Inhibition of Nuclear Transcription Factor- κ B and Activation of Peroxisome Proliferator-Activated Receptors in HepG2 Cells by Cucurbitane-Type Triterpene Glycosides from *Momordica charantia*

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ABSTRACT *Momordica charantia* is used to treat various diseases, including inflammatory conditions. Previous reports indicated that the extract of this plant inhibits activation of nuclear transcription factor- κ B (NF- κ B) but activates peroxisome proliferator-activated receptor (PPAR). Additionally, cucurbitane-type triterpene glycosides are the main bioactive components of the fruit of *M. charantia*. Therefore, we investigated the anti-inflammatory activity of 17 cucurbitane-type triterpene glycosides (**1–17**) isolated from this plant. Their inhibition of NF- κ B and activation of PPAR activities in HepG2 cells were measured using luciferase reporter and PPAR subtype transactivation assays. Compounds **6** and **8** were found to inhibit NF- κ B activation stimulated by tumor necrosis factor- α (TNF α) in a dose-dependent manner. With 50% inhibition concentration (IC₅₀) values of 0.4 μ M, compounds **6** and **8** were more potent inhibitors than the positive control, sulfasalazine (IC₅₀=0.9 μ M). Compounds **4**, **6**, and **8** also inhibited TNF α -induced expressions of inducible nitric oxide synthase and cyclooxygenase-2 mRNA. However, only compound **13** significantly increased PPAR γ transactivation.

KEY WORDS: • *Cucurbitaceae* • cucurbitane-type triterpene glycoside • *Momordica charantia* • nuclear transcription factor- κ B • peroxisome proliferator-activated receptor γ

INTRODUCTION

NUCLEAR TRANSCRIPTION FACTOR- κ B (NF- κ B) represents a family of Rel domain-containing proteins including RelA, RelB, c-Rel, NF- κ B1, and NF- κ B2. The activation of NF- κ B has been linked to multiple pathophysiological conditions such as cancer, arthritis, asthma, inflammatory bowel disease, and other inflammatory conditions.¹ NF- κ B can be activated by various stimuli, such as microbial and viral products, cytokines, DNA damage, and noxious chemicals. The induction of several pro-inflammatory mediators occurs as the results of increased inducible nitric oxide (NO) synthase (iNOS) and cyclooxygenase-2 (COX-2) activities.² Therefore, suppression of iNOS and COX-2 activities can be an important approach to preventing inflammation in organs. NF- κ B and the signaling pathways that regulate many physiological processes, including the innate and adaptive immune responses, cell death, and inflammation, have become a focal point for intense drug discovery and development efforts.³ Indeed, increasing evidence has validated NF- κ B as a target for anti-inflammatory and anticancer agents.

Peroxisome proliferator-activated receptor (PPAR) is a member of the nuclear receptor superfamily of ligand-dependent transcription factors that is predominantly expressed in adipose tissue,⁴ the adrenal glands, and the spleen.⁵ Originally identified as an essential protein in glucose metabolism, PPAR γ has since been shown to be important in cellular processes in endothelial cells, vascular smooth muscle cells, dendritic cells, and platelets.⁵ It can dampen macrophage inflammatory responses through inhibition of pro-inflammatory molecules, such as tumor necrosis factor- α (TNF α), interleukin (IL)-6, and iNOS. Developing specific inhibitors of COX-2 and iNOS with lower toxicity and higher anti-inflammatory activity in therapeutic applications is of great interest.

Bitter melon, the fruit of *Momordica charantia* L. (Cucurbitaceae), a traditional herbal medicine, is used worldwide. It is widely used as a bitter stomachic, a laxative, an antidiabetic agent, an anti-inflammatory agent, and an anticancer agent and in the treatment of rheumatoid arthritis.⁶ Herbal drugs have potential therapeutic application because of their effectiveness, lesser side effects, and relatively low cost. Therefore, investigation of agents from traditional medicinal plants has become more important, and researchers are competing to find new, effective, and safe therapeutic agents for the treatment of

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cancer, inflammatory disease, and diabetes. Bitter melon has various documented anti-inflammatory effects. It reduces prostaglandin E_2 , IL-1 β , IL-6, IL-7, TNF α , and lipopolysaccharide levels^{7–9} and increases the secretion of transforming growth factor- β , IL-10 in RAW 264.6 macrophages, Caco-2 cells, and THP-1 cells.^{8,10} Numerous cucurbitane-type triterpenes and their glycosides have been isolated from the roots,¹² fruits,^{13–23} seeds,^{24,25} leaves, and vines^{26–29} of this plant. However, the anti-inflammatory activity of these cucurbitane-type triterpene glycosides has not been evaluated and reported. Recently, one compound with the same skeleton, 23,24-dihydroxycucurbitacin D from the plant *Bryonia alba* L., was reported to exert anti-inflammatory activity by inhibiting NO generation through blocking NF- κ B activation and iNOS gene transcription.³⁰ Additionally, in previous studies, we isolated and identified 17 compounds from the methanol extract of *M. charantia* fruits, six of which were new and eleven were known.^{31,32}

Here we continued our previous work in screening active compounds for their effects against inflammation and type 2 diabetes. Their inhibitory effects on NF- κ B activation and iNOS and COX-2 expressions as well as transactivations of a PPAR response element (PPRE) and members of the PPAR family (PPAR α , PPAR β/δ , and PPAR γ) were also evaluated. This study provides new insights into the ways in which bitter melon modulates metabolic functions in human HepG2 cells.

MATERIALS AND METHODS

General experimental procedures

The optical rotation was determined on a Jasco (Easton, MD, USA) model DIP-370 digital polarimeter. Electrospray ionization mass spectra were obtained using an Agilent (Palo Alto, CA, USA) model 1200 LC-MSD Trap spectrometer. High-resolution electrospray ionization mass spectra were obtained using a JEOL (Tokyo, Japan) model JMS-T100LC spectrometer. The ¹H-nuclear magnetic resonance (600 MHz) and ¹³C-nuclear magnetic resonance (150 MHz) spectra were recorded on a JEOL model ECA 600 spectrometer, and trimethylsilane was used as an internal standard. Gas chromatography was performed on a Shidmazu (Kyoto, Japan) model 2010 instrument. Column chromatography was performed using silica gel (Kieselgel 60, 70–230 mesh and 230–400 mesh; Merck, Darmstadt, Germany) and YMC (Fujisilisa Chemical Ltd., Kasugai, Aichi, Japan) RP-18 resins.

Plant material

The fruit of *M. charantia* was collected in Vuthu, Thabinh Province, Vietnam, in June 2009 and identified by Dr. Ninh Khac Ban, Institute of Marine Biochemistry, Vietnam Academy of Science and Technology, Hanoi, Vietnam. A voucher specimen (IMBC MC0609) was deposited at the herbarium of the Institute of Marine Biochemistry, Vietnam Academy of Science and Technology.

Cell culture and reagents

Human hepatocarcinoma HepG2 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 10 μ g/mL streptomycin at 37°C and 5% CO₂. Human TNF α was purchased from ATgen (Seoul, Korea). Cells were counted with a hemocytometer, and the number of viable cells was determined through trypan blue dye exclusion.

Cytotoxicity assay

A 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt (MTS) assay (CellTiter 96[®] AQueous One Solution Assay, Promega, Madison, WI, USA) was performed to analyze the effect of the different compounds on cell viability. Cells were cultured overnight in 96-well plates (1 $\times$ 10⁴ cells per well). Cell viability was assessed after the incubation with the compounds at a concentration of 5 μ M for 24 h. The number of viable cells was determined by measuring the absorbance at 490 nm of the dissolved formazan product after addition of MTS for 30 min as described by the manufacturer.

Luciferase assay

Cells were seeded at 1.5 $\times$ 10⁵ cells per well in a 12-well plate and grown for 24 h. All cells were transfected using Lipofectamine[™] LTX (Invitrogen) according to the manufacturer's protocol. Luciferase (Luc) activity was assayed using an LB 953 Autolumat (EG&G Berthold, Nashua, NH, USA) as described previously.³³ NF- κ B-Luc and PPRE-Luc plasmids were kindly provided by Dr. Kyoon E. Kim (Chungnam National University, Daejeon, Korea). All experiments were performed in triplicate.

Reverse transcription–polymerase chain reaction

Total RNA was extracted from cells using easy-BLUE[™] (iNtRON Biotechnology, Seoul). Approximately 2 μ g of total RNA was reverse-transcribed using Moloney murine leukemia virus reverse transcriptase and oligo(dT) primers (Promega) for 1 h at 42°C. The resulting cDNA was polymerase chain reaction–amplified using Taq polymerase premixture (TaKaRa, Shiga, Japan). Polymerase chain reaction products were subjected to electrophoresis on 1% agarose gels and stained with ethidium bromide. Polymerase chain reaction was conducted with the following primer pairs: iNOS sense 5'-TCATCCGCTATGCTGGCTAC-3', iNOS antisense 5'-CTCAGGGTCACGGCCATTG-3', COX-2 sense 5'-GCCAGCACTTCACGCATCAG-3', COX-2 antisense 5'-GACCAGGCACCAGACCAAA GACC-3', glyceraldehyde 3-phosphate dehydrogenase sense 5'-TGTTGCCATCAATGACCCCTT-3', and glyceraldehyde 3-phosphate dehydrogenase antisense 5'-CTCCACGACGTACTCAGCG-3'. The specificity of the products generated using each set of primers was examined using gel electrophoresis and further confirmed using a melting curve analysis.

FIG. 1. Structures of cucurbitane-type triterpene glycosides 1–17 from the fruit of *M. charantia*.

PPAR subtype transactivation assay

The pFA-Gal4-PPAR α ligand binding domain (LBD), pFA-PPAR β/δ LBD, and pFA-PPAR γ LBD expression plasmids were provided by Dr. Young Yang (Sookmyung Women's University, Seoul). HepG2 cells were seeded at 1.5×10^5 cells in 12-well plates and grown for 24 h. All cells were transiently co-transfected with one of the expression vectors for pFA-Gal4-PPAR LBDs together with pFR-Luc using the WelFect-M™ GOLD transfection reagent (Well-GENE, Seoul) as described by the manufacturer. Following a 24-h incubation, the cells were treated with various concentrations of compounds and incubated for 20 h. Luc assays were performed using a dual-Luc reporter assay system according to the instructions of the manufacturer (Promega, Sunnyvale, CA, USA), and the activity was determined in a microplate luminometer (Centro LB 960, EG&G Berthold, Bad Wildbad, Germany) by measuring light emission for 5 s.

Statistical analysis

All results are expressed as mean $\pm$ SD values. Data were analyzed by one-factor analysis of variance. Quantification of polymerase chain reaction products was performed using

Image Lab™ software (Bio-Rad). If a statistically significant effect was found, the Newman–Keuls test was performed to isolate the difference between the groups. $P < .05$ was considered to be significant.

RESULTS

Chemistry

Seventeen compounds (**1–17**) were previously isolated from the methanol extract of *M. charantia* fruits (Figs. 1 and 2). These compounds included six new compounds—charantiosides A–C (**1**, **5**, and **6**) and momordicosides U–W (**13**, **14**, and **7**)—and 11 known compounds: karaviloside III (**2**),¹⁷ 7 β ,25-dihydroxycucurbita-5,23(*E*)-dien-19-al 3-*O*- β -D-allopyranoside (**3**),²¹ karaviloside II (**4**),¹⁷ momordicoside G (**8**),²² momordicoside F₂ (**9**),²² goyaglycoside-b (**10**),¹⁵ momordicoside F₁ (**11**),²² momordicoside I (**12**),²² momordicoside M (**15**),²⁰ momordicoside A (**16**),²⁴ and momordicoside C (**17**).²⁵

Biological activities

In our continuing study, the methanol extract of bitter melon has displayed the ability to markedly reduce

prostaglandin E₂-, IL-1 β -, IL-6-, IL-7-, and lipopolysaccharide-stimulated TNF α levels and increased PPAR α and PPAR γ expression.¹¹ Thus, the 17 compounds listed above were further evaluated for their effects on NF- κ B activation, iNOS and COX-2 expressions, and PPRE, PPAR α , PPAR β/δ , and PPAR γ transactivations.

To investigate cellular toxicity of the 17 isolated compounds, they were applied at various concentrations to HepG2 cells for 24 h, after which cell viability was measured in an MTS assay as described in Materials and Methods. None of the compounds displayed any cellular toxicity at the concentration of 5.0 μ M (data not shown). They were therefore used in subsequent experiments at concentrations of 0.05, 0.5, and 5.0 μ M.

Effect of compounds 1–17 on inhibition of NF- κ B activation

To evaluate the anti-inflammatory activity of 17 compounds listed above, we first examined their inhibitory effects on NF- κ B transcriptional activation in HepG2 cells (Fig. 3). Cells were treated with compounds at various concentrations prior to stimulation with TNF α (10 ng/mL). Compounds 6 and 8 were found to have potent effect on the inhibition of NF- κ B activation with both 50% inhibition concentration (IC₅₀) values of 0.4 μ M (Table 1).

Effects on iNOS and COX-2 expression

Seven compounds (2, 4, 6–8, 11, and 12) were chosen for further analysis effects of on iNOS and COX-2 mRNA ex-

TABLE 1. 50% INHIBITION CONCENTRATION VALUES OF COMPOUNDS 1–17 FOR NUCLEAR TRANSCRIPTION FACTOR- κ B ACTIVITY

Compound	IC ₅₀ (μ M)
1	ND
2	6.9 $\pm$ 1.1
3	9.1 $\pm$ 1.7
4	3.9 $\pm$ 0.3
5	ND
6	0.4 $\pm$ 0.1
7	7.2 $\pm$ 1.5
8	0.4 $\pm$ 0.1
9	> 10.0
10	> 10.0
11	4.9 $\pm$ 0.5
12	3.3 $\pm$ 0.4
13	> 10.0
14	> 10.0
15	ND
16	> 10.0
17	8.3 $\pm$ 1.3
Positive control ^a	0.9 $\pm$ 0.2

^aSulfasalazine was used as positive control compound.

IC₅₀, 50% inhibition concentration; ND, not determined.

pressions. Compounds 4, 6, and 8 dramatically reduced TNF α -induced iNOS and COX-2 mRNA expressions in a dose-dependent manner (Fig. 4).

PPAR

To study the modulation of PPAR activity by compounds 1–17, a PPRE cell-reporter system was used. Compounds 6, 8, 13, and 14 were found to activate the transcription of the PPRE-Luc reporter, compared with that of the positive control, troglitazone (Fig. 5). Consequently, compounds 6, 8, 13, and 14 were used to analyze PPAR subtype-specific reporter activity (PPAR α , PPAR β/δ , and PPAR γ). Compound 13 was found to significantly activate the transcription of PPAR γ but not PPAR α and PPAR β/δ (Figs. 6–8).

DISCUSSION

The pharmacological properties of local food plants are an attractive area for investigation. Accumulating evidence suggests that many locally consumed foods have functional properties, notably anti-inflammatory activity.³⁴ The results of the present study demonstrate the inhibitory effects of the tested compounds on NF- κ B transcriptional activation in HepG2 cells stimulated with TNF α . Compounds 6 and 8 strongly inhibited NF- κ B activation with both having IC₅₀ values of 0.4 μ M, compared with the positive control, sulfasalazine (0.9 μ M). Compounds 4, 11, and 12 also exhibited significant inhibitory activity with IC₅₀ values of 3.9, 4.9, and 4.3 μ M, respectively. The remaining compounds showed moderate activity. It is interesting that in the structure–activity relationship, compounds 4, 6, 8, and

FIG. 3. Effects of compounds 1–17 on tumor necrosis factor- α (TNF α)-induced nuclear transcription factor κ B activation in HepG2 cells. HepG2 cells transiently transfected with pNF- κ B-luciferase were pretreated for 1 h with vehicle (dimethyl sulfoxide [DMSO]) or one of the compounds, prior to 1 h of treatment with TNF α (10 ng/mL). Unstimulated HepG2 cells acted as a negative control. Cells were then harvested, and luciferase activities were assessed. Results are expressed as relative luciferase activity. Sulfasalazine was used as a positive (Pos.) control. Data are mean $\pm$ SD values ($n=3$). $P<.05$ versus control.

FIG. 4. Inhibitory effects of compounds 1–17 on TNF α -induced expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) mRNAs in HepG2 cells. Effects of compounds 2, 4, 6–8, 11, and 12 on COX-2 and iNOS mRNA expression in HepG2 cells were assessed. HepG2 cells were pretreated with one of the listed compounds for 1 h and then treated with TNF α (10 ng/mL) for 6 h. Total mRNAs were prepared from the cell pellets using TRIzol[®] (Invitrogen). Relative levels of mRNAs were measured by reverse transcription–polymerase chain reaction. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

FIG. 5. Effects of compounds 1–17 on peroxisome proliferator-activated receptor response element-mediated transcriptional activity. HepG2 cells were co-transfected with peroxisome proliferator-activated receptor response element luciferase and cytomegalovirus–peroxisome proliferator-activated receptor γ constructs and then incubated for 24 h with or without each compound. Troglitazone was used as a positive control. Data are mean $\pm$ SD values ($n=3$). Significant differences in treatments versus control are indicated: * $P < .05$, ** $P < .01$.

11, which showed potent and significant activity, have the same branch chain at C-17 (a double bond at C-23/24 and a methoxy group at C-25). Activation of NF- κ B and subsequent transcriptional induction of pro-inflammatory mediators play a critical role in inflammation.³⁵ In a previous report, an extract of bitter melon was shown to inhibit

FIG. 6. Effects of compounds 6, 8, 13, and 14 on activation of peroxisome proliferator-activated receptor (PPAR) α . HepG2 cells were transiently transfected with expression vectors for pFA-Gal4-PPAR α -ligand binding domain (LBD), pFR-luciferase, and pRL-cytomegalovirus and treated with various concentrations of each compound. Ciprofibrate was used as a positive control. Data are mean $\pm$ SD values ($n=3$). $P < .05$ was defined as significant.

FIG. 7. Effects of compounds 6, 8, 13, and 14 on activation of PPAR β/δ . HepG2 cells were transiently transfected with expression vectors for pFA-Gal4-PPAR β/δ -LBD, pFR-luciferase, and pRL-cytomegalovirus and treated with various concentrations of each compound. L-165,041 was used as a positive control. Data are mean $\pm$ SD values ($n=3$). $P < .05$ was defined as significant.

lipopolysaccharide-induced NF- κ B activation.⁸ Additionally, several studies have described the anti-inflammatory activity of cucurbitane-type triterpenes.³⁶ Therefore, we assessed the ability of seven compounds (**2**, **4**, **6–8**, **11**, and **12**) that significantly inhibited TNF α -mediated NF- κ B activation to modulate iNOS and COX-2 gene expressions. The results indicated that compounds **4**, **6**, and **8** (dramatically) and compounds **2**, **7**, and **11** slightly reduced TNF α -induced iNOS and COX-2 gene expressions in a dose-dependent manner.

FIG. 8. Effects of compounds 6, 8, 13, and 14 on activation of PPAR γ . HepG2 cells were transiently transfected with expression vectors for pFA-Gal4-PPAR γ -LBD, pFR-luciferase, and pRL-cytomegalovirus and treated with various concentrations of each compound. Troglitazone was used as a positive control. Data are mean $\pm$ SD values ($n=3$). $P < .05$ was defined as significant.

In a previous report, the ethyl acetate extract of *M. charantia* was found to activate both PPAR α and PPAR γ .¹¹ However, the effects of the cucurbitane-type triterpene glycosides **1–17** on PPARs have not previously been described. Therefore, we next evaluated the effects of compounds **1–17** on PPAR activity using a nuclear transcription PPARE cell-reporter system. The PPAR-responsive Luc reporter construct we used carries a copy of the firefly *Luc* gene under the control of a minimal cytomegalovirus promoter and tandem repeats of a PPARE sequence. Activated PPAR binds to the PPARE and activates transcription of the *Luc* reporter gene. Troglitazone, a well-known PPAR γ agonist, was used as a positive control.³⁷ HepG2 cells were co-transfected with the PPARE-Luc reporter and PPAR expression plasmids. As shown in Figure 5, compounds **6**, **8**, **13**, and **14** significantly activated transcription of the luciferase reporter, compared with that of the positive control, troglitazone. To determine how the cucurbitane-type triterpene glycosides **6**, **8**, **13**, and **14** influence the responses to inflammatory stimuli, their effects on the expressions of PPAR α , PPAR β/δ , and PPAR γ were also evaluated. The results of this study provide new insights into the ways in which bitter melon affects metabolic function in human HepG2 cells. PPAR γ , an important target in type 2 diabetes and metabolic syndrome, proved to be a key transcription factor.³⁸ Separately, a new feature of PPAR γ , namely its anti-inflammatory activity, was revealed and developed in parallel.³⁹ Our results showed that compound **13** significantly activated PPAR γ but not PPAR α or PPAR β/δ . The remaining tested compounds **6**, **8**, and **14** did not activate any PPAR subtypes.

In conclusion, the current study is a preliminary *in vitro* result of cucurbitane-type triterpene glycosides from bitter melon, which requires further investigations using *in vivo* and human studies to prove the efficacy.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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