Bifunctional Chemopreventive Effects of *Adenocaulon himalaicum* Through Induction of Detoxification Enzymes and Apoptosis

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ABSTRACT   Phase II detoxification enzymes are known to play essential roles in the detoxification and elimination of activated carcinogens during tumor initiation, while apoptosis is one of the most important chemopreventive targets for inhibiting tumor promotion in cancer. In this study, we investigated the cancer chemopreventive activity of two plant extracts, the ethanolic extract of *Adenocaulon himalaicum* (AHE) and the butanolic fraction of AHE (AHB). Both, the AHE and AHB induced NQO1 activity and had relatively high chemoprevention indices (CI = 12.4). The AHE and AHB were associated with increased NQO1 and HO-1 mRNA levels via Nrf2-ARE pathway activation. In addition, the AHB increased CYP1A1 activity through AhR-XRE pathway activation. We also found that the AHE and AHB induced apoptosis, as evidenced by phosphatidylserine external- ization, an increase in the sub-G0/G1 content, chromatin condensation, poly(ADP-ribose) polymerase cleavage, and p53 in- duction. These data suggest that AHE and AHB act as bifunctional inducers and that their chemopreventive effects result from the biphasic induction of phase II detoxification enzymes and apoptosis. Therefore, these results suggest that *A. himalaicum* plant extracts have potential for use as chemopreventive agents for the prevention and/or treatment of human cancers.

KEY WORDS: • Adenocaulon himalaicum • *apoptosis* • *colon cancer* • *heme oxygenase* • *Nrf2* • *quinone reductase*

INTRODUCTION

CARCINOGENESIS IS AN extremely complicated multistep process.[1] Environmental exposure to carcinogens is an important risk factor during the early stages of carcinogen- esis. Enhancing the body's carcinogen-detoxifying capabil- ity is a promising approach for cancer prevention.[2] Some chemicals are capable of inhibiting the metabolic activation of procarcinogens to their ultimate electrophilic species or inhibiting their subsequent interactions with DNA. These agents are known as "cancer-blocking agents" because they stimulate the detoxification of carcinogens during the initiation of carcinogenesis.[3] Other chemicals, known as "cancer-suppressing agents," suppress the promotion and progression of carcinogenesis by inducing cell cycle arrest and apoptosis, thereby eliminating preneoplastic cells.[4]

Sulforaphane, found in cruciferous vegetables, is a well- known phytochemical that prevents chemical carcinogene- sis by inducing phase II enzymes, including glutathione S-transferases (GSTs; EC 2.5.1.18) and quinone reductase (QR, also known as NAD(P)H:quinone oxidoreductase, NQO1; EC 1.6.5.2).[5] Because phase I enzymes are involved in both bioactivation and detoxification of carcinogens, monofunctional inducers, which upregulate only phase II enzymes, are thought to be more closely related to chemo- prevention than bifunctional inducers, which upregulate both phase I and II enzymes. However, bifunctional induc- ers have been shown to exert synergistic effects during cancer chemoprevention. Oltipraz, a plant-derived com- pound, is a promising bifunctional inducer that stimulates both phase II enzymes and phase I enzymes, such as cyto- chrome P450 (CYP).[6] Oltipraz is now undergoing clinical trials for use as a cancer chemopreventive agent. Its un- derlying mechanism of action is as follows: the aryl hydrocarbon receptor (AhR), which can be induced by bi- functional inducers such as Oltipraz, heterodimerizes with the aryl hydrocarbon receptor nuclear translocator (Arnt) and activates the *Nrf2* gene by binding to the xenobiotic response element (XRE) in the promoter region of *Nrf2*.[7]

Apoptosis is characterized by marked changes in cellular morphology, including chromatin condensation, membrane blebbing, the appearance of membrane-associated apoptotic bodies, internucleosomal DNA fragmentation, and poly (ADP-ribose) polymerase (PARP) cleavage.[8] The p53 tumor suppressor protein plays a key role in apoptosis and cell cycle arrest. Alterations in the p53 gene have been associ- ated with carcinogenesis in rodent models.[9] A recent in- vestigation into the relationship between apoptosis and Nrf2 in the *N*-nitrosobutyl (4-hydroxybutyl)-amine (BBN)– induced urinary bladder carcinogenesis model suggested

that p53 and Nrf2, and their respective gene batteries, cooperatively contribute to cancer prevention.[10]

Adenocaulon himalaicum (Asteraceae), a perennial herb, is mainly found in Southeast Asia. Its aerial parts have been used to treat abscesses, hemorrhage, and inflammation in Korean folk medicine,[11] and the sprouts of *A. himalaicum* are edible. However, its cancer preventive effects have not yet been reported. In this study, we have examined the cancer chemopreventive effects of an ethanolic extract of *A. himalaicum* (AHE) and its butanolic fraction (AHB) by investigating known anticancer mechanisms, including the induction of detoxification enzymes during the initiation stage and the induction of apoptosis during the promotion of carcinogenesis.

MATERIALS AND METHODS

Plant materials

Whole *A. himalaicum* plants were collected at the Wild Vegetable Experiment Station, Gangwon ARES, Korea, and a voucher specimen (D-039) was stored at the Functional Food Center, KIST Gangneung Institute, Gangneung, Korea.

Extraction, isolation, and identification of the AHE

The air-dried whole plant of *A. himalaicum* (1.2 kg) was extracted with 94% EtOH 4 times (4×19.0 L) under reflux for 4 h and evaporated *in vacuo* to yield the total extract (149.36 g). This extract was then suspended in distilled water and sequentially partitioned with *n*-hexane, ethyl acetate, and *n*-butanol. The *n*-butanol fraction (19.06 g) was chromatographed on a Sephadex LH-20 column and eluted using MeOH. Ten fractions (fractions 1–10) were obtained. Fraction 4 (383.3 mg) was further chromatographed over silica gel using a stepwise gradient of CH_2Cl_2-MeOH-Water (5:1:0.1 to 2:1:0.1) and yielded compounds **2** (1.7 mg), **3** (6.7 mg) and **5** (4.1 mg). Further purification of fraction 7 (268.6 mg) by repeated Sephadex LH-20 column chromatography with EtOH yielded compounds **1** (8.9 mg) and **4** (13.6 mg). These compounds were identified using NMR spectroscopy. The chemical structures are shown in Fig. 7A. The analytical HPLC system was equipped with an Agilent Series 1200 liquid chromatography and YMC Hydrosphere C-18 column (4.6 mm × 150 mm, 3 μm, Kyoto, Japan).

Cell culture

HCT116 cells, Hepa1c1c7 cells and BPrc1 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were maintained at subconfluence in 95% air and 5% CO_2 in a humidified atmosphere at 37°C. Minimum essential medium (MEM; Hyclone, Logan, UT, USA) was used for HCT116 cell cultivation and alpha–minimum essential medium (α-MEM; Hyclone) was used for Hepa1c1c7 and BPrc1 cell cultivation. The media were supplemented with 10% fetal bovine serum (FBS; Hyclone), penicillin (100 units/mL), and streptomycin (100 μg/mL).

Cell viability assay

The cytotoxicities of the AHE and its fractions were evaluated using a Cell Counting Kit (CCK-8; Dojindo Laboratories, Tokyo, Japan). In brief, the cells (5×10^3 cells/well) were plated on 96-well plates, incubated at 37°C for 24 h, and then given fresh medium. The cells were then treated with various concentrations of the AHE and its fractions and incubated at 37°C for an additional 24 h. Then, 10 μL of CCK-8 solution was added to 100 μL of media in the wells and incubated for another hour. 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, USA). The data were reported as the percentage of cell growth relative to the respective controls (cells treated with solvent only) for each sample concentration.

QR activity assay

Induction of QR activity was determined using the Prochaska modified bioassay, with slight modifications.[12,13] Hepa1c1c7 cells (5×10^3 cells/well) were plated on 96-well plates and incubated for 24 h prior to treatment. Sulforaphane (Calbiochem, San Diego, CA, USA) was used as a positive control. The absorbance at 610 nm was measured five times at 50 s intervals using a Synergy HT Multi-microplate Reader (Bio-Tek Instruments). Induction of QR activity was calculated by comparing the specific QR activity of the cells that had been treated with the extracts or fractions with the QR activity of vehicle-treated cells. Enzymatic activity was expressed as the concentration required to double QR activity (CD). The concentration resulting in a 50% inhibition of cell viability (chemoprevention index [CI]) was obtained by dividing the half-maximal cell growth inhibitory concentration (IC_{50}) values by the CD values.

EROD activity assay

The 7-ethoxyresorufin-O-deethylase (EROD) activity was determined as a measure of CYP1A1 activity. The EROD activity was determined as previously described, with minor modifications.[14] Hepa1c1c7 cells (10×10^4 cells/mL) were plated onto 24-well tissue culture plates and incubated for 24 h prior to treatment. The cells were then treated with various concentrations of the AHE or AHB and incubated for an additional 24 h. Fluorescence was measured using a Synergy HT Multi-microplate Reader at an excitation wavelength of 550 nm and an emission wavelength of 585 nm. Protein concentrations were determined according to the Bradford method using the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, USA). The fluorescence values were converted to picomoles using a calibration curve of resorufin fluorescence, and the specific activity was calculated as pmol of resorufin per mg of total protein per min.

Transient transfection and reporter gene assays

HCT116 cells (1×10^5 cells/mL) were cultured in 24-well tissue culture plates for 24 h and reached 40 to 50% confluence prior to transfection. The cells were transiently

co-transfected with 0.25 μg of ARE-QR-CAT or XRE-QR-CAT reporter constructs containing either an antioxidant response element (ARE) or a XRE that had been derived from the rat QR gene[15] and 0.02 μg of the pGL3-β-gal Vector using the Fugene 6 transfection reagent (Roche, Indianapolis, IN, USA) according to the manufacturer's recommended instructions. The pGL3-β-gal reporter plasmids were a gift from Dr. Son (Kangung-Wonju National University, Gangneung, Korea). After 24 h of treatment, the cells were lysed and assayed for CAT expression using a CAT-ELISA kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. CAT expression was normalized to β-galactosidase activity and the results are presented as fold induction over the control.

Total RNA extraction and real-time PCR analysis

Total RNA was extracted from the treated and untreated cell lines using RNeasy Mini Kit Columns (Qiagen, Valencia, CA, USA) according to the manufacturer's recommended protocol. The cDNA was prepared using random primers (Invitrogen, Carlsbad, CA, USA) and 200 U of SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Quantitative real-time PCR was performed in triplicate in 384-well plates as previously described.[13] Real-time PCR analysis was performed using an LC480 Detection System (Roche).

Flow cytometric cell cycle analysis

Cell cycle progression was evaluated using flow cytometric analysis, and the DNA content was measured using the propidium iodide (PI) labeling method, as previously described.[16] To quantitate apoptotic cell death, we measured the externalization of phosphatidylserine, a specific phospholipid marker for apoptosis, which can be detected using flow cytometric analysis, as previously described.[16] HCT116 cells (5×10^5 cells/dish) were seeded on 60 mm dishes and incubated at 37°C for 24 h and were then treated with each sample for 24 or 48 h. The data were obtained using Cell Quest Pro software.

Chromatin condensation

Chromatin condensation was observed using the Hoechst33342 staining method. HCT116 (3×10^5 cells/well) cells were plated on six-well plates, incubated at 37°C for 24 h, and treated with the AHE or AHB for 48 h. The cells were then treated with 20 μg/mL of Hoechst 33342 at 37°C for 15 min. After staining, both the detached and adherent cells were collected by trypsinization and washed with DPBS. The stained cells were visualized using a fluorescent microscope (Nikon TE2000U, Nikon, Kanagawa, Japan).

Western blot analysis

To prepare the nuclear and cytoplasmic fractions, the cells were treated with the AHE or AHB for 4 h, and the nuclear proteins were extracted using a Nuclear Extraction

Kit (Sigma Chemical, St. Louis, MO, USA) according to the manufacturer's instructions. To prepare the total lysates, the cells were treated with either the AHE or AHB and then lysed and analyzed using Western blotting, as previously described.[13]

Immunofluorescence assay

HCT116 cells were grown on glass coverslips in 24-well plates. The cells were treated with either the AHE or AHB for 4 h, fixed with 4% paraformaldehyde at room temperature for 20 min, fixed with 100% methanol at −20°C for 15 min, and blocked with phosphate-buffered saline (PBS) containing 5% (v/v) goat serum and 0.3% Triton X-100 for 1 h. The cells were then incubated with the primary antibody at a 1:100 dilution in PBS containing 5% (v/v) goat serum and 0.3% Triton X-100 at 4°C overnight. The cells were washed with PBS and incubated with Alexa Fluor 488-conjugated anti-rabbit and Alexa Fluor 594-conjugated anti-goat secondary antibodies (Invitrogen) at 1:200 dilutions for another hour. The cells were then washed and mounted on glass slides with VECTASHIELD Mounting Medium and DAPI (Vector Laboratories, Inc., Cambridgeshire, United Kingdom). Images were obtained using a Leica TCS SP5 Confocal System (Leica, Wetzlar, Germany).

Statistics

Statistical analyses were performed using one-way ANOVA followed by either Dunnett's test or Bonferronis's test for multiple comparisons using GraphPad Prism 4 software (GraphPad Software, Inc., La Jolla, CA, USA). Differences were considered to be statistically significant at $P < .05$.

RESULTS

The AHE and AHB increase phase II detoxification enzymes, QR and HO-1

QR activity was measured in an effort to investigate the potential chemopreventive effects of the AHE using mouse hepatocarcinoma Hepa1c1c7 cells as a model cell line. Treatment with the AHE induced QR activity in a dose-dependent manner (Fig. 1A) and produced a doubling of QR activity (CD) at a concentration of 19.1 μg/mL with low cytotoxicity (IC$_{50}$ = 197.7 μg/mL), resulting in a relatively high CI value (10.4; Table 1). Because treatment with the AHE stimulated high QR activity, we subjected the AHE to further fractionation with different organic solvents, which resulted in four fractions: *n*-hexane, ethyl acetate, *n*-butanol, and water. These four fractions also increased the QR activity (Fig. 1C) to varying degrees. The AHB displayed the highest CI value (12.4) of the four fractions, with an IC$_{50}$ of 177.7 μg/mL and a CD value of 14.3 μg/mL (Table 1). Thus, the AHB was chosen for further investigation of the mechanisms governing the cancer chemopreventive effects.

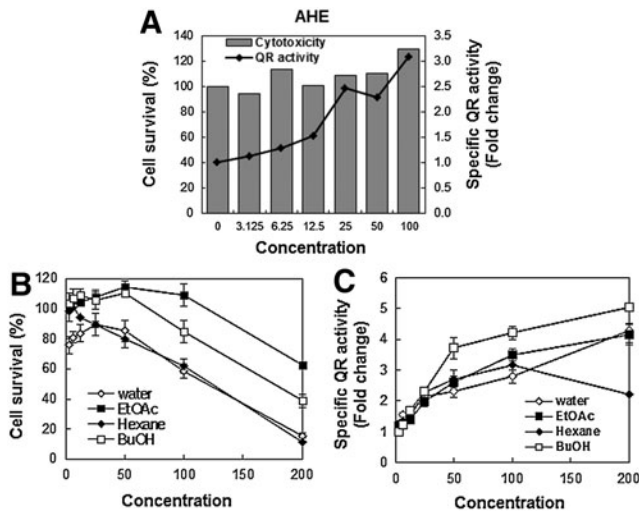**FIG. 1.** NQO1 activity is induced by the AHE and its fractions in Hepa1c1c7 cells. **(A)** Measurement of AHE-induced QR activity in Hepa1c1c7 cells. The cells were treated with the indicated concentrations of the AHE (0–100 $\mu g/mL$) for 24 h. The cell survival ratio (%) **(B)** and specific QR activity **(C)** of the four AHE fractions in Hepa1c1c7 cells. Each value represents the mean ± SD from three independent experiments. NQO1, NAD(P)H dehydrogenase (quinone 1); AHE, ethanolic extract of *Adenocaulon himalaicum*; QR, quinone reductase; SD, standard deviation.

To determine whether the AHE and AHB could affect NQO1 (which is known as a QR gene) expression in HCT116 cells, we treated the cells for 24 h with various concentrations of the AHE or AHB. We observed a marked dose-dependent induction of NQO1 activity and mRNA expression (Fig. 2A–C). HO-1, another detoxification enzyme, was also dose-dependently increased following treatment with both fractions, although treatment with the AHB produced a stronger induction of both *NQO1* and *HO-1* mRNA expression compared to treatment with the AHE (Fig. 2D). These results suggest that AHE and AHB induce the transcription and translation of detoxification

TABLE 1. INDICATORS OF THE EFFECTS
OF ETHANOLIC EXTRACT OF *ADENOCAULON HIMALAICUM*
AND ITS FRACTIONS IN HEPA1C1C7 AND HCT116 CELLS

	Hepa1c1c7			*HCT116*
	CD ($\mu g/mL$)	IC$_{50}$ ($\mu g/mL$)	CI	IC$_{50}$ ($\mu g/mL$)
AHE	19.1	197.7	10.4	151.7
Hexane fraction	17.5	102.2	5.8	73.2
Ethyl acetate fraction	24.7	>200.0[a]	8.1	>200.0[a]
AHB	14.3	177.0	12.4	134.5
Water fraction	27.7	106.6	3.8	136.7
Sulforaphane (μM)	1.17	14.96	12.8	

[a]The highest limit of test concentration was 200 $\mu g/mL$. If the extract had no cytotoxicity, 200 $\mu g/mL$ was used to calculate the CI value.

CD, concentration required to double QR activity; IC$_{50}$ concentration required to inhibit cell growth by 50%; CI (chemoprevention index) = IC$_{50}$/ CD; AHE, ethanolic extract of *Adenocaulon himalaicum*; AHB, butanolic fraction of AHE; QR, quinone reductase.
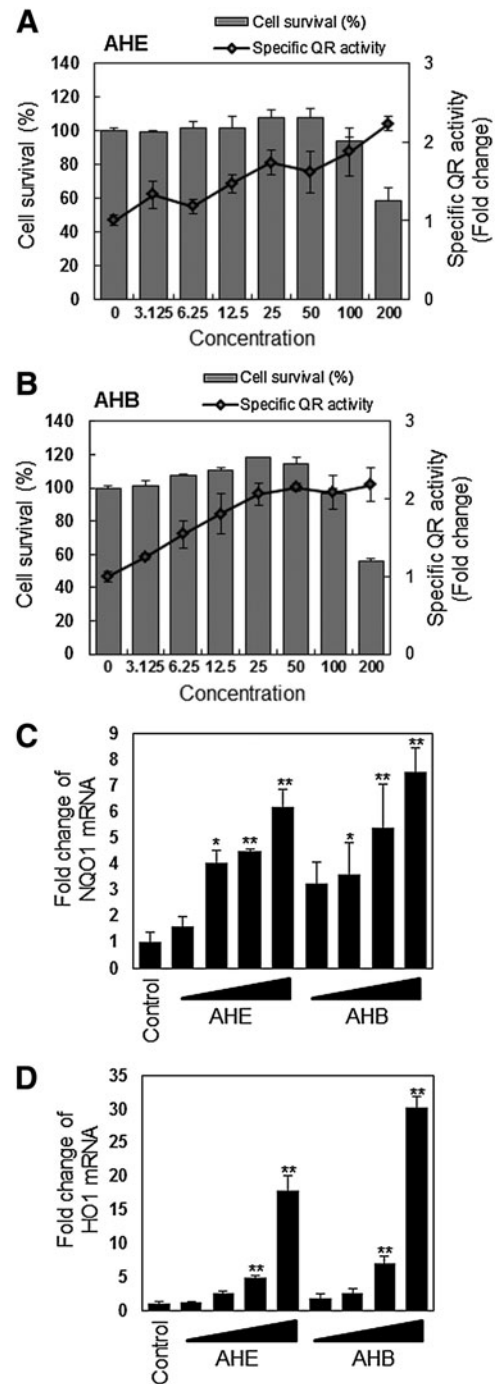

FIG. 2. NQO1 and HO-1 are induced by the AHE and its fractions in HCT116 cells. **(A)** Measurement of AHE-induced QR activity in HCT116 cells. The cells were treated with the indicated concentrations of the AHE (0–200 $\mu g/mL$) for 24 h. **(B)** Measurement of butanolic fraction of AHE (AHB)–induced QR activity in HCT116 cells. The cells were treated with the indicated concentrations of the AHB (0–200 $\mu g/mL$) for 24 h. Each value represents the mean ± SD from three independent experiments. *NQO1* **(C)** and *HO-1* mRNA expression **(D)** in response to treatment with the AHE or AHB in HCT116 cells. The cells were treated with 25, 50, 100, and 200 $\mu g/$ mL of the AHE or AHB for 24 h. The results represent the mean ± SD; $n = 3$. Asterisks denote significant differences compared to the control (*$P < .05$; **$P < .01$).
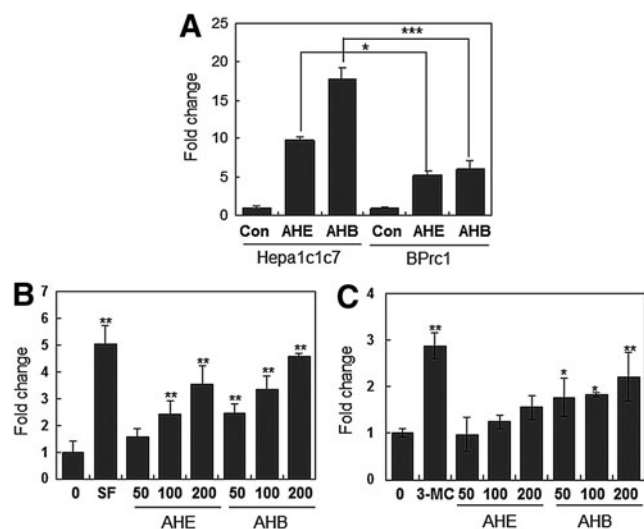
FIG. 3. Treatment with the AHE and the AHB induces ARE and XRE activation. **(A)** Real-time PCR analysis of AHE- or AHB-induced *Nqo1* mRNA expression in Hepa1c1c7 and BPrc1 cells. The cells were treated with 50 μg/mL of the AHE or AHB for 48 h. The data are expressed as the mean $\pm$ SD ($n=3$). Asterisks indicate significant differences compared to the vehicle-treated group ($*P < .05$; $***P < .001$). **(B, C)** Activation of the ARE **(B)** and XRE **(C)** by the AHE or AHB was measured using an ARE- or XRE-containing reporter construct. The cells were treated with the indicated concentrations (50, 100, and 200 μg/mL) of the AHE for 24 h. A 5 μM concentration of sulforaphane (SF) or a 0.1 μM concentration of 3-methylcolanthrene (3-MC) was used as a positive control for ARE or XRE activation, respectively. The results represent the mean $\pm$ SD; $n=4$. Asterisks denote significant differences as compared to the control ($*P < .05$; $**P < .01$). ARE, antioxidant response element; XRE, xenobiotic response element.

enzymes in human colorectal cancer cells and that this induction may occur as a result of bioactive components of the AHE and AHB.

The AHE and AHB induce ARE and XRE activation

The majority of the genes encoding phase II detoxification and antioxidant enzymes contain two essential *cis*-acting elements, known as the ARE and XRE, in their promoter regions.[15,17] To determine whether the induction of NQO1 by the *A. himalaicum* extracts was mediated via the ARE and/or XRE motifs, we examined *Nqo1* mRNA levels in two different mouse hepatocarcinoma cell lines: Hepa1c1c7 (wild-type) and BPrc1 (Arnt-deficient). Treatment with the AHE and AHB significantly increased *Nqo1* mRNA levels in Hepa1c1c7 cells. However, *Nqo1* mRNA levels in BPrc1 cells were markedly decreased after treatment (Fig. 3A), suggesting that full induction of *Nqo1* mRNA by the AHE and AHB requires a functional XRE system. To verify whether AHE- and/or AHB-inducible expression of NQO1 was mediated by both the ARE and XRE motifs, we performed reporter gene analysis using a construct containing the ARE or XRE core sequences from the upstream region of the rat *Nqo1* gene. We found that treatment with the AHE and AHB induced activation of the reporter gene via the ARE and XRE in a dose-dependent manner (Fig. 3B, C).
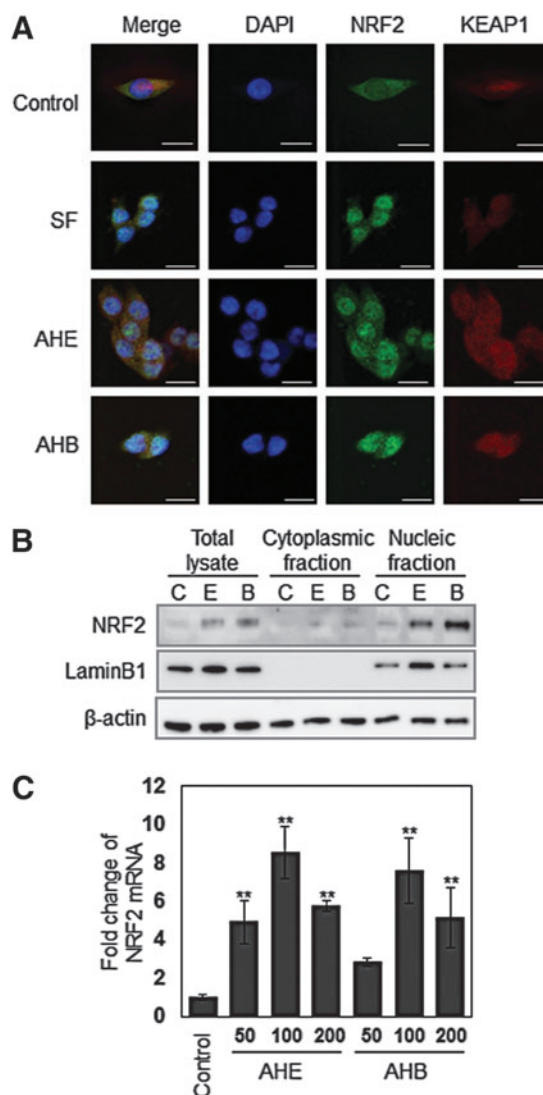

FIG. 4. The AHE and AHB induce Nrf2 nuclear translocation. **(A)** Cellular distribution of Nrf2 in response to treatment with 100 μg/mL of the AHE or AHB in HCT116 cells. The cells were treated with 100 μg/mL of the AHE or AHB for 4 h, after which point an immunofluorescence assay was performed, followed by detection with confocal microscopy (bar = 20 μm). **(B)** Nuclear Nrf2 accumulation in response to treatment with the AHE or AHB in HCT116 cells as assessed using Western blotting. The cells were treated with 100 μg/mL of the AHE or AHB for 4 h. The figure shows a representative image from three independent experiments. **(C)** *Nrf2* mRNA expression in response to treatment with the AHE or AHB in HCT116 cells. The cells were treated with 50, 100, and 200 μg/mL of the AHE or AHB for 24 h. The results represent the mean $\pm$ SD; $n=3$. An asterisk denotes a significant difference as compared to the control ($**P < .01$). Color images available online at www.liebertpub.com/jmf

Although the AHE and AHB activated both the ARE and XRE motifs, they had a much stronger effect on the ARE than on the XRE, and showed a difference of approximately 25-fold in activation over the control.

The AHE and AHB induce the nuclear translocation of Nrf2

Nrf2 is an important transcription factor that regulates ARE-driven genes such as *NQO1, GST,* and *HO-1.* The
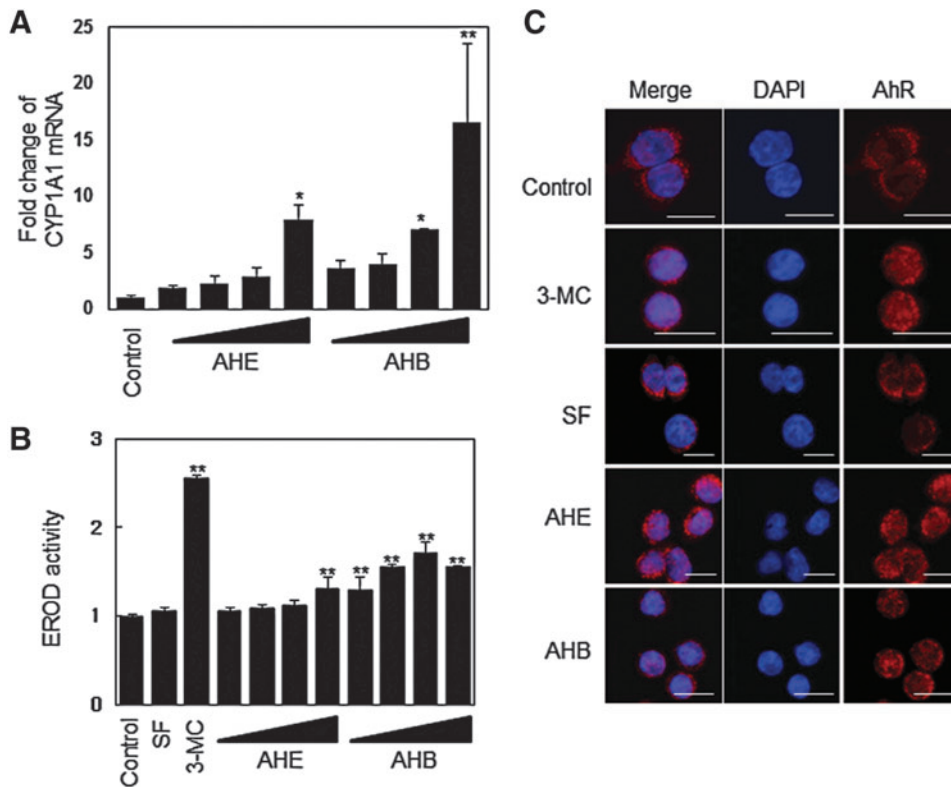
FIG. 5. The AHE and AHB increase *CYP1A1* mRNA levels and induce EROD activity by inducing AhR accumulation in the nucleus. **(A)** *CYP1A1* mRNA expression in response to treatment with the AHE or AHB in HCT116 cells. HCT116 cells were treated with 25, 50, 100, and 200 μg/mL of the AHE or AHB for 24 h. **(B)** EROD activity in response to treatment with the AHE or AHB in Hepa1c1c7 cells. 3-MC (0.1 μM) was used as a positive control. HCT116 cells were treated with 25, 50, 100, and 200 μg/mL of the AHE or AHB for 24 h. **(C)** Cellular distribution of the AhR in response to treatment with 100 μg/mL of the AHE or AHB in HCT116 cells. The cells were treated with 100 μg/mL of the AHE, 100 μg/mL of the AHB, 5 μM of SF, or 0.1 μM of 3-MC for 3 h, after which point an immunofluorescence assay was performed, followed by detection by confocal microscopy (bar = 20 μm). The results represent the mean ± SD; $n = 3$. Asterisks denote significant differences compared to the control (*$P < .05$; **$P < .01$). EROD, 7-ethoxyresorufin-O-deethylase; AhR, aryl hydrocarbon receptor; 3-MC, 3-methylcolanthrene. Color images available online at www.liebertpub.com/jmf

nuclear translocation of activated Nrf2 is an important upstream step in the mechanism underlying NQO1 expression.[13] To investigate whether the AHE and AHB can induce Nrf2 translocation in HCT116 cells, we used an immunofluorescence assay to examine the intracellular distribution of Nrf2. In untreated HCT116 cells, Nrf2 fluorescence was distributed throughout the cells, including the cytoplasms and nuclei of the cells. However, following treatment with the AHE or AHB for 4 h, Nrf2 fluorescence was concentrated in the nuclei (Fig. 4A). In an effort to further confirm that Nrf2 nuclear translocation is induced by the AHE and AHB, we obtained the cytoplasmic and nucleic fractions of the cells and detected Nrf2 protein using Western blot analysis. The amount of Nrf2 protein in the nucleus was markedly increased, supporting the results of immunofluorescence (Fig. 4B). Treatment with the AHE and AHB also increased *Nrf2* mRNA levels, as assessed using real-time PCR analysis (Fig. 4C). These data not only indicate that the AHE and AHB regulate Nrf2 through transcriptional activation and translocation to the nucleus but also that AHE- and AHB-mediated NQO1 induction involves the activation of the ARE promoter motif by Nrf2 in human colorectal carcinoma cells. In all of the experiments, the AHB showed much stronger effects than the AHE.

The AHE and AHB induce the phase I detoxification enzyme CYP1A1

Phase I detoxification enzymes are regulated by the XRE,[15,17] whereas phase II detoxification enzymes are regulated by both the ARE and XRE.[18] In the present study,

because the AHE and AHB activated the XRE in a dose-dependent manner, with the AHB fraction displaying greater effects than the AHE extract (Fig. 3C), we investigated the effects of the AHE and AHB on the expression of CYP1A1, an important phase I enzyme. We found that the AHE and AHB increased *CYP1A1* mRNA levels in a dose-dependent manner (Fig. 5A), while only the AHB significantly induced CYP1A1 activity at 25–100 μg/mL, as measured by the EROD assay in Hepa1c1c7 cells (Fig. 5B).

We used an immunofluorescence assay to determine whether the AhR, a key transcription factor that directly binds to the XRE, accumulates in the nucleus in response to treatment with the AHE or AHB. AhR fluorescence was distributed in the cytoplasm of untreated cells and in cells treated with sulforaphane (SF), a well-known monofunctional inducer. However, after treatment with the AHE or AHB for 3 h, the AhR was detected in both the cytoplasm and the nucleus (Fig. 5C). Similar results were shown in cells that had been treated with 3-methylcolanthrene (3-MC), a powerful AhR inducer. These results suggest that the AHE and AHB also exert a chemopreventive effect through the AhR-mediated induction of phase I enzymes in addition to the induction of phase II detoxification enzymes.

The AHE and AHB induce antiproliferative activity and apoptosis

Treatment with 100 or 200 μg/mL of the AHE increased the proportion of the cell population in the G2/M phase, which was accompanied by a slight decrease in the cell population at S phase (Fig. 6A). The AHE caused G2/M arrest
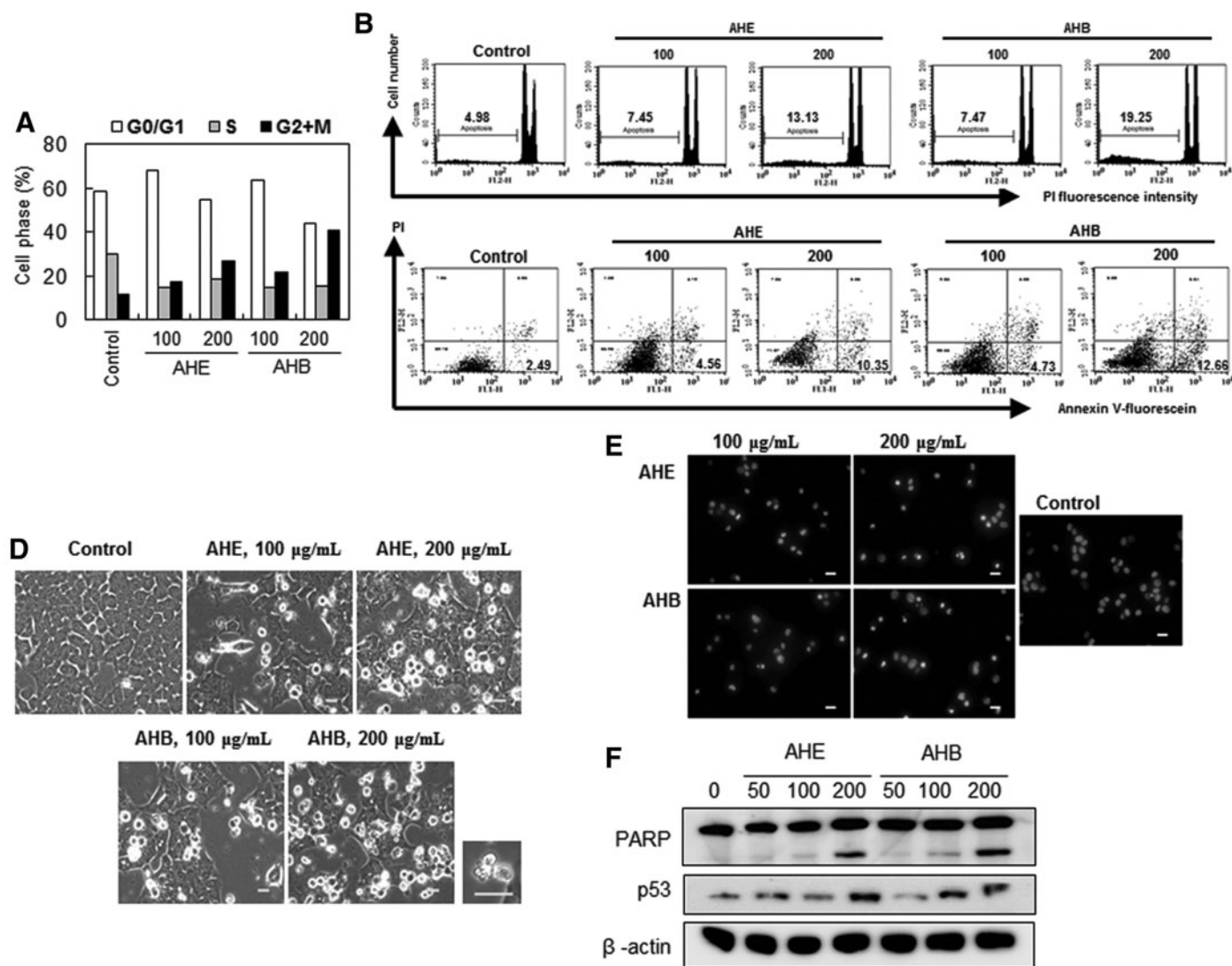
FIG. 6. The AHE and AHB induce apoptosis in HCT116 cells. HCT116 cells were treated with the AHE or AHB (100 and 200 μg/mL) for 48 h. **(A)** The cell cycle distribution and **(B)** sub G0/G1 content (%) were evaluated using flow cytometric analyses measuring the DNA content according to the PI labeling method. **(C)** Phosphatidylserine externalization was measured using flow cytometric analysis after annexin V-fluorescein and PI staining. The cell population (%) for each quadrant is shown. The lower right quadrant (annexin V-fluorescein positive and PI negative) represents the early apoptotic cell population (%), indicated in bold. **(D)** Cellular morphology was observed using a phase contrast microscope. Phase contrast microscopy images were then taken. A magnified picture of apoptotic cells treated with 200 μg/mL of the AHB is shown on the right hand side of the original picture (bar = 20 μm). **(E)** Chromatin condensation was observed using fluorescence microscopy after Hoechst33342 staining (bar = 20 μm). **(F)** PARP cleavage and p53 levels were observed using Western blot analysis. The cells were treated with the indicated concentrations (50, 100, and 200 μg/mL) of the AHE or AHB for 48 h. All data are representative of three independent experiments. PI, propidium iodide; PARP, poly(ADP-ribose) polymerase.

in 17.2% and 26.7% of cells, and the AHB caused even greater G2/M arrest (21.6%, and 40.5% of cells) at concentrations of 100 and 200 μg/mL, respectively. To determine whether AHE- and AHB-mediated inhibition of cell proliferation was due to apoptosis, we measured the levels of phosphatidylserine in the outer membrane. Apoptosis occurred in 4.98% of the solvent-treated control cells, while treatment with 200 μg/mL of the AHE and AHB caused apoptosis in 13.13% and 19.25% of the treated cells, respectively (Fig. 6B). These results suggested that treatment with the AHE and AHB potentiates apoptosis in HCT116 cells. A

double labeling assay, in which annexin V was combined with PI, revealed that HCT116 cells that had been treated with 200 μg/mL of the AHE or AHB exhibited a dose-dependent increase in annexin V$^+$/PI$^-$ (Fig. 6C), indicating that both the AHE and AHB induce apoptosis in HCT116 cells.

Morphological changes due to apoptosis were also observed in the cells after treatment with 100 and 200 μg/mL of the AHE or AHB (Fig. 6D). These changes included the appearance of apoptotic bodies (Fig. 6D) and chromatin condensation (Fig. 6E) in cells that had been treated with 200 μg/mL of the AHB. In addition, both the AHE and AHB
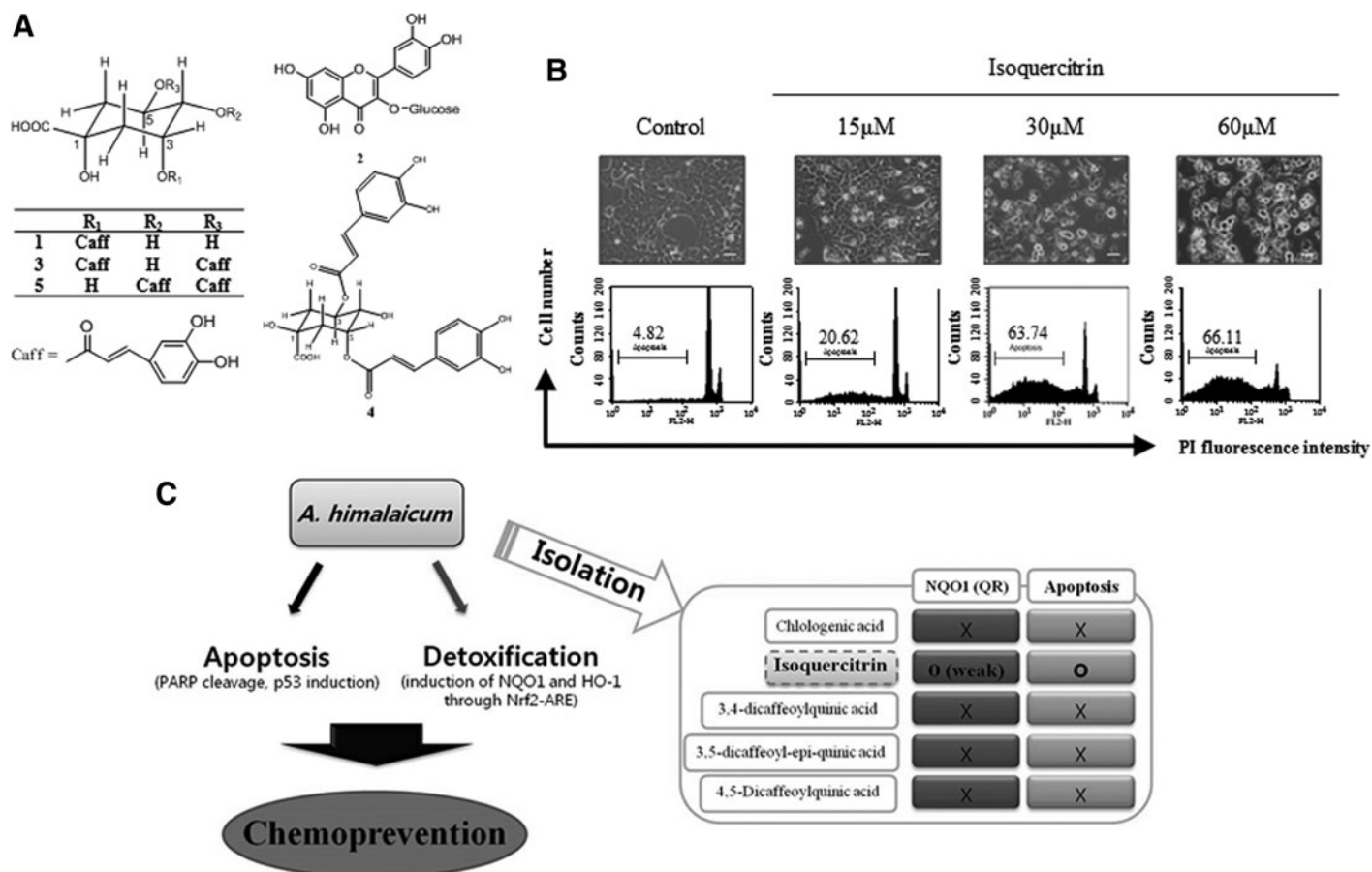
FIG. 7. Isoquercitrin, a major active compound of the AHB that was isolated from the AHE, induces apoptosis in HCT116 cells. **(A)** Chemical structures of the compounds that were isolated from the AHB. **(B)** HCT116 cells were treated with isoquercitrin (15, 30, and 60 μM) for 48 h. Cellular morphology was observed using a phase contrast microscope (*above*) and the sub G0/G1 content (%) was evaluated via flow cytometric DNA content analysis using the PI labeling method (*below*; bar = 20 μm). **(C)** Schematic diagram illustrating the elucidation of the bifunctional chemopreventive effects of an *A. himalaicum* extract and the compounds that were isolated from it.

induced PARP cleavage, as indicated by the diminution of the parent PARP band and the concomitant dose-dependent accumulation of the PARP cleavage fragment (Fig. 6F). We also found that p53, an upstream regulator of apoptosis, became upregulated as the concentrations of the AHE or AHB were increased (from 50 to 200 μg/mL; Fig. 6F). These results suggested that p53 contributes to the antiproliferative effects and apoptosis induced by the AHE and AHB.

Isoquercitrin, a major compound isolated from the AHB, induces apoptosis but does not induce QR activity

We analyzed the chemical composition of the active fraction, the AHB, and isolated 5 major compounds: chlorogenic acid (**1**), isoquercitrin (**2**), 3,4-dicaffeoylquinic acid (**3**), 3,5-dicaffeoyl-*epi*-quinic acid (**4**), and 4,5-dicaffeoylquinic acid (**5**) (Fig. 7A). We further investigated the induction of apoptosis and QR activity. Isoquercitrin (**2**) displayed potent antiproliferative activity, with an IC$_{50}$ value of 29.61 μM for 48 h in HCT116 cells. It also appeared to increase apoptosis in HCT116 cells, as indicated by the increase in the sub G0/

G1 content (%) and apoptotic morphological changes (Fig. 7B). However, isoquercitrin (**2**) only very weakly induced QR activity (data not shown). The other compounds (**1**, **3–5**) did not induce apoptosis or QR activity (data not shown).

DISCUSSION

In the present study, we investigated the cancer chemopreventive actions of the *A. himalaicum* extracts, AHE and AHB, on human cancer cells. Induction of phase II detoxification enzymes, such as QR and GST, is well known to block tumor initiation. Therefore, we chose to measure QR activity as a biomarker in an effort to screen the tumor-blocking effects of the target fractions of an *A. himalaicum* extract. The Hepa1c1c7 mouse hepatocarcinoma cell line is a popular model for use in the QR assay because of its low basal levels, and high inducibility, of QR. In HCT116 cells, QR activity was induced by the AHE or AHB, but the inducibility was not high (Fig. 2A, B).

In the present study, we have defined the AHE and AHB as bifunctional inducers because they induce not only phase II detoxification enzymes, including NQO1 (also known as

QR) and HO-1, by activating the ARE and XRE promoter elements but also the phase I enzyme, CYP1A1, through XRE activation. We also observed synergistic induction of *NQO1* and *HO-1* mRNA as a result of significant ARE and XRE promoter motif activation by the AHB fraction but not the AHE extract (Fig. 2C, D). These results support the results of a previous report that stated that the transcriptional activation of *NQO1* gene promoters can be regulated by two key *cis*-acting elements, the ARE and XRE.[19]

The ARE motif is known to be activated by Nrf2 and its binding complex, whereas the XRE is known to be activated by the AhR-Arnt system.[6,19] In our experiments, Nrf2 rapidly accumulated in the nucleus when the cells were treated with the AHE or AHB (Fig. 4A). Our results also imply that the AHB activates the AhR-Arnt system because the AHB induced CYP1A1 by activating the XRE motif, leading to nuclear accumulation of the AhR (Figs. 3C and 5). Nrf2 plays an essential role in protecting against oxidative/electrophilic stress.[20] In contrast, the AhR-Arnt system has two functions, which are classified as ''adaptive'' (the enhanced metabolism of xenobiotic metabolizing enzymes) and ''toxic'' (the adverse effects of dioxin-like compounds via the induction of CYP1A1 enzymes).[21] Despite its toxic function, activation of the AhR-Arnt may facilitate detoxification by transcriptional regulation of Nrf2 depending on the balance of its adaptive and toxic functions within the cell.[7] *Nrf2* is known to contain three XRE-like elements that are regulated by the AhR-XRE pathway, and multiple copies of the ARE.[7] Therefore, most of the bifunctional inducers, including the AHE and AHB, may not only induce Nrf2 nuclear accumulation but may also regulate Nrf2 transcription levels (Fig. 4C). One of the explanations for this phenomenon is that the AHE and AHB may increase *Nrf2* mRNA by AhR-XRE binding in the Nrf2 promoter region. Therefore, Nrf2 could be one of the downstream targets of the AhR. It is possible that phase II detoxification enzymes may be synergistically activated by the AHE or AHB through the activation of both the ARE and XRE motifs, thus facilitating cancer prevention.

The AHE and AHB also stimulated Nrf2 translocation into the nucleus by releasing Nrf2 from the Keap1–Nrf2 complex (Fig. 4A, Supplementary Fig. S1; Supplementary Data are available at www.liebertpub.com/jmf). Nrf2 cellular protein levels were increased concurrently with Nrf2 translocation into the nucleus. This change in the cellular distribution of Nrf2 is a key event in the induction of phase II detoxification enzymes. However, it remains unclear whether the AHE and AHB inhibit KEAP-1 by directly interacting to protect Nrf2 from the ubiquitin-dependent degradation pathway, or whether they activate Nrf2 directly by phosphorylation through the PI3K or p38 pathways.[22]

Tumorigenesis is a multistep process that results from responses to various factors. Foods contain many components that can act as effective cancer preventive agents due to their synergistic actions. *A. himalaicum* is an abundant plant in Asia, and it has been traditionally consumed as food. We have focused on the chemopreventive effects of both *A. himalaicum* whole extract and its fractions. In this study,

a range of concentrations of the AHE extract and AHB fraction induced detoxification enzyme activity, while they inhibited cancer cell growth at high concentrations by inducing apoptosis. These results illustrate that the AHE and AHB prevent cancer very efficiently by blocking and suppressing different steps in the carcinogenesis process.

The well-known p53 tumor suppressor gene plays a key role in a cell's decision to enter cell cycle arrest or undergo apoptosis.[23] In our study, both the AHE and AHB induced cell cycle arrest in the G2/M phase and induced apoptosis at high concentrations. The role played by p53 in G2/M cell-cycle arrest is complex and involves multiple targets that affect either the cell cycle or the mitotic machinery.[24] In a recent study, synergistic chemoprevention was shown to occur as a result of crosstalk between Nrf2 and p53.[10] The synergistic induction observed in this study also suggests that the AHE and AHB might exert dual chemopreventive effects through the cooperation between Nrf2 and p53.

According to previous reports, activation of the AhR pathway could increase the induction of apoptosis via caspase-3 activation.[25,26] In our case, the AHE and AHB induced the AhR pathway and its detoxification enzymes, including CYP1A1, and inducing p53-mediated apoptosis at 100–200 μg/mL. We speculated that AHE- and AHB-mediated induction of p53-mediated apoptosis might originate from AhR pathway activation.

Many flavonoids have been shown to activate chemoprevention and these results have been verified in manyarticles.[27,28] A flavonoid, isoquercitrin, the *O*-glycoside of quercetin, displayed antiproliferative activity,[29] but its QR-inducing activity was very weak.[30] Our data were in agreement with the results of the previous study. Therefore, we concluded that isoquercitrin is a major active component of the AHB that contributes to the antiproliferative and apoptosis-inducing activity of the AHB. According to the results of our recent study, chlorogenic acid (**1**) and 3,5-dicaffeoylquinic acid (**5**) have antigenotoxic activity.[31] Therefore, isoquercitrin (**2**), chlorogenic acid (**1**), and 3,5-dicaffeoylquinic acid (**5**) can be used as marker compounds for the standardization of functional materials (such as the AHE and AHB) for cancer chemoprevention. However, we could not decipher the active components of the AHB that were responsible for inducing QR activity. Therefore, future detailed chemical analyses may be required to uncover the active compounds that are responsible for the induction of QR activity.

Taken together, our data indicate that the *A. himalaicum* extracts, the AHE and AHB, exert their chemopreventive effects through the induction of detoxification enzymes as a result of Nrf2-ARE and AhR-XRE pathway activation and through the induction of apoptosis. These bifunctional effects could potentially be applied to the development of cancer chemopreventive agents for the prevention of human colorectal cancer. The chemopreventive effects of the AHE and AHB should first be confirmed with *in vivo* efficacy and cytotoxicity assays prior to further application.

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AUTHOR DISCLOSURE STATEMENT

The authors do not have any competing financial interests.

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