were recorded on a FT-IR 4100 (JASCO Inc., Easton, USA). Mass spectra were obtained on a PolarisQ Ion Trap GC/MS^{n} (Thermo Fisher Scientific Inc., Waltham, USA). Vacuum liquid chromatography (VLC) separations were carried out on silica gel (Macherey-Nagel Kieselgel, Darmstadt, Germany). The analytical properties of the compound were as follows.

Alantolactone: IR υ_{max} cm^{-1}: 1744 (γ-lactone), 1459, 1260. EI-MS 70 eV, *m/z* (rel. int.): 232 [M^{+}] (85), [M-CH_3]$^{+}$ (100), 171 (38), mp: 77–79 °C (lit. 78.5–80 °C) ^{1}H NMR ($CDCl_3$) : δ1.02 (3H, d, J = 8.0 Hz, H-15), 1.08 (1H, dd, J = 13.6, 4.0 Hz, H-1), 1.22 (3H, s, H-14), 1.37 (1H, dt, J = 13.6, 3.6 Hz, H-3), 1.46 (2H, m, H-2), 1.51 (1H, m, H-9), 1.54 (1H, m, H-1), 1.73 (1H, m, H-3), 2.34 (1H, dd, J = 7.6, 2.4 Hz, H-9), 2.39 (1H, m, H-4), 3.52 (1H, m, H-7), 4.76 (1H, m, H-8), 5.09 (1H, d, J = 4.0 Hz, H-6), 5.55 (1H, d, J = 2.0 Hz, H-13), 6.11 (1H, d, J = 2.0 Hz, H-13). ^{13}C NMR ($CDCl_3$): δ16.6 (C-2), 22.4 (C-14), 28.4 (C-15), 32.5 (C-1, 10), 37.4 (C-4), 39.3 (C-7), 41.5 (C-3), 42.5 (C-9), 76.3 (C-8), 118.7 (C-6), 121.4 (C-13), 139.7 (C-11), 148.7 (C-5), 170.2 (C-12).

Biochemical assays. QR activity was measured by a spectrophotometric assay in which the rate of reduction of 2,6-dichlorophenolindophenol was monitored at 600 nm (Benson *et al.*, 1980). GST activity was assayed by the method described by Habig *et al.* (1974), with 1-chloro-2,4-dinitrobenzene as a substrate. *tert*-Butylhydroquinone (TBHQ, 20 μM or 3.3 μg/mL), a known QR inducer, was used as a positive control in all biochemical assays. The specific activity of enzymes was normalized to the protein concentration, which was determined in triplicate using the Lowry assay (1951). All values are reported as mean ± standard deviation (SD) whenever possible.

Assay of reporter gene activity. HepG2-C8 cells were plated in 6-well plates at a density of 5×10^5 cells/well. After 16 h incubation, the cells were cultured in fresh modified DMEM with high glucose containing 0.5% FBS for 12 h before sample treatment. After the cells were cultured for another 16 h in the presence of various concentrations of sample, the cells were collected and the luciferase activity was determined according to the protocol provided by the manufacturer (Promega Corp., Madison, WI) (Kim *et al.*, 2003). Briefly, after sample treatment, the cells were washed twice with ice-cold PBS and harvested in reporter lysis buffer. The homogenates were centrifuged at 12 000 × **g** for 2 min at 4 °C. A 20 μL supernatant was assayed for luciferase activity using TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Luciferase activity was normalized against protein concentration.

Preparation of nuclear protein extract. Nuclear and cytosolic protein extracts were prepared according to the method as described (Nguyen *et al.*, 2005). Briefly, the cells were cultured on 100 mm dishes to 90% confluence and treated with alantolactone for various times. After being washed, the cells were harvested by scraping in ice-cold PBS and collected by centrifugation at 500 × **g** for 5 min. The cells were lysed with buffer A (10 mM HEPES-KOH (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonylfluoride) on ice for 20 min and then centrifuged at 14 000 × **g** for 15 min at 4 °C. The supernatants were saved as the cytoplasmic fractions.

DOI: 10.1002/ptr

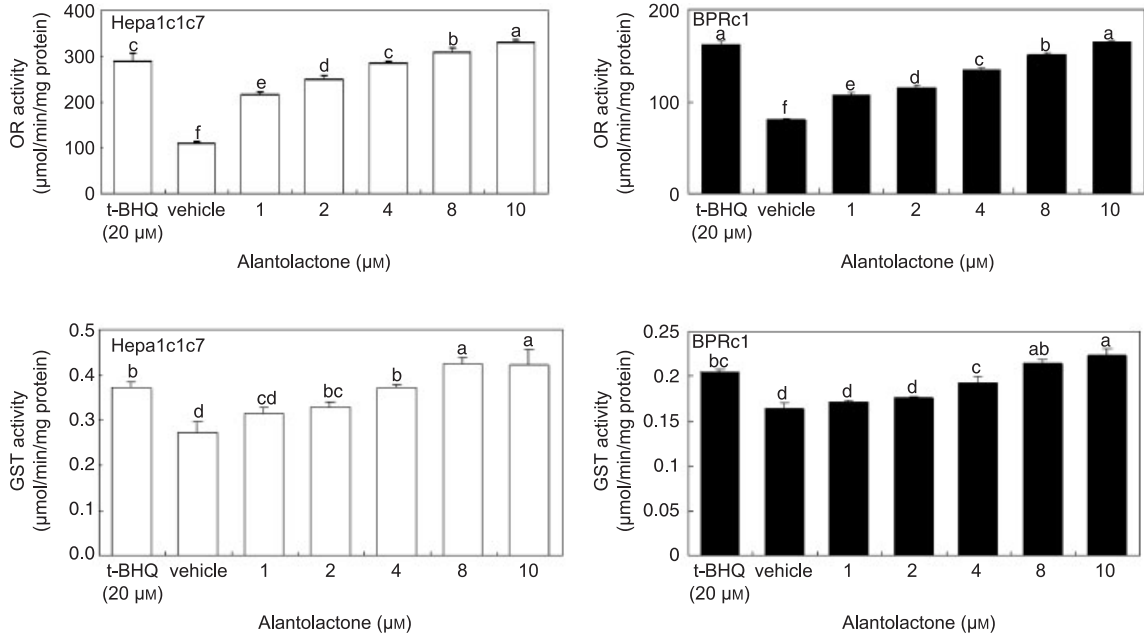

Figure 2. QR and GST-inducing activities of alantolactone in hepa1c1c7 and BPRc1 cells. (A) Quinone reductase activity. (B) Glutathione *S*-transferase activity. Hepa1c1c7 (□) and BPRc1 (■) cells were treated with various doses of alantolactone for 24 h, followed by assaying for QR (A) and GST (B) activities as described in 'Materials and Methods'. Bars represent mean ± SD. Means without common letter differ, $p < 0.05$.

The nuclear pellets were washed three times with buffer A and resuspended in buffer B (20 mM HEPES, 0.5 M KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, pH 7.9) for 30 min at 4 °C on a rotating wheel and then centrifuged at 14 000 × **g** for 15 min at 4 °C. The nuclear fraction was subjected to immunoblot analysis using anti-Nrf2 or anti-SAM antibody.

Western blot. This was performed on cytosolic fractions prepared from cultured cells to estimate the level of detoxifying enzymes according to a protocol described previously (Kim *et al.*, 2004; Im *et al.*, 2007). The primary antibodies including anti-NQO1, anti-GST-pi, anti-HO1, anti-γGCS, anti-GR, anti-Nrf2, anti-β-tubulin and horseradish peroxidase-conjugated secondary antibody anti-goat or anti-rabbit IgG were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

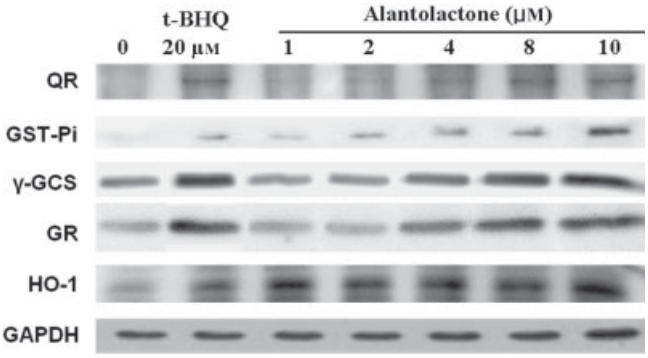

Figure 3. Effect of alantolactone on the expression of phase 2 detoxifying enzymes including QR, GST-pi, γ-GCS, GR, HO-1 in hepa1c1c7 cells. The cells were treated with various doses of alantolactone for 24 h, followed by immunoblot analyses as described in 'Materials and Methods'. QR, NAD(P)H:(quinone acceptor) oxidoreductase 1; GST, glutathione *S*-transferase; HO-1, heme oxygenase; γ-GCS, γ-glutamylcysteine synthase; GR, glutathione reductase.

RESULTS

Effect of alantolactone on QR and GST activities in murine hepatoma cells

The preliminary study demonstrated that the methanol extract of *Inula helenium* caused significant QR activity induction, one of the well-known biomarkers for chemopreventive potential. The methanol extract was further fractionated according to polarity using silica gel chromatography and VLC, and alantolactone identified as a phase 2 enzyme inducer. As shown in Fig. 2, alantolactone increased the activities of QR and GST in a dose-dependent manner in both hepa1c1c7 cells and its mutant BPRc1 cells lacking aryl hydrocarbon receptor nuclear translocator (ARNT), which are typical murine hepatoma cell lines highly responsive to phase 2 enzyme inducers and thereby widely used for screening phase 2 enzyme inducers (Seidal and Denison, 1999; Zhang *et al.*, 1992; Chandra and de Mejia, 2004). In both cases, the induction of QR and GST in hep1c1c7 cells was quantitatively more pronounced than in BPRc1 cells.

Effect of alantolactone on expression of phase 2 detoxifying enzymes in hepa1c1c7 cells

As alantolactone enhanced the activities of QR and GST significantly, we were also interested in examining its effect on the expression of other phase 2 or antioxidant enzymes including γ-glutamylcysteine synthetase (γ-GCS), glutathione reductase (GR) and heme oxygenase 1 (HO1) by western blot. As shown in Fig. 3, alantolactone increased the dose-dependent induction

DOI: 10.1002/ptr

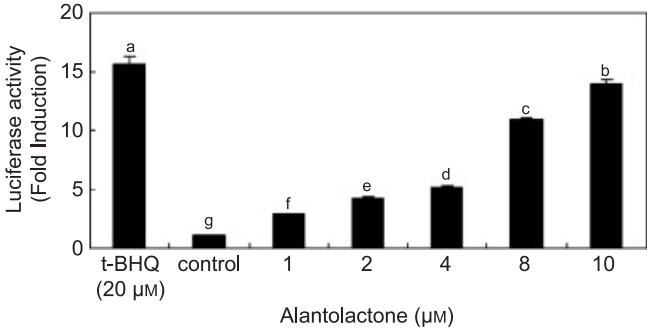

Figure 4. Induction of ARE-luciferase activities by alantolactone in HepG2-C8 cells which were generated by transfecting plasmid containing ARE-luciferase gene into HepG2 cells. Bars represent mean ± SD. Means without common letter differ, $p < 0.05$.

of γ-GCS, GR, HO1, QR and GST-pi in hepa1c1c7 cells exposed for 24 h. In particular, the expression of HO-1 most sensitively responded to alantolactone, increasing the enzyme level in cells exposed to as low as 1 μM of the compound.

Dose-response on the expression of pARE-TI-luciferase

We investigated whether induction of phase 2 detoxifying enzymes is mediated via antioxidant response element (ARE) on respective genes by using HepG2-C8 cells transfected with pARE-TI-luciferase construct. Alantolactone, which has shown significant induction of some phase 2 enzymes, was added to HepG2-C8 cells at the indicated concentrations as described in Fig. 4. The luciferase activity of HepG2-C8 cells was assayed as described in Materials and Methods and normalized by the protein concentration of the cells. The fold induction of the luciferase activity was calculated by dividing the treated samples by the control. The induction of luciferase activity was increased in a dose-dependent manner, and the induction level at a concentration of 10 μM alantolactone was 14-fold over the control. This result suggests that the induction of phase 2 detoxifying enzymes by alantolactone is mediated by the *cis*-acting ARE in the promoter regions of the encoding genes.

Translocation of Nrf2 into nucleus by alantolactone

There is good evidence that ARE in the genes of phase 2 enzymes is bound by transcriptional factors such as Nrf2 for transcriptional activation (Sporn and Liby, 2005; Kim *et al.*, 2003). Also most phase 2 enzyme inducers release Nrf2 from Kelch-like ECH-associated protein 1 (Keap1), facilitating the translocation of Nrf2 into the nucleus, where Nrf2 plays a pivotal role in the transcriptional activation of enzymes involved in the removal of toxic chemicals including reactive oxygen species. When hepa1c1c7 cells were treated with alantolactone, the cells accumulated Nrf2 in the nucleus in a dose-dependent manner (Fig. 5A). The nuclear Nrf2 accumulation reached a maximum level when exposed to alantolactone (8 μM) for 6 to 8 h, and the longer exposure decreased the nuclear Nrf2 level (Fig. 5B), may be due to cytotoxicity.

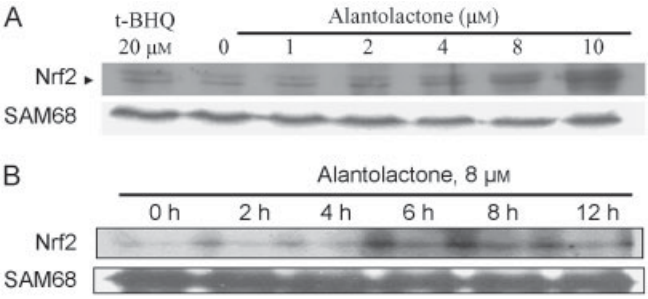

Figure 5. Translocation into nucleus of Nrf2 by alantolactone in hepa1c1c7 cells. (A) Cells were treated with different concentrations of alantolactone for 24 h. (B) Cells were treated with 8 μM alantolactone for different periods. Antibody against Sam 68 was used as loading control of nuclear extract.

Regulation of nuclear translocation of Nrf2 by kinase inhibitors

To determine whether nuclear translocation of Nrf2 by alantolactone is associated with some kinases such as protein kinase C (PKC), Jun *N*-terminal kinase (JNK), extracellular signal-regulated protein kinase 1/2 (ERK 1/2), p38 mitogen-activated protein kinase (p38 MAPK) and phosphatidylinositol 3-kinases (PI3K), hepa1c1c7 cells were treated with alantolactone with and without kinase inhibitors such as PD-98059 (ERK1/2 inhibitor), SB203580 (p38 MAPK inhibitor), SP600125 (JNK inhibitor), staurosporine (PKC inhibitor), LY29004 and wortmannin (PI3K inhibitors), followed by observing the nuclear accumulation of Nrf2 using western blot. As shown in Fig. 6, PI3K inhibitors, LY29004 and wortmannin significantly suppressed the nuclear accumulation of Nrf2 stimulated by alantolactone while SP600125, a JNK inhibitor, caused a weak yet significant repression of the compound-induced accumulation of Nrf2 in the nuclear compartment.

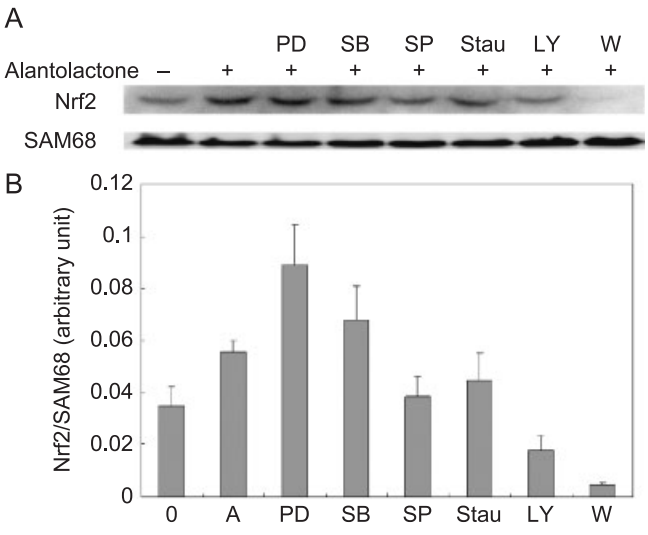

Figure 6. Effect of kinase inhibitors on alantolactone-induced nuclear translocation of Nrf2. Hepa1c1c7 cells were treated with alantolactone in the absence and presence of kinase inhibitors, PD (20 μM PD98059, ERK1/2 inhibitor), SB (20 μM SB203580, p38 MAPK inhibitor), SP (20 μM SP600125, JNK inhibitor), Stau (0.3 μM staurosporine, PKC inhibitor), LY (40 μM LY29004, PI3K inhibitor) or W (2 μM wortmannin, PI3K inhibitor) and subjected to western blot. (A) A representative blot showing Nrf2 in the nuclear fraction. (B) Densitometry of blots showing means from three separate experiments normalized to SAM68.

DOI: 10.1002/ptr

DISCUSSION

Our previous study showed that the methanol extract of *Inula helenium* (Compositae) has strong potential to induce quinone reductase (Im *et al.*, 2007). In this study the methanol extract was further fractionated using silica gel chromatography and VLC and two pure compounds isolated as QR inducers. Among them, alantolactone showed strong potential to induce phase 2 detoxifying enzymes, mediated by nuclear translocation or accumulation of Nrf2, leading to transcriptional activation via the interaction between the Nrf2 and ARE sequence in the relevant genes. Nrf2 is a member of the cap'n'collar family of transcription factors and possesses a highly conserved basic region leucine zipper structure (Chan and Kan, 1999; Moi *et al.*, 1994; Kang *et al.*, 2007). The roles of Nrf2 in the regulation of the expression of many detoxifying and antioxidant enzymes under oxidative stress have been verified in experiments using Nrf2-deficient mice in which the expression of these enzymes is dramatically attenuated, and these mice are much more susceptible to carcinogen-induced toxicity and carcinogenesis (Chan and Kwong, 2000). Nrf2 is sequestered by the inhibitory protein, Keap1, until inducers react with cysteine thiol residues on Keap1, which causes conformational change in the Keap1-Nrf2 complex and the release of Nrf2, allowing Nrf2 activate responsive genes (Begleiter and Fourier, 2004; Kwak *et al.*, 2002). The Nrf2-keap1-ARE pathway was reported to be activated by numerous chemopreventive agents ranging from those naturally found in food, such as resveratrol, curcumin and sulforaphane, to synthetic molecules such as oltipraz, which are being tested in clinical trials (Talalay *et al.*, 2003; Zhang and Goedon, 2004; Keum *et al.*, 2006). Furthermore, the α-methylene-γ-butyrolactone structural unit, which characterizes a group of naturally occurring sesquiterpene lactones, is known to possess numerous biological activities including the induction of heme oxygenase 1 (Jeong *et al.*, 2007). The data from our study and other groups (Im *et al.*, 2007; Jeong *et al.*, 2007; Kim *et al.*, 2003) suggest the involvement of the nuclear translocation of Nrf2 in the regulation of phase 2 detoxifying enzymes.

Multiple signaling kinases have been reported to regulate ARE, which include p38 MAPK, ERK, JNK, PI3K, PKC and the pancreatic endoplasmic reticulum kinase (PERK). All these kinases have been reported to positively regulate ARE-mediated gene expression with the exception of p38 MAPK which was shown to phosphorylate Nrf2 and promotes the association between Nrf2 and Keap1 proteins, thereby potentially inhibiting the nuclear translocation of Nrf2 (Keum *et al.*, 2006).

This study demonstrated that PI3K among the kinases might play an important role in the nuclear translocation of Nrf2 by alantolactone because the stimulation of nuclear Nrf2 accumulation by alantolactone was significantly attenuated by JNK (SP600125) and PI3K inhibitors (LY29004 and wortmannin) (Fig. 6). However, nuclear translocation of Nrf2 by alantolactone was not suppressed by some other inhibitors of Erk1/2 (PD-98059), p38 MAPK (SB-203580), and PKC (staurosporine), suggesting that induction of phase 2 enzymes by alantolactone might be caused independently of ERK1/2, PKC and p38 MAPK.

In conclusion, the data strongly suggest that induction of phase 2 enzymes by alantolactone isolated from *Inula helenium* might be mediated by the Nrf2-ARE pathway. More specifically, the stabilization of Nrf2 might be brought about when alantolactone or its reactive intermediates stimulated Nrf2 phosphorylation through PI3K and JNK, which triggered its dissociation from Keap1 and dampened its rate of degradation by the proteasome. In addition, alantolactone and/or its reactive intermediates may also cause direct modifications of Keap1, inhibiting its activity and thus contributing to the stabilization of Nrf2 (Nguyen *et al.*, 2005; Tanigawa *et al.*, 2007). There is also a possibility that alantolactone modulates the GSH/GSSG ratio and thereby activates the Nrf2-ARE pathway (Kim *et al.*, 2003). Therefore, future studies focusing on the cross-talk between signaling pathways in phase 2 enzyme expression will lead to a better understanding of the action mechanism and efficacy of alantolactone in cancer prevention.

Acknowledgements

This study was supported by a grant from the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2005-F00055) and a grant (20070301-034-039-007-03-00) from BioGreen 21 Program, Rural Development Administration, Republic of Korea.

REFERENCES

Begleiter A, Fourie J. 2004. Induction of NQO1 in cancer cells. *Methods Enzymol* **382**: 320–351.

Benson AM, Hunkeler MJ, Talalay P. 1980. Increase of NAD(P)H: quinone reductase by dietary antioxidants: possible role in protection against carcinogenesis and toxicity. *Proc Natl Acad Sci USA* **77**: 5216–5220.

Cantrell CL, Abate L, Fronczek FR, Franzlau SG, Quijano L, Fischer NH. 1999. Antimycobacterial eudesmanolides from *Inula helenium* and *Rudbeckia subtomentosa. Planta Med* **65**: 351–355.

Chan K, Kan YW. 1999. Nrf2 is essential for protection against acute pulmonary injury in mice. *Proc Natl Acad Sci USA* **96**: 12731–12736.

Chan JY, Kwong M. 2000. Impaired expression of glutathione synthetic enzyme genes in mice with targeted deletion of the Nrf2 basic-leucine zipper protein. *Biochim Biophys Acta* **1517**: 19–26.

Chandra S, de Mejia EG. 2004. Polyphenolic compounds, antioxidant capacity, and quinone reductase activity of an aqueous extract of *Ardisia compressa* in comparison to mate (*Ilex paraguariensis*) and green (*Camellia sinensis*) teas. *J Agric Food Chem* **52**: 3583–3589.

Chevallier A. 2001. *Encylopedia of Medicinal Plants*. Dorling Kindersley: London, 109.

Habig WH, Pabst MJ, Jakoby WB. 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem* **249**: 7130–7139.

Im SS, Kim JR, Lim HA *et al.* 2007. Induction of detoxifying enzyme by sesquiterpenes present in *Inula helenium. J Med Food* **10**: 100–106.

Jaiswal AK. 2000. Regulation of genes encoding NAD(P)H: quinone oxidoreductases. *Free Radic Biol Med* **29**: 254–262.

Jeong GS, Pae HO, Jeong SO *et al.* 2007. The alpha-methylene-gamma-butyrolactone moiety in dehydrocostus lactone is

responsible for cytoprotective heme oxygenase-1 expression through activation of the nuclear factor E2-related factor 2 in HepG2 cells. *Eur J Pharmacol* **565**: 37–44.

Kang ES, Woo IS, Kim HJ *et al.* 2007. Up-regulation of aldose reductase expression mediated by phosphatidylinositol 3-kinase/Akt and Nrf2 is involved in the protective effect of curcumin against oxidative damage. *Free Radic Biol Med* **43**: 535–545.

Kaur B, Kalsi PS. 1985. Stereostructures of inunal and isoalloalantolactone, two biologically active sesquiterpene lactones from *Inula racemosa*. *Phytochemistry* **24**: 2007–2010.

Keum YS, Yu S, Chang PP *et al.* 2006. Mechanism of action of sulforaphane: Inhibition of p38 mitogen-activated protein kinase isoforms contributing to the induction of antioxidant response element-mediated heme oxygenase-1 in human hepatoma HepG2 Cells. *Cancer Res* **66**: 8804–8813.

Kim BR, Hu R, Keum YS *et al.* 2003. Effects of glutathione on antioxidant response element-mediated gene expression and apoptosis elicited by sulforaphane. *Cancer Res* **63**: 7520–7525.

Kim JH, Lee JS, Song KS, Kwon CS, Kim YK, Kim JS. 2004. Polyozellin isolated from *Polyozellus multiplex* induces phase 2 enzymes in mouse hepatoma cells and differentiation in human myeloid leukaemic cell lines. *J Agric Food Chem* **52**: 451–455.

Kwak M-K, Wakabayashi N, Kensler TW. 2002. Chemoprevention through the Keap1-Nrf2 signaling pathway by phase 2 enzyme inducers. *Mutat Res* **555**: 133–148.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275.

Marshall JA, Cohen N. 1964. The structure of alantolactone. *J Org Chem* **29**: 3727–3729.

Moi P, Chan K, Asunis I, Cao A, Kan YW. 1994. Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. *Proc Natl Acad Sci USA* **91**: 9926–9930.

Nguyen T, Sherratt PJ, Nioi P, Yang CS, Pickett CB. 2005. Nrf2 controls constitutive and inducible expression of ARE-driven genes through a dynamic pathway involving nucleocytoplasmic shuttling by Keap1. *J Biol Chem* **280**: 32485–32492.

Seidel SD, Denison MS. 1999. Differential gene expression in wild-type and arnt-defective mouse hepatoma (Hepa1c1c7) cells. *Toxicol Sci* **52**: 217–225.

Sporn MB, Liby KT. 2005. Cancer chemoprevention: scientific promise, clinical uncertainty. *Nat Clin Pract Oncol* **2**: 518–525.

Talalay P, Dinkova-Kostova AT. 2004. Role of nicotinamide quinone oxidoreductase 1 (NQO1) in protection against toxicity of electrophiles and reactive oxygen intermediates. *Methods Enzymol* **382**: 355–364.

Talalay P, Dinkova-Kostova AT, Holtzclaw WD. 2003. Importance of phase 2 gene regulation in protection against electrophile and reactive oxygen toxicity and carcinogenesis. *Adv Enzyme Regul* **43**: 121–134.

Tanigawa S, Fujii M, Hou DX. 2007. Action of Nrf2 and Keap1 in ARE-mediated NQO1 expression by quercetin. *Free Radic Biol Med* **42**: 1690–1703.

Zhang Y, Gordon GB. 2004. A strategy for cancer prevention: stimulation of the Nrf2-ARE signaling pathway. *Mol Cancer Ther* **3**: 885–893.

Zhang Y, Talalay P, Cho CG, Posner GH. 1992. A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. *Proc Natl Acad Sci USA* **89**: 2399–2403.