Bi-Functional Induction of the Quinone Reductase and Cytochrome P450 1A1 by Youngiasides *via* Nrf2-ARE and AhR-XRE Pathways

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Many phytochemicals are known to exert cancer chemopreventive activity by eliminating chemical carcinogens or toxic xenobiotics through the action of detoxification enzymes. In this study, we investigated the cancer chemopreventive effects of youngiasides isolated from *Crepidiastrum denticulatum*. These youngiasides significantly induced quinone reductase (QR) activity in mouse hepatoma Hepa-1c1c7 cells, and showed a relatively high chemoprevention index (CI; divided IC_{50} value with CD value). The youngiasides also significantly induced transcriptional activation of QR in Hepa-QR-secreted alkaline phosphatase (SEAP) cells, which is a stable cell line containing the intact promoter region of QR. In order to determine if upregulation of QR by the youngiasides was mediated through a mono-functional or bi-functional mechanism, we examined the nuclear factor-E2 p45-related factor 2(Nrf2)-antioxidant response element (ARE) and aryl hydrocarbon receptor (AhR)-xenobiotic response element (XRE) pathways, which are two major pathways, involved in regulation of Phase I and/or Phase II detoxification enzymes. The youngiasides increased the cytochrome P450 1A1 (CYP1A1) mRNA and protein levels in human colorectal cancer Caco-2 cells and also increased the QR mRNA and protein levels in Caco-2 cells through ARE and XRE activation which resulted from translocation of Nrf2 and AhR into the nucleus. These results suggest that regulation of QR by the youngiasides was due to bi-functional induction through the Nrf2-ARE and AhR-XRE pathways. Thus, these youngiasides as bi-functional inducers of QR have potential as cancer chemopreventive agents.

Key words youngiaside; quinone reductase; cytochrome P450 1A1; bi-functional inducer; cancer chemoprevention

Cancer chemoprevention is defined as the use of phytochemicals (naturally occurring compounds or their synthetic derivatives) from dietary foods or natural products to inhibit, delay or reverse carcinogenesis or prevent the development of cancer.[1,2] The important strategy of cancer chemoprevention aims to reduce the risk of cancer through modulation of detoxification enzymes involved in metabolic activation. The detoxification/excretion of carcinogens is one of the representative mechanisms of action of chemopreventive agents.[3]

Dietary phytochemicals can be classified into two groups, termed mono- and bi-functional inducers. Mono-functional inducers upregulate a number of phase II detoxification enzymes, including quinone reductase (QR, also known as NAD(P)H: quinone oxidoreductase, NQO1) and glutathione S-transferases (GSTs). The bi-functional inducers upregulate a similar array of phase II enzymes as well as a number of phase I enzymes including cytochrome P450 1A1 (CYP1A1).[4] Since phase I enzymes are involved in both bioactivation and detoxification of carcinogens, the mono-functional inducers, which upregulate only phase II enzymes, are thought to be more closely associated with chemoprevention than are bi-functional inducers that upregulate both phase I and II enzymes. However, the bi-functional inducers can in some cases exert synergistic effects in cancer chemoprevention. Oltpraz, a plant-derived compound, is a promising bi-functional inducer and is found to increase levels of not only phase II, but also phase I, enzymes.[5]

These inducers are known to regulate genes that encode detoxification enzymes and which involve two important promoter sites. One site is known as the antioxidant response element (ARE) associated with induction of phase II detoxification enzymes (mono-functional induction),[6,7] while the second is the xenobiotic response element (XRE) located in the promoters of genes encoding phase II, and some phase I, cytochrome P450 enzymes (bi-functional induction).[8] ARE- and XRE-containing promoters can be activated by binding of specific transcription factors such as nuclear factor E2 (NF-E2) p45-Nrf2 and AhR, respectively. Nrf2 is a member of the CNC (cap'n'collar) basic leucine zipper (bZIP) transcription factor family and AhR is a member of the basic helix–loop–helix/Per-Arnt-Sim (bHLH/PAS) family.[9] Nrf2 is known to translocate into the nucleus and recruit Maf proteins, which is followed by the activation of transcription of ARE-dependent genes.[10] The ligand-bound AhR translocates into the nucleus where it binds as a heterodimer with the AhR nuclear translocator (Arnt) and activates the transcription of XRE-dependent genes.[11] In addition, it was recently revealed that Nrf2 is one of the AhR target genes.[12,13]

In Korea, early sprouts have been used of the compositae plant *Crepidiastrum denticulatum* (HOUTT.) PAK & KAWANO; however, induction of QR by this plant has not yet been reported. Further, the chemical structures of youngiaside A, B, and C isolated from *C. denticulatum* have been elucidated,[14] but their possible physiological uses such as in cancer chemoprevention have not been identified. In this study, we first identified a possible molecular mechanism for the cancer chemopreventive effects of these youngiasides, which showed the most potent chemopreventive activity in Caco-2 human colorectal cells. The application of their cancer chemopreventive effects is also discussed in this manuscript.

METERIALS AND METHODS

Chemicals Whole compositae plants (aerial component

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and roots) of *C. denticulatum* (HOUTT.) PAK & KAWANO were collected in August, 2007, at the Wild Vegetable Experiment Station, Gangwon ARES, Korea, and the voucher specimen (D-043) was stored at the Natural Products Research Center, KIST Gangneung Institute, Gangneung, Korea. Dried plants were extracted by refluxing with 94% EtOH for 3 h. After evaporating the EtOH under reduced pressure, the extract was suspended in distilled water and partitioned sequentially with *n*-hexane, ethyl acetate, and butanol. The youngiasides (youngiaside A: YA; youngiaside B: YB; and youngiaside C; YC) were isolated from the ethyl acetate fraction of *C. denticulatum* and identified by ^{1}H- and ^{13}C-NMR. Their chemical structures are depicted in Fig. 1. Sulforaphane (SFN; 5 μM), β-naphthoflavone (BNF; 5 μM) and 3-methylcholanthrene (3MC; 0.1 μM) were used as positive controls were obtained from Calbiochem (San Diego, CA, U.S.A.), and Sigma chemicals (St. Louis, MO, U.S.A.), respectively.

Cell Viability Test The cytotoxicity of youngiasides was evaluated using the Cell Counting kit (CCK-8, Dojindo Laboratories, Tokyo, Japan) as described previously with slight modifications.[15] Hepa1c1c7 cells (1×10^4 cells per well) were plated onto 96-well tissue culture plates. The absorbance at 450 nm was measured and the absorbance at 600 nm was subtracted using a Synergy HT Multi-microplate reader (Bio-Tek Instruments, Winooski, VT, U.S.A.).

Analysis of Detoxification Enzyme Activity The CYP1A1-dependent 7-ethoxyresorufin-*O*-deethylase (EROD) activity was determined in Hepa1c1c7 cells using ethoxyresorufin (ERF). EROD activity was measured as previously described with minor modifications,[15,16] and QR activity was measured as described previously, also with slight modifications.[17—19] Hepa1c1c7 cells (1×10^4 cells per well) were plated onto 96-well plates and incubated for 24 h prior to treatment. Media containing various concentrations of youngiasides was then added and incubated for an additional 24 h. The absorbance at 610 nm was measured five times at 50 s intervals using a Synergy HT Multi-microplate reader.

RNA Extraction and Real-Time Polymerase Chain Reaction (PCR) Analysis Total RNA was extracted using RNeasy mini kit columns (Qiagen, Valencia, CA, U.S.A.), according to the manufacturer's instructions. The cDNA was prepared from DNase I treated total RNA by random primer (Invitrogen, Carlsbad, CA, U.S.A.) polymerization using 200 U SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's protocol. Real-time quantitative PCR was performed in triplicate in 384-well plates as previously described.[20] The primers designed for Real-time PCR are summarized in Table 1 and were confirmed with the sequences in the NCBI data base. Real-time PCR analysis was performed using an LC480 Detection System (Roche, Indianapolis, IN, U.S.A.).

Transient Transfection and Reporter Gene Assay Caco-2 cells (5×10^4 cells per ml) were cultured in 24-well tissue culture plates for 24 h before transfection at 40—60% confluency. For the measurement of either ARE or XRE activation by youngiasides, Caco-2 cells were transiently co-transfected with 0.5 μg of the pGL3-β-gal construct, and with 2.5 μg of one of two different reporter constructs containing either ARE-QR-CAT or XRE-QR-CAT, derived from the rat QR gene.[6] All CAT (chloramphenicol acetyltransferase) reporter gene constructs were a kind gift from Dr. Cecil Pickett (Schering-Plough Research Institute, NJ, U.S.A.). After 24 h of treatment, cells were lysed and assayed for CAT expression using a CAT-ELISA kit (Roche, Indianapolis, IN, U.S.A.), following the manufacturer's instructions. CAT expression was normalized to β-galactosidase activity.[21]

Table 1. Primer Sequences Used in Real-Time PCR Analysis

Gene name	Accession no.	Primer sequence
mQR	NM008706	5′-CAAGTTTGGCCTCTCTGTGG-3′
		5′-GATCTGGTTGTCAGCTGGAAT-3′
mGAPDH	NM008084	5′-AGCTTGTCATCAACGGGAAG-3′
		5′-TTTGATGTTAGTGGGGTCTCG-3′
QR	NM000903	5′-ATGTATGACAAAGGACCCTTCC-3′
		5′-TCCCTTGCAGAGAGTACATGG-3′
CYP1A1	NM000499	5′-CCCAGCTCAGCTCAGTACCT-3′
		5′-GGAGATTGGGAAAAGCATGA-3′
GAPDH	NM002046	5′-AGCCACATCGCTCAGACAC-3′
		5′-GCCCAATACGACCAAATCC-3′

The assay for QR-secreted alkaline phosphatase (SEAP) activity was based on the original secreted SEAP enzymatic reporter assay described previously.[22,23] The transcriptional activity of QR was measured in Hepa-QR-SEAP cells (1×10^5 cells per ml) plated onto 24-well tissue culture plates, incubated for 24 h, and then treated with various concentrations of youngiasides and incubated for an additional 24 h. The measurement of activity using the Great EscAPe^{TM} SEAP Chemiluminescence Kit 2.0 (Clontech, Palo Alto, CA, U.S.A.), following the manufacturer's instructions, was normalized to the cellular protein content.

Western Blot Analysis The youngiaside-treated cells were lysed in cell lysis buffer (Cell Signaling Technology, Beverly, MA, U.S.A.) with a protease inhibitor cocktail (Sigma, St. Louis, MO, U.S.A.) by sonication. Cytoplasmic and nuclear fractions were isolated using the Nuclear extraction kit (Sigma, St. Louis, MO, U.S.A.), according to the manufacturer's instructions. Proteins were then analyzed by Western blotting as previously described.[20]

Immunofluorescence Assay Caco-2 cells were grown on glass coverslips in 24-well plates. Cells were treated with youngiasides for various periods, then fixed with 4% paraformaldehyde at room temperature for 20 min and with 100% methanol at −20 °C for 15 min, and blocked with phosphate buffered saline (PBS) containing 5% (vol/vol) goat serum and 0.3% Triton X-100 for 1 h. Cells were then incubated overnight at 4 °C with primary antibodies recognizing Nrf2 (1 : 100) and AhR (1 : 50), and diluted in PBST containing 5% (vol/vol) goat serum and 0.3% Triton X-100. Cells were washed with PBS and incubated with Alexa Flour 488-conjugated anti-rabbit or Alexa Flour 594-conjugated anti-goat, secondary antibody (Invitrogen, Carlsbad, CA, U.S.A.) at a 1 : 200 dilution for a further 1 h. Cells were washed and mounted on glass slides with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, U.S.A.). Images were obtained using a Leica TCS SP5 confocal system (Leica, Wetzlar, Germany).

Statistics The results were expressed as fold-induction relative to control. The mean values were obtained from comparison between each treated group and the control, and were analyzed using the one-way ANOVA with Dunnett's

post-hoc test. Differences were considered statistically significant at $p<0.05$.

RESULTS

Increase in QR Activity Exerted by Youngiasides In this study, we found that the extracts and specific fractions of *C. denticulatum* significantly induced QR activity. Among the fractions, the ethyl acetate fraction showed the highest chemoprevention index (CI) value, and so we further isolated three active compounds: YA, YB, and YC (Table 2, Fig. 1). We first investigated the ability of YA, YB, and YC to induce QR specific activity, and dose-dependent experiments were performed in Hepa1c1c7 cells, a model cell line for measuring QR activity and evaluating the CI. The QR activity was measured after treatment with YA, YB, or YC (7.81—500 μM) for 24 h. YA, YB, and YC each induced QR activity in a dose-dependent manner in the non-toxic concentration range of 7.81—250 μM, and decreased QR activity at higher concentrations (250—500 μM) due to cytotoxic effects (Fig. 2A).[20] Furthermore, YA, YB, and YC showed a relatively high CI arising from low cytotoxicity ($IC_{50}>500\ \mu$M) and low CD values (Table 3). The CI was determined by dividing the IC_{50} (concentration required to inhibit cell growth by 50%) with the CD value (concentration required to double the QR activity).[24]

Effects of Youngiasides on Bi-functional Induction We also examined the effects of YA, YB, and YC on transcriptional activation of QR by using a SEAP assay in Hepa-QR-SEAP cells, which is a stable cell line generated by transfection with a phQR-SEAP construct containing the full promoter region of QR. YA, YB, and YC each significantly induced the transcription of QR in a dose-dependent manner at concentrations of 10, 50, and 100 μM (Fig. 2B). The expression of phase II detoxifying genes and antioxidant enzymes is known to be through two essential *cis*-acting elements, ARE and XRE.[6,7] Therefore, we examined QR mRNA levels in two different mouse hepatocarcinoma cell lines, Hepa1c1c7 (wild-type) and BPrc1 (Arnt-deficient), to determine whether the transcriptional activation of QR by YA, YB, and YC was through ARE and/or XRE. The level of induction of QR mRNA expression by YA, YB, and YC was slightly lower in BPrc1 cells than in Hepa1c1c7 cells, while induction by SFN, a mono-functional inducer, was the same between both cell lines (Fig. 2C). The well-known bi-functional inducer β-naphthoflavone (BNF), synthesized from flavonoids, produced the same results as did the youngiasides (data not shown). The activity of the detoxification enzymes supported the concept of youngiasides as bi-functional inducers. We had observed a significant increase in QR activity, as mentioned above, and CYP1A1 enzyme activity was also increased by YA, YB, and YC in Hepa1c1c7 cells relative to controls (Figs. 2A, 4A). To more specifically delineate the mechanism in human cells, Caco-2 cells were used for the next experiments because of their high induction ability, and we tested QR protein expression in several human colorectal cancer cell lines (data not shown).

In Caco-2 cells, QR (Fig. 3) and CYP1A1 (Figs. 4B, C) mRNA and protein expression levels were increased by YA, YB, and YC. SFN, a potent ARE inducer, markedly induced QR but was only a weak activator of CYP1A1, while 3MC (a carcinogen that is a known activator of CYP1A1 through the AhR-XRE pathway) highly induced CYP1A1 but weakly activated QR (Figs. 3, 4). These results suggest that YA, YB, and YC can induce phase I and phase II detoxification enzymes through regulation of their transcription in human colorectal cancer cells, and that YA, YB and YC act in a similar way to other bi-functional inducers, which modulate both phase I and phase II enzymes to enhance the detoxification of carcinogens.

Effects of Youngiasides on QR Activation through the

Table 2. Cytotoxicity and Chemoprevention Index (CI) of Extract and Fractions from *C. denticulatum* in Hepa1c1c7 cells

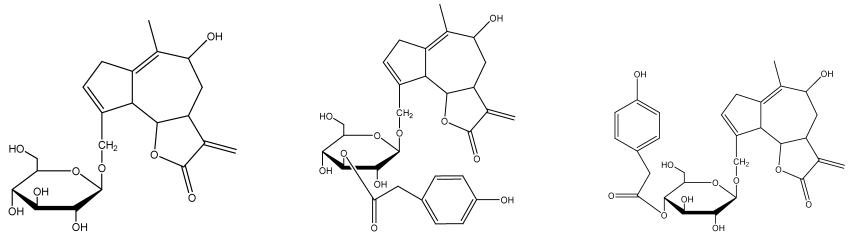
		CD[a]	IC_{50}[b]	CI[c]
Extract (μg/ml)		9.43	>200[d]	21.20
Fractions (μg/ml)	*n*-Hexane	26.26	89.12	3.39
	Ethyl acetate	9.90	151.10	15.27
	n-Butanol	16.81	>200	11.90
	Water	123.82	>200	1.62

a) Concentration required to double the QR activity. *b*) Concentration required to inhibit cell growth by 50%. *c*) The chemoprevention index is obtained by dividing the IC_{50} value with the CD value. *d*) The highest concentration used in these experiments and the minimum range of non-toxic concentration.

Table 3. Cytotoxicity and Chemoprevention Index (CI) of Youngiasides from the Ethyl Acetate Fraction of *C. denticulatum* in Hepa1c1c7 cells

	CD[a]	IC_{50}[b]	CI[c]
Youngiaside A	43.80	>500[d]	11.42
Youngiaside B	52.68	>500	9.49
Youngiaside C	40.01	>500	12.50

a) Concentration required to double QR activity. *b*) Concentration required to inhibit cell growth by 50%. *c*) The chemoprevention index is obtained by dividing the IC_{50} value with the CD value. *d*) The highest concentration used in these experiments and the minimum range of non-toxic concentration.



youngiaside A (YA), MW 424 youngiaside B (YB), MW 558 youngiaside C (YC), MW 558

Fig. 1. Chemical Structures of the Three Youngiasides Used in This Study

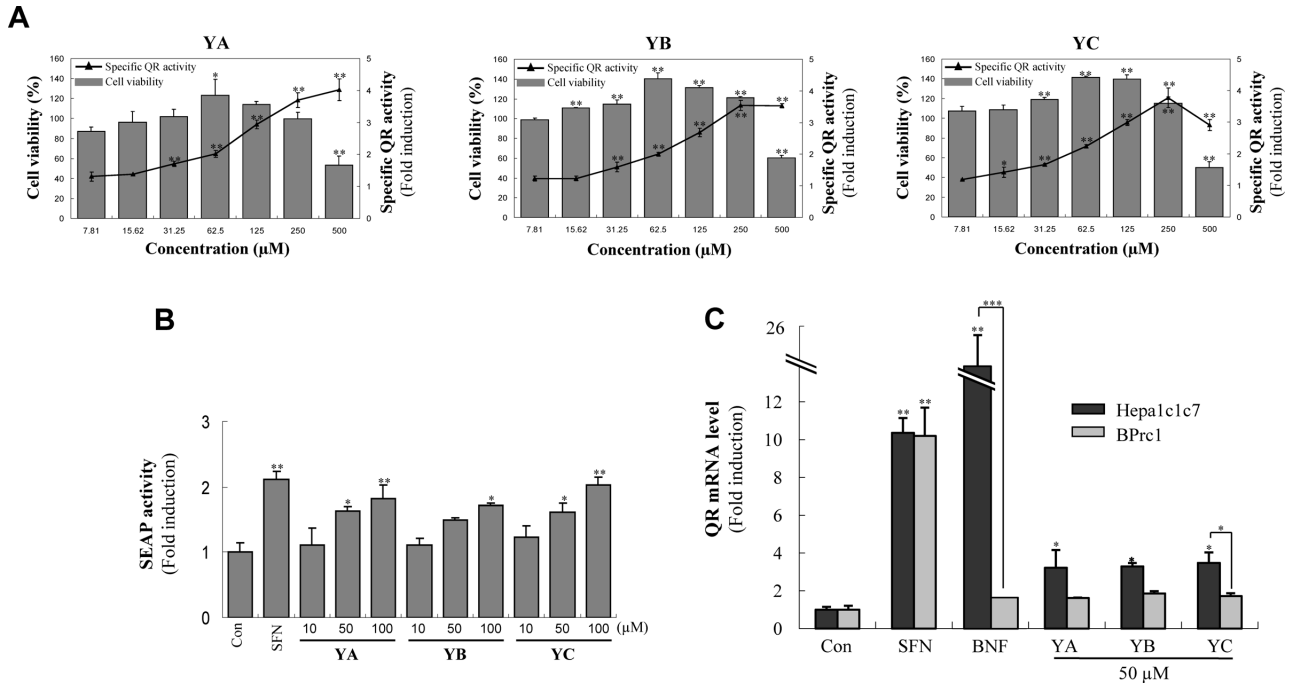


Fig. 2. Induction of QR Activity, mRNA, and Protein Levels by Three Youngiasides, YA, YB and YC, for 24 h in Hepa1c1c7 cells

(A) QR activity induced by three youngiasides at 7.81—500 μM in Hepa1c1c7 cells. Specific QR activity (triangles) was expressed as fold-induction relative to control, and cell viability (bars) was expressed as percentage of untreated control. (B) Transcriptional activation of QR induced by three youngiasides at 10—100 μM in Hepa-QR-SEAP cells. (C) QR mRNA expression induced by three youngiasides at 50 μM in Hepa1c1c7 or BPrc1 cells using reverse transcriptase (RT)-PCR analysis. Each bar represents fold-induction of QR-SEAP activity (B) or QR mRNA levels (C) relative to control. The results represent the mean±S.D.; $n=3$. An asterisk denotes significant difference from control (* $p<0.05$; ** $p<0.01$).

Nrf2-ARE Pathway Bi-functional inducers upregulate phase II enzymes including QR as well as a number of phase I enzymes including CYP1A1.[4] Since we demonstrated that YA, YB, and YC induced both phase I and II enzymes, as outlined above, we further tested activation of the Nrf2-ARE pathway as one of the bi-functional mechanisms of induction of QR. First, we performed a transient transfection assay in Caco-2 cells with a CAT reporter construct containing the ARE consensus (ARE-QR-CAT). CAT activity was measured after treatment with YA, YB, or YC (10—100 μM) for 24 h and, as anticipated, the ARE was significantly activated (Fig. 5A).

It is known that Nrf2 is a key regulator of induction of Phase II detoxification enzymes. When an activator disrupts its complex with Keap1, Nrf2 translocates into the nucleus and induces transcription of Phase II-associated genes.[25] To confirm translocation to the nucleus, we performed Western blot analysis of the cytoplasmic and nuclear fractions of Caco-2 cells after treatment with YA, YB, or YC. A significant increase in Nrf2 protein levels in the nuclear fraction was observed after treatment with YA, YB, or YC for 6 h at 50 μM, while Nrf2 protein levels were very low in the cytoplasmic fraction at 6 h (Fig. 5B). To further confirm the distribution of Nrf2, we performed an immunofluorescence assay in Caco-2 cells. As shown Fig. 5C, in untreated cells, Nrf2 fluorescence was found to be distributed throughout the cells, in both the cytoplasm and nucleus. After treatment with YA, YB, or YC for 3 h, most of the Nrf2 fluorescence was localized to the nuclei, as was also observed for SFN treatment. These results suggest that the induction of QR by YA, YB, or YC in Caco-2 cells was mediated *via* the Nrf2-

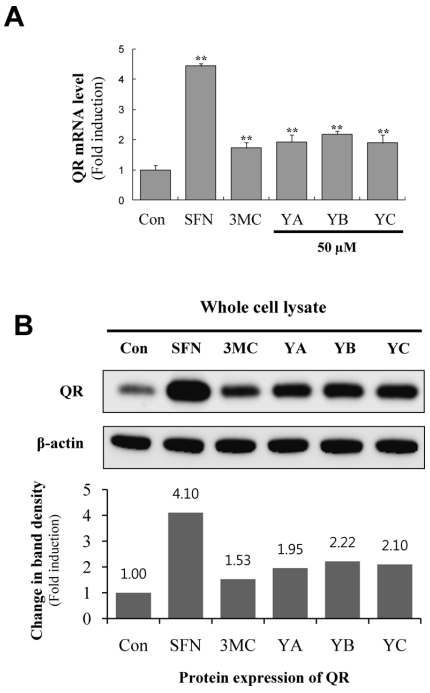


Fig. 3. Induction of QR mRNA and Protein Levels by Three Youngiasides, YA, YB, and YC, for 24 h in Caco-2 cells

(A) QR mRNA levels induced by three youngiasides at 50 μM in Caco-2 cells analyzed using RT-PCR. Each bar represents fold-induction of QR mRNA levels relative to control and the results represent the mean±S.D.; $n=3$. An asterisk denotes significant difference from control (** $p<0.01$). (B) QR protein expression induced by three youngiasides at 50 μM in Caco-2 cells analyzed by Western blotting. The image shows the results of a representative experiment from three independent experiments performed.

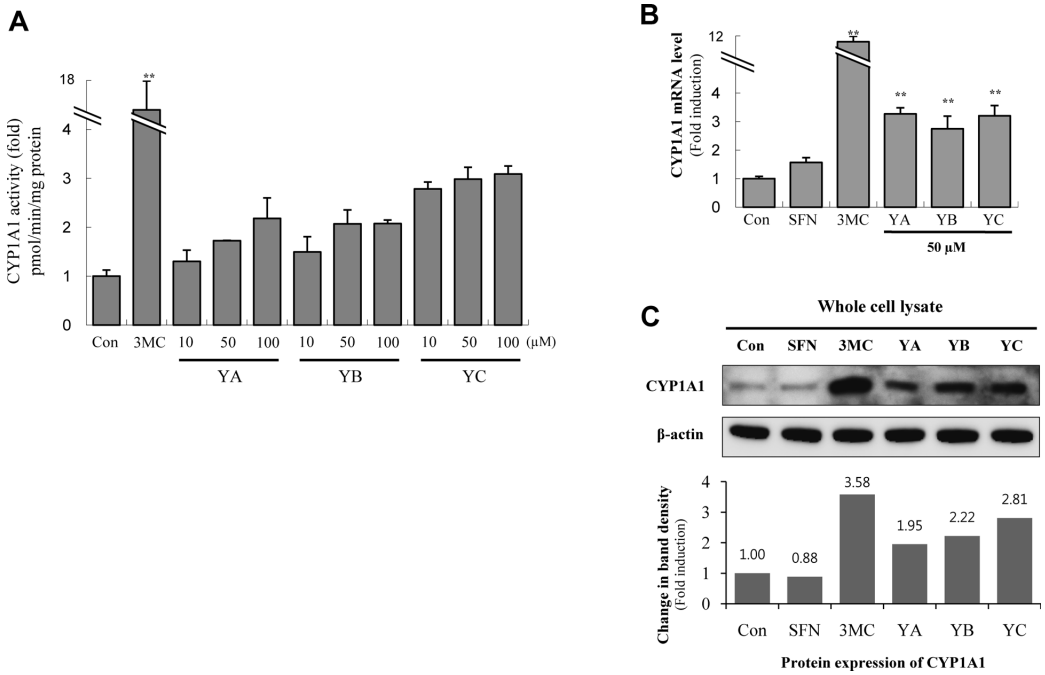


Fig. 4. Induction of CYP1A1 Activity, mRNA, and Protein Levels by Three Youngiasides, YA, YB, and YC for 24 h

(A) CYP1A1 (EROD) activity induced by three youngiasides at 10—100 μM in Hepa1c1c7 cells. (B) CYP1A1 mRNA levels induced by three youngiasides at 50 μM in Caco-2 cells analyzed using RT-PCR. Each bar represents fold-induction of CYP1A1 mRNA levels relative to control and the results represent the mean±S.D.; $n=3$. An asterisk denotes significant difference from control (** $p<0.01$). (C) CYP1A1 protein expression induced by three youngiasides at 50 μM in Caco-2 cells. The image shows the results of a representative experiment from three independent experiments performed.

ARE pathway through translocation of Nrf2 from the cytoplasm to the nucleus.

Effects of Youngiasides on QR Activation through the AhR-XRE Pathway To investigate whether the activation of the AhR-XRE pathway by YA, YB, and YC was also a bifunctional mechanism of induction of QR, reporter gene analysis was performed using a CAT reporter construct containing the XRE from the human QR gene. Caco-2 cells were transiently transfected with the reporter construct containing only the XRE consensus, and XRE-driven CAT activity was measured after treatment with YA, YB, or YC (10—100 μM) for 24 h. As shown in Fig. 6A, YA, YB, and YC each activated the XRE in a dose-dependent fashion.

In order to determine whether the activation of XRE was related to translocation of AhR from the cytoplasm to the nucleus, Western blotting of cytoplasmic and nuclear fractions from YA-, YB-, and YC-treated Caco-2 cells was performed. Compared with control cells, we observed a significant decrease in AhR protein levels in the cytoplasmic fraction, and an increase in AhR protein levels in the nuclear fraction, after treatment with 50 μM YB or YC for 1 h. Interestingly, relative to control cells, AhR protein levels were increased in the nuclear fraction, but were not decreased in the cytoplasmic fraction, following treatment with 50 μM YA for 1 h (Fig. 6B). We also examined levels of Arnt, a dimeric partner with activated AhR in the nucleus,[26] and found a significant increase in Arnt expression relative to control in the nuclear fraction after treatment with 50 μM YA, YB, or YC for 1 h (Fig. 6B). Moreover, following exposure of Caco-2 cells to 50 μM YB or YC for 3 h, AhR fluorescence was primarily localized to the nuclei, while nuclear AhR fluorescence was weak after treatment with 50 μM YA (Fig. 6C). These results indicate that activation of XRE by YA, YB, and YC is mediated through translocation of AhR from the cytoplasm to the nucleus, which leads to induction of QR and thus cancer chemoprevention.

DISCUSSION

The development of many types of cancer is closely related to exposure to environmental carcinogens. Recently, a number of studies have demonstrated inhibition and/or delay of cancer development using dietary phytochemicals such as sulforaphane, lycopene, resveratrol, curcumin, gingerol, and diallyl sulfide.[2,27] Therefore, identifying phytochemicals for cancer chemoprevention is a highly promising perspective in the natural product research area. Of these natural products, compositae plants having many types of terpenes (terpenes are classified by the number of terpene units in the molecule)[28] are known to exert various chemopreventive effects.[29] In particular, youngiasides isolated from *C. denticulatum* and as used in this study are sesquiterpene glucosides (a type of terpene). Several studies have reported induction of detoxification enzymes by sesquiterpenes.[30—32] In this study, we found that the youngiasides activated QR through the Nrf2-ARE pathway as one of the known specific mechanisms for induction of detoxification enzymes. There was almost no difference in induction ability among the youngiasides, indicating that the structural differences among them did not markedly affect QR induction. The situation could be different *in vivo* because the glycosidic bonds of the youngiasides are more likely to be broken during metabolic processes, and this topic will require further study.

Three kinds of cell lines (Hepa1c1c7, BPrc1, Caco-2) were used in the present study. For measurement of enzymatic activity, we used Hepa1c1c7 cells because they are

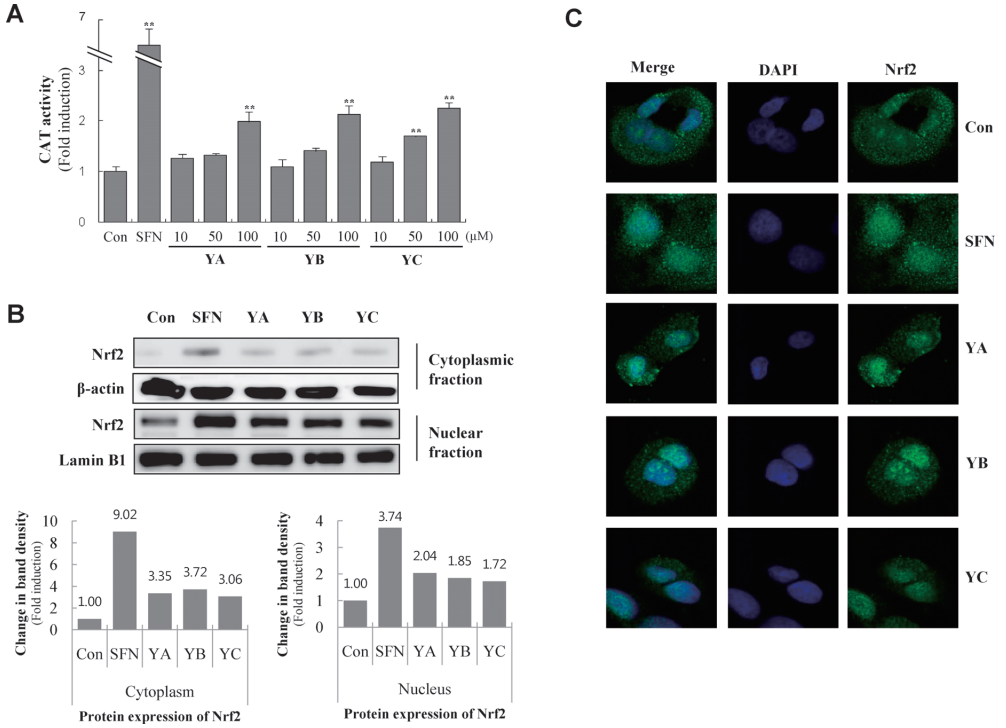


Fig. 5. ARE Activation and Nrf2 Nuclear Accumulation Induced by Three Youngiasides, YA, YB, and YC

(A) ARE activation by three youngiasides. The Caco-2 cells transiently transfected with the ARE-QR-CAT construct for 24 h. After 24 h treated with three youngiasides at 10—100 μM. CAT expression was normalized to β-galactosidase expression from the co-transfected pGL3-β-gal plasmid. All data were expressed as fold-induction relative to control. The results represent the mean±S.D.; $n=3$. An asterisk denotes significant difference from control (** $p<0.01$). (B) Nuclear translocation and accumulation of Nrf2 following 6 h treatment with three youngiasides 50 μM. Lamin B1 was used as the nuclear envelope marker. (C) Cellular distribution of Nrf2 following 3 h treatment with three youngiasides 50 μM. An immunofluorescence assay was performed followed by detection with confocal microscopy. The image shows the results of a representative experiment from three independent experiments performed.

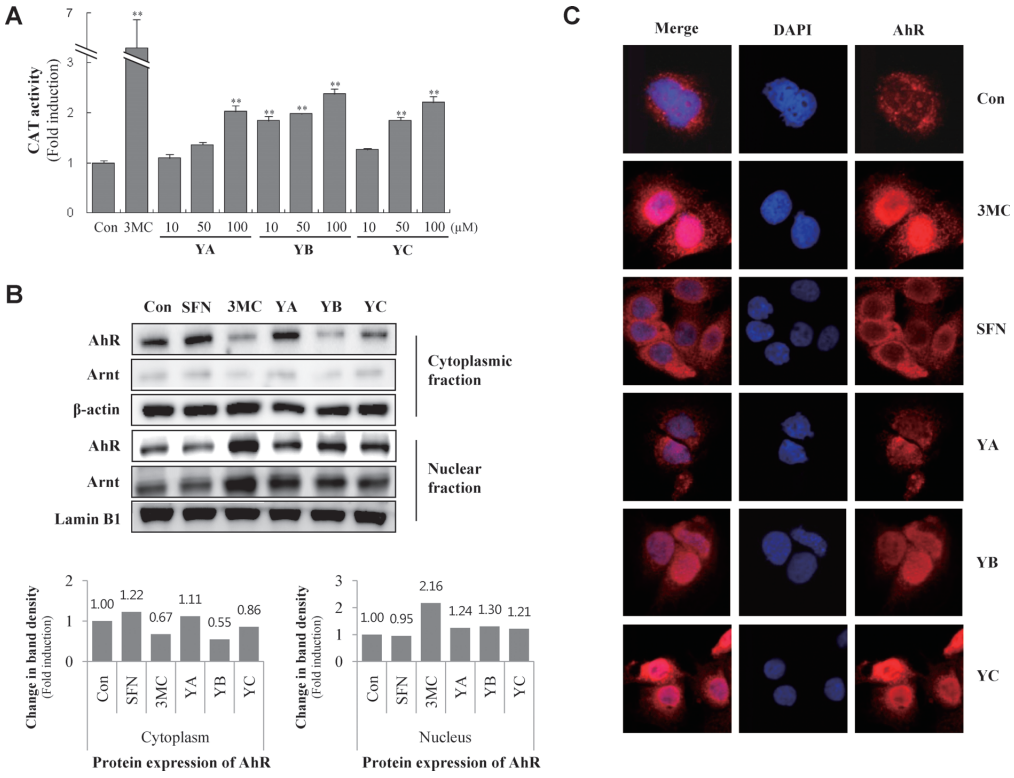


Fig. 6. XRE Activation and AhR Nuclear Translocation and Accumulation Induced by Three Youngiasides, YA, YB, and YC

(A) XRE activation by three youngiasides. The Caco-2 cells transiently transfected with the XRE-QR-CAT construct for 24 h. After 24 h treated with three youngiasides at 10—100 μM. CAT expression was normalized to β-galactosidase expression from the co-transfected pGL3-β-gal plasmid. All data were expressed as fold-induction relative to control. The results represent the mean±S.D.; $n=3$. An asterisk denotes significant difference from control (** $p<0.01$). (B) Nuclear translocation and accumulation of AhR following 1 h treatment with three youngiasides 50 μM. Lamin B1 was used as the nuclear envelope marker. (C) Cellular distribution of AhR following 3 h treatment with three youngiasides 50 μM. An immunofluorescence assay was performed followed by detection with confocal microscopy. The image shows the results of a representative experiment from three independent experiments performed.

known as the best model cell line for measuring QR and EROD activity, due to the low basal expression levels and richness of hepatic detoxification enzymes. The BPrc1 cell line mutated from Hepa1c1c7 (known paired cell lines) was required to test whether youngiasides are bi-functional inducers or not. The remainder of the experiments, aiming to elucidate the underlying molecular mechanisms, were performed in a human cell line system since eventually these will be applied to development of chemopreventive agents in humans. Among the various human cell lines available, human colorectal cancer cells were used for further study. Natural products and their phytochemicals have been reported to have protective effects against a wide range of cancers, but especially colon cancer.[33] For that reason, we used youngiasides to treat several human colorectal cancer cell lines that have been identified as showing sensitive protein expression of QR. The Caco-2 cell line was chosen for this study because of the low basal expression level and high induction ability of QR (data not shown).

The youngiasides showed differential induction ability for QR between Hepa1c1c7 and BPrc1 cells (Fig. 2C), which suggests that, besides the Nrf2-ARE pathway, another pathway such as the AhR-XRE pathway is involved. AhR is known to be present in the cytoplasm in a latent complex with two Hsp90s, and related chaperones. When the cells are ligand-activated, this leads to nuclear translocation followed by release of the chaperones in exchange for the AhR partner protein Arnt. The AhR-Arnt heterodimer then binds to XRE and transactivates phase I and/or phase II detoxifying genes.[9,13,34] CYP1A1, a phase I detoxification enzyme, also has XRE sequences in the promoter region and is usually regulated by AhR-XRE pathway. In our data, basal and inducing levels of CYP1A1 mRNA were not significantly increased by youngiasides in BPrc1 cells although they were increased in Hepa1c1c7 cells. These data can be indirect evidence that CYP1A1 increase by the treatment of youngiasides are through AhR-XRE pathway.

As shown in Fig. 6B, we observed that AhR was translocated from the cytoplasm into the nucleus in the YB-, and YC-treated cells. However, YA treatment of cells neither decreased the AhR levels in the cytoplasmic fraction or increased it as great as in the cells treated with YB or YC observed in the nuclear fraction. These results suggest that the absence of decrease in cytoplasmic AhR after treatment of YA is occurred by concomitant increase in AhR expression. Furthermore, we did not observe AhR in the nuclear fraction even after treatment for 6 h (data not shown). Arnt protein levels were also significantly increased in the nuclear fraction of Caco-2 cells treated with youngiasides (Fig. 6B), indicating translocation of AhR into the nucleus and dimerization with Arnt followed by binding to the XRE sequence. While YA had a relatively more marked effect on phase II enzymes than on phase I enzymes, YB and YC induced both phase I and II enzymes. We assumed that this could result from the different structures of the individual compounds, indicating that the structure of youngiasides affects activities in the AhR-XRE pathway, but not the Nrf2-ARE pathway.

Activators of AhR and Nrf2 have been termed bi- and mono-functional inducers, respectively. Interestingly, many phytochemicals are mono- and bi-functional inducers.[35,36] Our results showed that the bioactive youngiasides induced QR through both Nrf2-mediated ARE activation and AhR-mediated XRE activation, which indicates that they are acting as typical bi-functional inducers. Bi-functional inducers such as oltipraz and indole-3-carbinol (I3C) are known to modulate both phase I and phase II enzymes to enhance detoxification of carcinogens. Numerous studies have reported the cancer chemopreventive effects of oltipraz[37,38] and I3C.[39,40] It has been demonstrated that oltipraz, a derivative of 1,2-dithiole-3-thione from cruciferous vegetables, has excellent cancer chemopreventive effects in many target organs challenged by various carcinogens, as evidenced by a very large-scale clinical trial in China.[37,38] I3C is also known to show chemopreventive activity in a number of animal models.[39] In general, the induction of phase I detoxification enzymes such as CYP1A1 can ultimately produce electrophilic species and/or initiate carcinogenesis through the bioactivation of carcinogens.[41] However, it can also facilitate the elimination and excretion of carcinogens before the action of phase II enzymes, as occurs for oltipraz and I3C.[42,43] Therefore, the youngiasides identified as bi-functional inducers in this study show promise as potent chemopreventive agents, subject to further analysis *in vivo* and in clinical studies. In addition, we are currently investigating the hepato-protective effects of youngiasides in *in vitro* and *in vivo* models, towards identification of further health-promoting functions.

Recently, a close relationship between AhR and Nrf2 has been reported, described as "cross-talk".[27] It is interesting to note that some cross-talk appears to take place between the Nrf2 and AhR pathways, as AhR requires the presence of Nrf2 in order to induce expression of QR exerted by dioxin or 3MC.[12,44] As mentioned above, the mouse Nrf2 promoter contains several XREs[13] and the mouse AhR promoter contains an ARE.[45] In view of the results so far reported, that AhR regulates Nrf2 mRNA levels and Nrf2 regulates AhR mRNA levels, this results in CYP1A1 induction at the same time as induction of Nrf2. In addition, AhR has dual functions classified as 'adaptive' (enhanced metabolism of xenobiotics) and 'toxic' (adverse effects of dioxin-like compounds) in induction of CYP1 enzymes.[35] We are currently examining the relationship between AhR-XRE and Nrf2-ARE using youngiasides in colorectal cancer cells.

In summary, in this study, we have investigated the molecular mechanism of action of youngiasides in induction of detoxification enzymes. The youngiasides from *C. denticulatum* are active components responsible for induction of QR and exhibiting high CI values. In further study of the molecular mechanisms, we confirmed the youngiasides as bi-functional inducers since they induced QR as well as CYP1A1 at the mRNA and protein levels, resulting from both AhR-mediated XRE activation and Nrf2-mediated ARE activation. Thus, we conclude that the youngiasides examined here have considerable potential for development as nutraceuticals or cancer chemopreventive agents.

Acknowledgements This work was supported by a KIST-Gangneung Institute intramural research grant (2Z03411).

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