약학 석사 학위논문

Elucidation of the molecular mechanism for adiponectin secretion-promoting phenylethylchromones from *Aquilaria malaccensis*–derived agarwood

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최 정 민

Elucidation of the Molecular Mechanism for Adiponectin Secretion-Promoting Phenylethylchromones from *Aquilaria Malaccensis*-Derived Agarwood

지도교수 노 민 수

이 논문을 약학 석사 학위논문으로 제출함

2019 년 8 월

서울대학교 대학원

약학과 천연물 전공

최 정 민

최 정 민의 석사 학위논문을 인준함

2019 년 7 월

위 원 장 이 상 국 (인)

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ABSTRACT

Elucidation of the molecular mechanism for adiponectin secretion-promoting phenylethylchromones from *Aquilaria malaccensis*–derived agarwood

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Agarwood has been traditionally used to treat various human diseases, although its underlying pharmacological mechanism of action has not yet been fully elucidated. Recently, the potential of adiponectin regulators has received attention because they are associated with various human disease conditions such as obesity, type 2 diabetes, atherosclerosis, and cancer. The aim of this study was to evaluate whether *Aquilaria malaccensis*-derived agarwood altered adiponectin production during adipocyte formation in human BM-MSCs. *A. malaccensis*-derived agarwood methanol extract significantly induced adiponectin release during adipocyte formation in hBM-MSCs. Bioactivity-guided isolation of adiponectin secretion-promoting compounds from agarwood revealed phenylethylchromones as major active compounds and 6-methoxy -2- (2-phenylethyl) chromone and 7-methoxy-2- (2-phenylethyl) chromone as the most potent compounds. In target deconvolution experiments, phenylethylchromones, were found to be present in notable quantities in *A. malaccensis*-derived agarwood, and shown to act as PPARγ partial agonists. This result supports the hypothesis that the therapeutic effects of agarwood on metabolic diseases are associated with a PPARγ-partial agonism-dependent adiponectin secretion

mechanism. Additionally, we demonstrated that phenylethylchromones can be exploited as a pharmacophore when designing a novel PPARγ partial agonist.

Key words : *Aquilaria malaccensis*–derived agarwood; adiponectin; human bone marrow mesenchymal stem cells; phenylethylchromones; PPARγ partial agonism

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Ⅰ. Introduction

Agarwood is a secondary metabolite formed during plant defense responses against pathogen-induced injuries in the bark of Aquilaria species, which taxonomically belong to Thymelaeaceae.[1] When trees of *Aquilaria* species are infected by various fungi or wounded by insects, animals or lightning strikes, resinous volatile organic compounds are synthesized to suppress further fungal growth or trigger the healing process at wounded sites.[2] The habitats of *Aquilaria* species that produce agarwood are distributed in the tropical rainforests of Southeastern Asia. This fragrant resin-containing natural product has been widely used in conventional medicines in Asian countries, including China, India, Korea, and Japan, to relieve the symptoms of various diseases, such as gastrointestinal disorders, asthma, pain, and high fever.[1] Agarwood extracts or agarwood-derived compounds have been suggested to include the diverse potential therapeutic effects such as anti-diabetic,[3] anti-cancer,[3-5] anti-allergic,[6, 7] anti-nociceptive,[8] and sedative activities.[9, 10]

Adiponectin, also referred to as Acrp30/AdipoQ, is an adipocyte secreting protein hormone, involved with cellular metabolism. It has attracted therapeutic attention because of its

significant association with diverse diseases such as obesity, diabetes, atherosclerosis, and cancer.[11- 13] Commonly, the serum AdipoQ levels in these patient populations are lower compared to those of a healthy population. In obese and diabetic patients, the serum ratio of adiponectin to leptin is lower than that of healthy individuals.[14, 15] Moreover, insulin sensitivity was improved when exogenous adiponectin was administered to diabetic mice.[16, 17] Adiponectin also improved the pathogenic outcome of atherosclerosis in mice.[18] The administration of adiponectin also inhibited pathogenic fibrosis in mice, inducing alcoholic and nonalcoholic fatty liver diseases.[19] Because adiponectin has therapeutic potential in numerous human diseases, such as obesity, type 2 diabetes, atherosclerosis, and fatty liver, novel adiponectin secretion regulators are being actively sought for the development of new drugs.

In mammalian adipocytes, adiponectin release is stimulated by the activation of peroxisome proliferator-activated receptor (PPAR) γ.[11, 12] PPARγ agonists, such as rosiglitazone and pioglitazone, significantly upregulate adiponectin production in 3T3-L1 adipocytes.[20] Adiponectin secretion can also be induced by PPARα, PPARδ, glucocorticoid receptor (GR), GPR109A, liver X receptor (LXR),

and protein kinase D (PKD).[21-24] However, it may be possible that these proteins indirectly trigger PPARγ activation. Interestingly, aspirin, a major non-steroidal anti-inflammatory drug (NSAID), promotes adiponectin secretion during adipocyte differentiation in human bone marrow mesenchymal stem cells (hBM-MSCs), although its molecular targets responsible for promoting adiponectin secretion have not yet been clearly identified.[25] Because numerous molecular mechanisms regulate adiponectin secretion, it is advisable to use cell-based phenotype assays when screening and studying adiponectin secretagogues. Phenotypic assays for the discovery of adiponectin secreting modulators have been developed using 3T3-L1 preadipocytes or hMSCs.[26- 28] Because MSCs can give rise to cells of multiple differentiation lineages[24], adipogenesis in a hMSCs-based assay system includes earlier differentiation processes such as the lineage commitment of MSCs to preadipocytes compared to that of murine preadipocyte cell lines. In this regard, phenotypic assays using hMSCs have more molecular target coverage for the discovery of novel adiponectin secretagogues.

In a preliminary screen of various natural products, we found that the methanol extract of an agarwood formed in *A. malaccensis*

enhanced adiponectin production during adipocyte differentiation in hBM-MSCs. Because an *A. malaccensis*-derived agarwood methanol extracts increased adiponectin production, pharmacological mechanisms that explain the traditional uses of an agarwood in diverse human metabolic and inflammatory conditions may be associated with its adiponectin secretion-promoting activity. In view of this, we performed a bioactivity-guided isolation study of adiponectin secretion-inducing compounds using *A. malaccensis*-derived agarwood.

Ⅱ. Materials and Methods

1. Collection of agarwood materials.

Agarwood chips of *A. malaccensis* were purchased from Industrial Plantation Co. (Vientiane, Laos) in January 2010. A voucher specimen (AM-2010-01) was deposited at the herbarium of the Natural Products Research Institute, Seoul National University (Seoul, Korea).

2. Collection of agarwood extractions and isolated compounds.

Dr. Chi Thanh Ma from Prof. Jeong Hill Park's lab provided the extractions and purified compounds of *A. malaccensis*, as well as the structure of the fifteen phenylethylchromone compounds.

3. Cell culture and differentiation.

The hBM-MSCs were obtained from Lonza (Walkersville, MD, USA) and cultured in Dulbecco Modified Eagle's Medium (DMEM; glucose 1 g/L) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA). When maintaining hBM-MSCs in culture, media was replaced every 2nd or 3rd day during cell differentiation. hBM-MSC differentiate upon exposure to the growth medium that is: DMEM with 4.5 g/L of glucose and supplemented with

10% FBS, 10 μg/mL insulin, 0.5 μM dexamethasone, and 0.5 mM IBMX. Dexamethasone, insulin, IBMX, glibenclamide, pioglitazone, and other chemicals were purchased from Sigma-Aldrich.

4. Oil Red O and Hematoxylin staining

The level of adipocyte differentiation in hBM-MSCs was measured using an Oil Red O (ORO, Sigma-Aldrich) staining method. Cells were washed twice with phosphate-buffered saline (PBS) and then fixed with 10% formalin in PBS (pH 7.4) for 30 min. Fixed cells were washed with 60% isopropanol and dried completely. Lipid droplets were stained with 0.2% ORO reagent in 60% isopropanol for 10 min at 25 °C, and then were washed with tap water four times. To stain cell nuclei, slides were incubated with hematoxylin reagent for 1 min and then washed three times with tap water. Differentiated cells were observed and photographed using an Eclipse TS100 inverted microscope (Nikon Co., Tokyo, Japan).

5. Adiponectin measurements: Enzyme-linked immunosorbent assay (ELISA)

For quantitative measurements of adiponectin in cell culture supernatants, a Quantikine^{TM} immunoassay kit (R&D Systems,

Minneapolis, MN, USA) was used, and adiponectin concentrations were determined according to the manufacturer's instructions

6. Nuclear receptor (NR) assays.

The TR-FRET-based NR binding assay was performed using Lanthascreen™ competitive binding assay kits (Invitrogen) to analyze the binding of phenylethylchrome compounds to GR, PPARα, PPARγ, and PPARδ. The Lanthascreen™ coactivator assay kits were used as manufacturer's instructions to determine the receptor activation of LXRα. All assay measurements were performed using a CLARIOstar (BMG LABTECH, Ortenberg, Germany) according to the manufacturer's instructions.

7. Total RNA isolation and quantitative real-time PCR (q-RT-PCR).

Total RNA from hBM-MSCs was harvested using Trizol™ (Invitrogen). RNA concentrations were determined spectrophotometrically at 260 and 280 nm using an Epoch Microplate Spectrophotometer (BioTeK, Winooski, VT, USA). A total of 2 μg RNA from each sample was reverse transcribed into cDNA using a Maxima First Strand cDNA Synthesis Kit for q-RT-PCR (Thermo Scientific™, Waltham, MA, USA). TaqMan Universal Master Mix II and q-RT-PCR primer sets (Applied Biosystems, Foster City, CA, USA) were used to determine the

transcript levels of *PPARG* (Hs00234592_m1), and *FABP4* (Hs00609791_m1). The housekeeping gene *Human glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*, 4333764F) was used to normalize sample variations. All q-RT-PCR was performed with an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems). Relative gene expression levels were quantified using equations from a mathematical model developed by Pfaffl.[55]

8. Molecular docking model study. Molecular docking models of phenylethylchromones to PPARγ-LBD were generated using both AutoDock Vina 1.1.2 software (The Scripps Research Institute, La Jolla, CA, USA) and Accelrys Discovery Studio (Dassault Systems, BIODIVA Corporation, San Diego, CA, USA). The protein structural coordinates of PPARγ were obtained from the Protein Data Bank (PDB code number 5TWO) for the rivoglitazone-bound structure.[40] The crystal structure was prepared for docking by removing the native ligand from PPARγ-LBD, followed by the addition of polar hydrogens using MGLTools 1.5.6 (The Scripps Research Institute). The center and size of the grid box (docking space) was calculated and confined by a rivoglitazone space. We focused on key amino acid residues of the three important branches of PPARγ-LBD for successful docking.[41] Docking success

was analyzed based on the lowest affinity value or free energy score.

9. Statistical analysis. Statistical analyses were performed with RStudio® for Windows (RStudio Inc., Boston, MA, USA). Means ± standard deviation (SD) were used to describe experimental values (n = 3 or 4). Statistical significance was calculated using one-way analysis of variance (ANOVA) analysis and post-hoc tests. Pearson's correlation was used to measure the correlation coefficient. P-values of less than 0.05 and 0.01 were regarded as statistically significant.

Ⅲ. Results

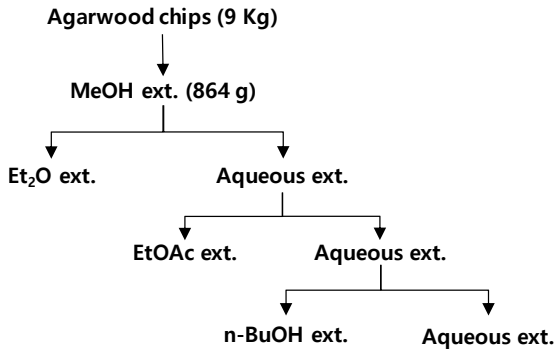
1. Bioactivity-guided isolation of adiponectin secretion-promoting activity from *A. malaccensis*-derived agarwood chips.

The methanol extract, diethyl ether (Et_2O), ethyl acetate (EtOAc), and n-butanol of *A. malaccensis*-derived agarwood chips was provided by Chi Thanh Ma from Jeong Hil Park's lab (Fig. 1A). To set the nontoxic range concentration before adipogenesis experiment was performed, the concentration to cause cell death and the concentration to release inflammatory cytokines were measured at 1 ppm to 30 ppm of agarwood extracts using CCK-8 and PGE2 ELISA(Fig.1B).

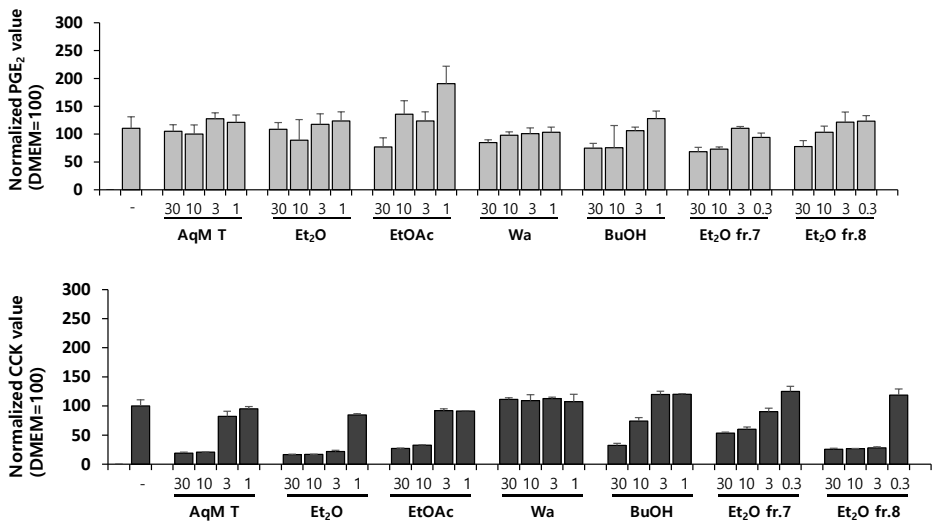
Adiponectin production efficacy during adipocyte differentiation in hBM-MSCs of organic solvent and aqueous fractions were evaluated by adding 10 μg/ml of each extract to the adipogenesis-inducing medium (IDX medium), that included insulin, dexamethasone, and 3-isobutyl-1-methylxanthine (IBMX) (Fig. 1C). The methanol, Et_2O, and EtOAc extracts of agarwood chips enhanced adiponectin production by 68.0%, 153.3%, and 61.5%, respectively, compared to that by the IDX

control. The effect of 10 μg/ml Et_2O extract on adiponectin production and lipid droplet formation during adipocyte differentiation in hBM-MSCs was as potent as that of 300 μM aspirin although it was lower than those of glibenclamide and pioglitazone.

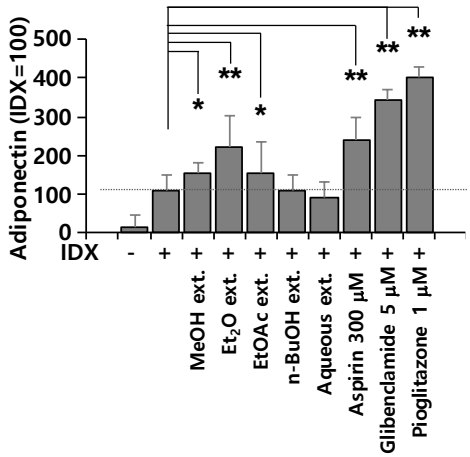
A


Agarwood chips (9 Kg)
MeOH ext. (864 g)
Et2O ext.
Aqueous ext.
EtOAc ext.
Aqueous ext.
n-BuOH ext.
Aqueous ext.

B


Normalized PGE2 value (DMEM=100)
300
250
200
150
100
50
0
- 30 10 3 1 30 10 3 1 30 10 3 1 30 10 3 1 30 10 3 1 30 10 3 0.3 30 10 3 0.3
AqM T
Et2O
EtOAc
Wa
BuOH
Et2O fr.7
Et2O fr.8
Normalized CCK value (DMEM=100)
300
250
200
150
100
50
0
- 30 10 3 1 30 10 3 1 30 10 3 1 30 10 3 1 30 10 3 1 30 10 3 0.3 30 10 3 0.3
AqM T
Et2O
EtOAc
Wa
BuOH
Et2O fr.7
Et2O fr.8

C



D

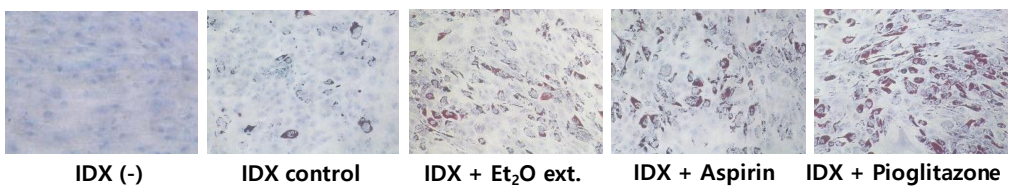
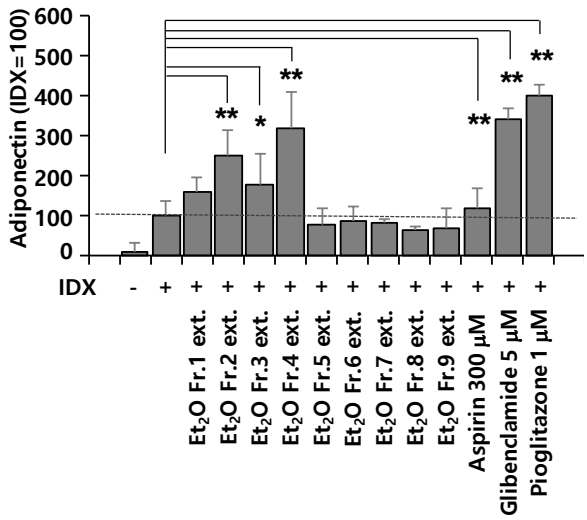


Figure 1. Effects of *A. malaccensis*-derived agarwood extracts on adiponectin production during adipocyte formation in hBM-MSCs. (A) A brief extraction scheme of diethylether (Et_2O), ethylacetate (EtOAc), and n-butanol (BuOH) of agarwood chips provided by Dr. Chi Thanh Ma. (B) CCK-8 and PGE2 ELISA were performed at 1 ppm to 30 ppm to set the nontoxic range concentration before adipogenesis experiment was performed. (C) Organic solvent extracts were co-treated with hBM-MSCs when adipocyte differentiation was induced with an IDX medium. On the third day of the differentiation process, adipogenic media containing the extracts were replaced. On the fifth day, adiponectin levels of cell culture supernatants were quantified using

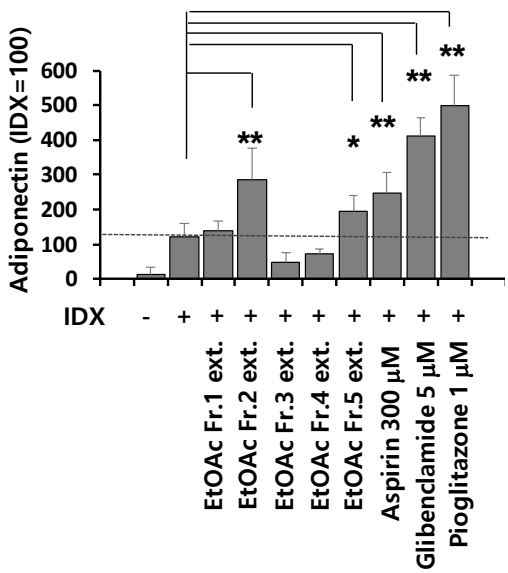
Adiponectin ELISA. (C) Oil red O staining was performed to visualize lipid droplets that formed during adipogenesis in hBM-MSCs. Values represent means ± SD (n = 3); * $p \leq 0.05$ and ** $p \leq 0.01$.

Therefore, the Et_2O and EtOAc extracts were further fractionated by silica gel column chromoatography (Fig. 2C). Among the seven fractionated Et_2O samples, fractions 2, 3, and 4 significantly increased adiponectin production unlike the IDX control (Fig. 2A–B). In EtOAc extracts, EtOAc fractions 2 and 5 showed adiponectin secretion-promoting activity during adipocyte differentiation in hBM-MSCs. From the bioactive Et_2O and EtOAc fractions, a novel 5,6-dihyroxy-2-(2-phenylethyl)chromone (**4**) and 14 known compounds (**1-3**, **5-15**) were purified by using semi-quantitative preparative liquid column (LC) chromatography (Fig. 2C). The structure of compounds were elucidated by Chi Thanh Ma(Fig. 2D).

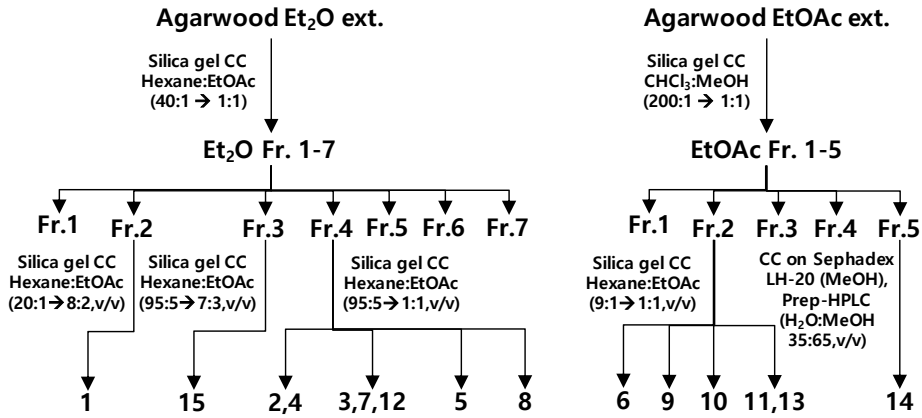
A


Adiponectin (IDX=100)
600
500
400
300
200
100
0
**
*
**
**
**
**
IDX - + + + + + + + + + + + + +
Et2O Fr.1 ext.
Et2O Fr.2 ext.
Et2O Fr.3 ext.
Et2O Fr.4 ext.
Et2O Fr.5 ext.
Et2O Fr.6 ext.
Et2O Fr.7 ext.
Et2O Fr.8 ext.
Et2O Fr.9 ext.
Aspirin 300 µM
Glibenclamide 5 µM
Pioglitazone 1 µM

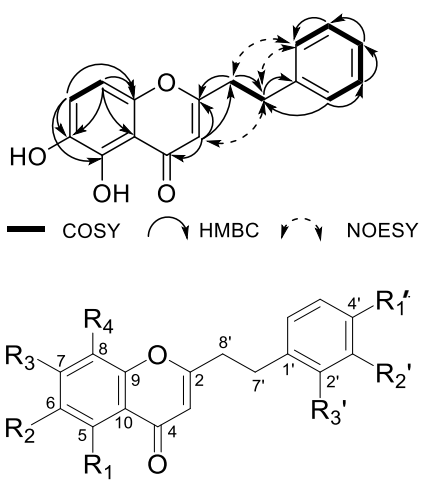
B


Adiponectin (IDX=100)
600
500
400
300
200
100
0
**
*
**
**
**
IDX - + + + + + + + + + +
EtOAc Fr.1 ext.
EtOAc Fr.2 ext.
EtOAc Fr.3 ext.
EtOAc Fr.4 ext.
EtOAc Fr.5 ext.
Aspirin 300 µM
Glibenclamide 5 µM
Pioglitazone 1 µM

C



D



	R1	R2	R3	R4	R1'	R2'	R3'
1	H	H	H	H	H	H	H
2	H	MeO	H	H	H	H	H
3	H	H	MeO	H	H	H	H
4	OH	OH	H	H	H	H	H
5	H	H	H	H	MeO	H	H
6	H	MeO	MeO	H	H	H	H
7	H	MeO	H	H	H	MeO	H
8	OH	MeO	H	H	H	H	H
9	H	OH	H	H	MeO	H	H
10	H	OH	H	H	H	H	H
11	H	H	H	OH	H	H	H
12	H	H	H	H	H	H	OH
13	H	OH	H	Cl	H	H	H
14	- O -		OH	OH	H	H	H
15	OH	H	H	H	H	H	H

Figure 2. Bioactivity-guided isolation of adiponectin-promoting compounds from agarwood chips derived from *A. malaccensis.* (A and B) Et_2O and EtOAc extracts exhibiting increased adiponectin production were fractionated using silica gel column chromatography (cc) and the fractionated extracts were co-

cultured with hBM-MSCs during adipocyte formation. On the third day of the adipogenesis induction process, media were replaced. On the fifth day of the process, On the fifth day, adiponectin levels of cell culture supernatants were quantified using Adiponectin ELISA. (C) Adiponectin secretion-promoting compounds were purified by applying the bioactive Et_2O and EtOAc fractions to column chromatography by Dr. Chi Thanh Ma, and the chemical structure of bioactive compounds were elucidated as phenylethylchromones (D). Values represent means ± SD (n = 3); * $p \leq 0.05$ and ** $p \leq 0.01$.

2. Chemical structure determination of adiponectin secretion-promoting compounds in diethyl ether fractions from *A. malaccensis*-derived agarwood

When fifteen purified phenylethylchromone compounds from the bioactive Et_2O fractions 2, 3, and 4 were analyzed for adiponectin secretion promoting activity in the hBM-MSC differentiation model, 12 compounds at 30 μM were active and reasonably increased adiponectin secretion during adipocyte differentiation in hBM-MSCs compared to that induced by the IDX control (Fig. 3A). However, compounds **9**, **11**, and **12**, did not alter adiponectin production in hBM-MSCs. Among 12 bioactive compounds, the concentration-dependent effects of the top four bioactive compounds were measured (Fig. 3B). To calculate effective concentration 50 (EC_{50}) values, pioglitazone, a clinically available PPARγ agonist, was used as a reference agonist to determine the 100% response value. In dose-response curve analysis, EC_{50} values of compounds **1**, **2**, and **3** were 25.27, 16.18, and 20.26 μM, respectively. Compound **4** significantly enhanced adiponectin production by 1.95-fold compared to that of the negative control. However, the adiponectin secretion-promoting efficacy of **4** by 60 μM was 48% of the maximal pioglitazone-induced response (Fig. 3B).

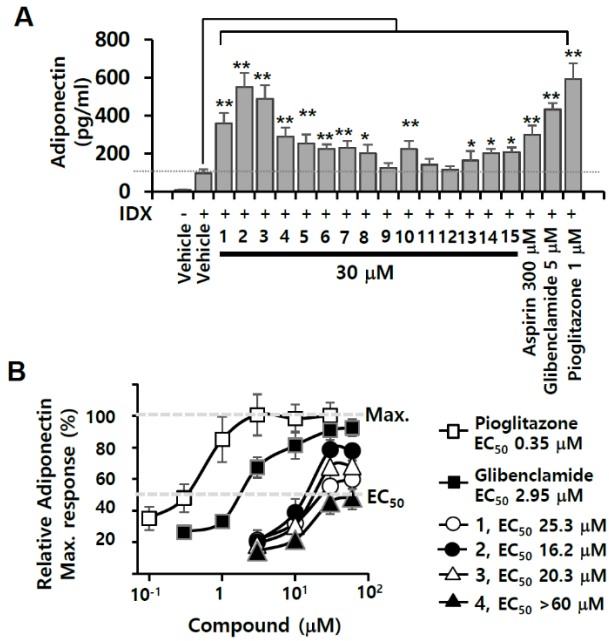


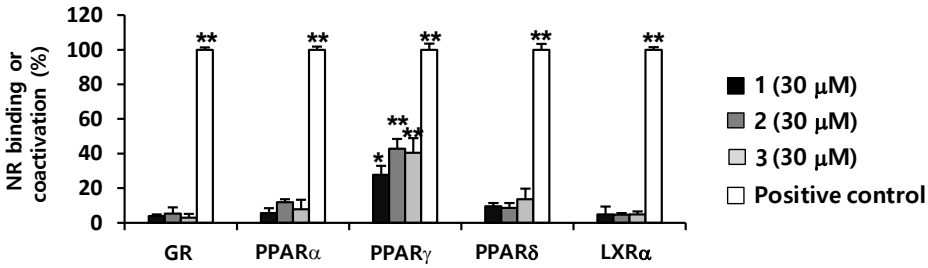
Figure 3. Adiponectin secretion-promoting activity of phenylethylchromone compounds purified from *A. malaccensis*-derived agarwood during adipocyte formation in hBM-MSCs. (A) Phenylethychromone compounds (30μM) were added to IDX medium when adipogenesis was induced in hBM-MSCs. On the third day of the differentiation process, adipogenic media with the phenylethylchromone compounds were replaced. On the fifth day, cell culture supernatants were harvested and adiponectin concentrations were measured. (B) The concentration dependency of phenylethylchromine compounds **1**, **2**, **3**, and **4** on adiponectin secretion-promoting activity was determined. Values represent means ± SD (n = 3); * $p \leq 0.05$ and ** $p \leq 0.01$.

3. Target identification of adiponectin secretion-promoting phenylethylchromones in the *A. malaccensis*-derived agarwood.

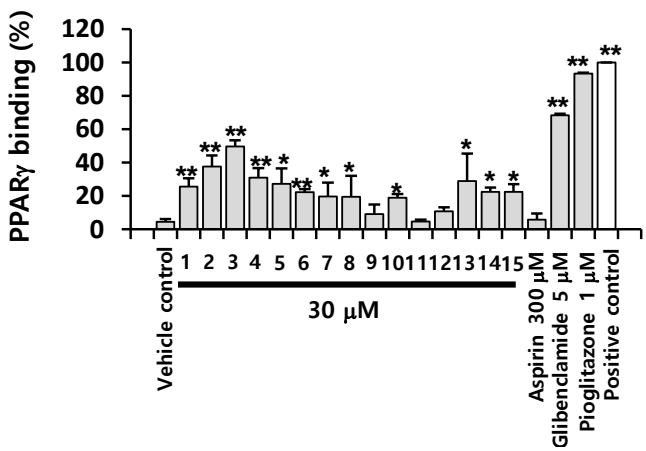
Adiponectin secretion during adipocyte differentiation in hBM-MSCs is primarily regulated by the activation of nuclear hormone receptors, such as GR, PPARα, PPARδ, PPARγ, and LXR.[24, 38] Chromone compounds **1**, **2**, and **3** were measured for the ability to directly bind to GR, PPARα, PPARδ, PPARγ, or LXR. In a time-resolved fluorescence resonance energy transfer (TR-FRET)-based receptor binding assay, compounds **1**, **2**, and **3** significantly replaced the binding of the labeled PPARγ ligand by 37.7%, 52.8%, and 50.3%, respectively (Fig. 4A). Phenylethylchromone compounds **1**, **2**, and **3** does not influence GR, PPARα, PPARδ, or LXRα. Next, we measured the PPARγ binding activity of the fifteen chromone compounds isolated from the *A. malaccensis*-derived agarwood and found that 12 compounds showed significant PPARγ binding activity (Fig 4B). In the Pearson correlation analysis between the level of competitive binding to PPARγ and adiponectin secretion-promoting activity at 30 μM of chromone compounds, the r^2 value was 0.73 ($p < 0.01$) (Fig. 4C). Regarding the correlation coefficient, the adiponectin secretion-promoting activity of chromone compounds isolated from *A. malaccensis*-derived agarwood

was reasonably associated with PPARγ binding affinity. Next, we analyzed the concentration-dependent PPARγ binding activity of chromone compounds **1**, **2**, **3**, and **4**, as they showed potency for both adiponectin secretion-promoting activity and receptor binding unlike the other chromone derivatives. Compounds **1**, **2**, and **3** bound PPARγ in a concentration-dependently with K_i values for **1**, **2**, and **3** of 54.0, 18.1, and 14.9 μM, respectively; however, these compounds were not as potent as the clinically available PPARγ agonist pioglitazone (K_i = 0.062 μM) or a PPARγ binding sulfonylurea antidiabetic drug glibenclamide (K_i = 0.66 μM) (Fig. 4D).[39] The value for compound **4** was incalculable because it replaced only 41.1% of the labeled PPARγ ligand binding at 60 μM, which was the highest concentration tested in the TR-FRET-based concentration-dependency assay. Both the correlation coefficient and dose-response results supported that chromone compounds participated in the release of adiponectin during adipocyte differentiation in hBM-MSCs via a PPARγ dependent mechanism.

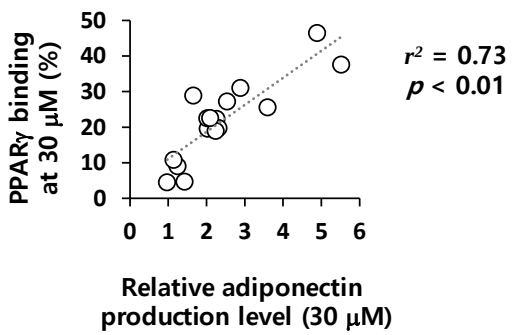
A


NR binding or coactivation (%)
120
100
80
60
40
20
0
GR
PPARα
PPARγ
PPARδ
LXRα
1 (30 μM)
2 (30 μM)
3 (30 μM)
Positive control

B


PPARγ binding (%)
120
100
80
60
40
20
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Vehicle control
30 μM
Aspirin 300 μM
Glibenclamide 5 μM
Pioglitazone 1 μM
Positive control

C


PPARγ binding at 30 μM (%)
50
40
30
20
10
0
0 1 2 3 4 5 6
Relative adiponectin production level (30 μM)
r² = 0.73
p < 0.01

D

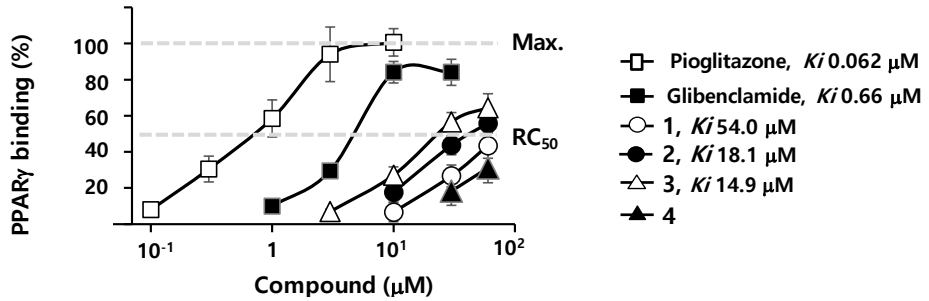


Figure 4. PPARγ binding activity of adiponectin secretion-promoting phenylethylchromone compounds. (A) TR-FRET competitive binding assays with phenylethylchromone compounds **1**–**3** used as ligand for GR, PPARα, PPARγ, and PPARδ were performed. The receptor co-activation analysis for LXRα was determined as described in the Experimental section (B) TR-FRET-based competitive binding activities of phenylethylchromones were determined. (C) Pearson's correlation coefficient (r^2) between the PPARγ binding affinities and relative adiponectin secretion-promoting activities of phenylethylchromone compounds was calculated. (D) *Ki* values for compounds **1**–**3** based on the Cheng and Prusoff equation. Values represent means ± SD (n = 3); * $p \leq 0.05$ and ** $p \leq 0.01$.

4.Molecular docking simulation study to identify interactions of phenylethylchromone compounds 2 and 3 with the ligand-binding domain of PPARγ

To further understand the binding mode of phenylethylchromone compounds **2** and **3** to the PPARγ ligand binding domain (LBD), molecular docking experiments were performed using the crystal structure, the rivoglitazone-bound structure of PPARγ(PDB code 5TWO).[40] The docking models of compounds **2** and **3** to the PPARγ-LBD were compared to that of pioglitazone, a full PPARγ agonist (Fig. 5). The binding free energy of the pioglitazone docking model was -9.1 kcal/mol and the CDOCKER energy for pioglitazone was -39.0 kcal/mol (Fig. 5A). Similar to the rivoglitazone-bound 5TWO crystal structure, pioglitazone adopts a horseshoe-shaped binding conformation that is, centered around helix 3 (H3) of the PPARγ-LBD (Fig. 5A). In Autodock Vina analysis, the free energy scores of optimized docking models for compounds **2** and **3** were -8.5 and -8.4 kcal/mol, respectively (Fig. 4B-C). The CDOCKER free energy levels for compounds **2** and **3** were -28.3 kcal/mol and -26.4 kcal/mol, respectively.

The ligand binding pocket (LBP) of PPARγ has been described

to have three branches that give rise to a Y-shaped cavity with the potential to accommodate chemically diverse endogenous or exogenous ligands.[41] Ligand interactions with the first branch that consists of H3, H5, H11, and H12 are hydrophilic and those with the second branch contributed by H3, H6, H7, and the β-sheet region are generally hydrophobic. Helices H2, H3, and H5, as well as a part of the β-sheet region form the third branch exhibit both hydrophilic and hydrophobic regions.[41, 42] Pioglitazone forms hydrogen bonds and hydrophobic interactions with various amino acid residues, such as isoleucine (Ile) 262 (near Ω-loop), lysine (Lys) 263 (Ω-loop), cysteine (Cys) 285 (H3), phenylalanine (Phe) 287 (H3), arginine (Arg) 288 (H3), histidine (His) 323 (H5), leucine (Leu) 330 (H5), Ile341 (β-sheet s3), and tyrosine (Tyr) 473 (H12) in both the hydrophilic and hydrophobic branches of PPARγ-ligand-binding pocket (LBP) (Fig. 5A). The docking model showed that the thiazolidinedione group of pioglitazone and tyrosine (Tyr) 473 residue in H12 formed a π-sulfur interaction as well as hydrogen bonding. Ligand interactions with Tyr473 (H12) are known to induce the stabilization of the coactivator protein interaction surface of PPARγ, which is known as the activation function-2 (AF-2) surface and is important in the recruitment of transcriptional

coactivators.[40, 43] In contrast to the pioglitazone-PPARγ-LBD docking model, compounds **2** and **3** did not interact with Tyr473 in H12. The docking models with the lowest favorable free energy level showed that compounds **2** and **3** occupy the hydrophobic PPARγ-LBP (Fig. 5B-C). In the internal hydrophobic LBP, compounds **2** and **3** form hydrogen bonds with serine 289 residue in H3 and also assume hydrophobic interactions with Cys285 (H3), alanine (Ala) 292 (H3), Ile326 (H5), Leu330 (H5), and Ile341 (s3). This result indicated that compounds **2** and **3** occupied the region between H3 and the β-sheet where most of PPARγ partial agonists are found to occupy in ligand-PPARγ LBD structures.[28, 42, 44]

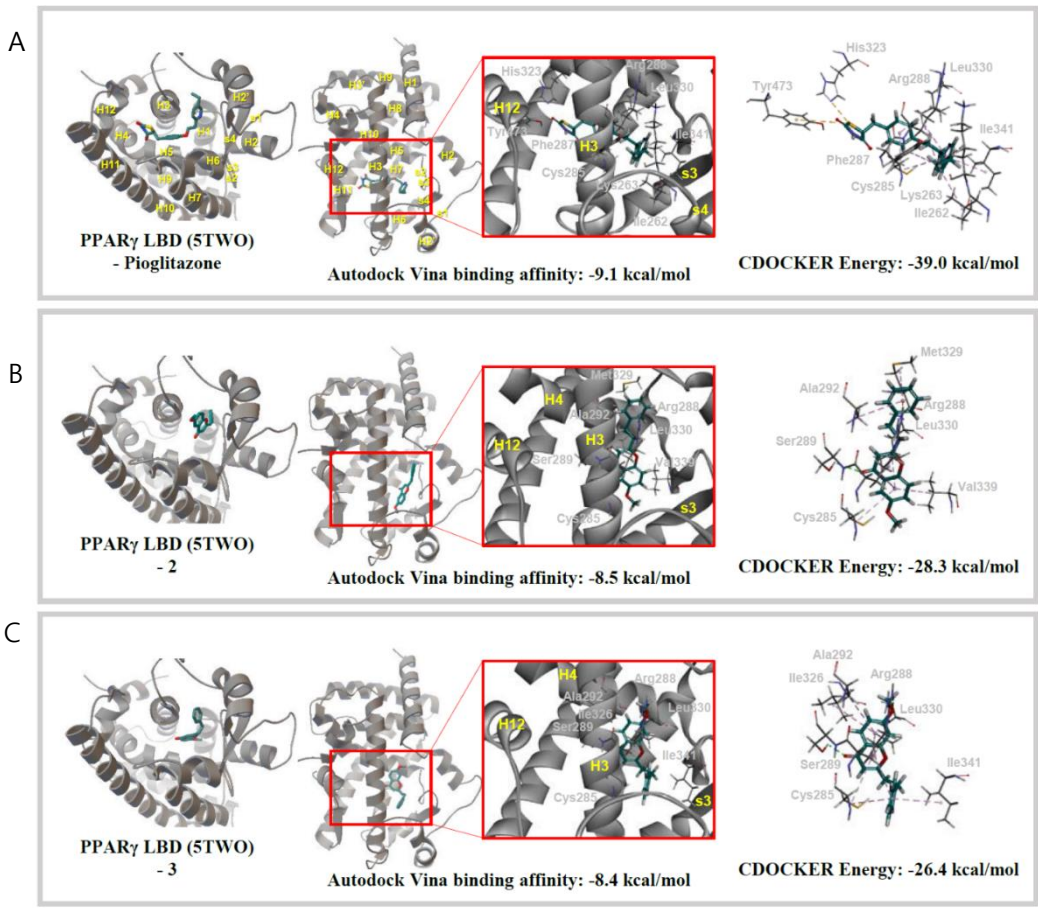


Figure 5. Molecular docking simulation of the interaction between adiponectin secretion-promoting phenylethylchromone compounds and PPARγ-LBD. Molecular docking simulations of pioglitazone (A), **2** (B), and **3** (C) docked to the structure of PPARγ-LBD (PDB 5TWO) were performed using AutoDock Vina version 1.1.2. Free energy scores were calculated using both the Autodock Vina and Accelrys Discovery Studio software. Helix numbering follows the convention used for PPARγ-LBD.[40, 41]

5.Experimental validation of phenylethylchromone compounds 2 and 3 as PPARγ partial agonists.

Although the docking model implied the PPARγ partial agonism of phenylethylchromones, experimental validation was needed. To validate the docking model-predicted PPARγ partial agonism, pioglitazone was co-administered with compounds **2** or **3** during adipocyte differentiation in hBM-MSCs. By definition, a partial agonist plays a role as an antagonist against a full agonist.[45] When adiponectin production was measured in hBM-MSCs, compounds **2** and **3** functionally antagonized the effects of pioglitazone on adiponectin production during adipocyte differentiation, demonstrating that these adiponectin secretion-promoting chromone compounds are PPARγ partial agonists (Fig. 6A-B). Because compounds **2** and **3** enhanced adiponectin production simultaneously antagonizing the effect of a PPARγ full agonist, phenylethylchromones can be classified as a PPARγ partial agonist.

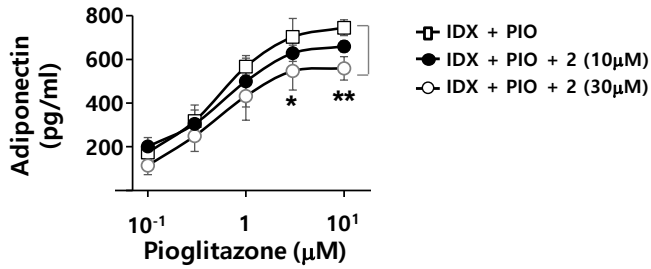
In addition to efficacy of adiponectin production, we also elucidated whether phenylethylchromone compounds **2** and **3** antagonized the pioglitazone-activated transcriptional changes during adipocyte differentiation in hBM-MSCs. The transcriptional changes

of the genes encoding PPARγ and fatty acid binding protein 4 (FABP4) showed that the pioglitazone-activated upregulation of mRNA levels of both *PPARγ* and *FABP4* was reasonably attenuated by the co-administration of compound **2** or **3**, further supporting the role of compounds 2 and 3 as a PPARγ partial agonists (Fig. 6C–D). Therefore, phenylethylchromone compounds purified from *A. malaccensis*-derived agarwood influenced adiponectin secretion during adipocyte differentiation in hBM-MSCs through PPARγ partial agonism.

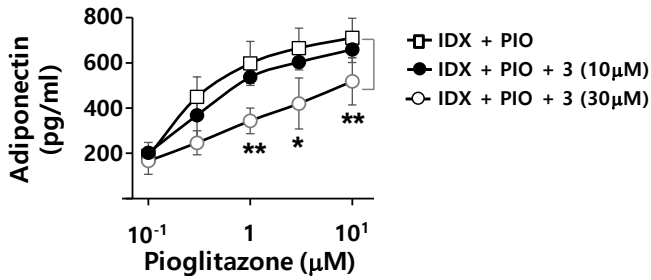
Thiazolidinedione (TZD) PPARγ full agonists such as rosiglitazone, troglitazone, and pioglitazone are PPARγ full agonists that can improve insulin sensitivity. The adverse ourcomes of TZD PPARγ full agonists that ultimately led to market withdrawal include weight gain, renal fluid retention, hepatitis, and an increased risk of cardiovascular events.[46-48] Fatty acids and prostanoids, major endogenous PPARγ ligand candidates, have a far weaker agonistic activity than TZD PPARγ full agonists. Therefore, selective PPARγ modulators that are as potent as endogenous ligands have been suggested as a potential therapeutic drug that may exhibit reduced adverse effects.[47] In this regard, the adiponectin secretion-promoting phenylethylchormones identified from *A. malaccensis*-derived

agarwood provide a pharmacophore for selective PPARγ modulators, which could mitigate or avoid the adverse outcomes of therapeutic PPARγ full agonists.

A



B



C

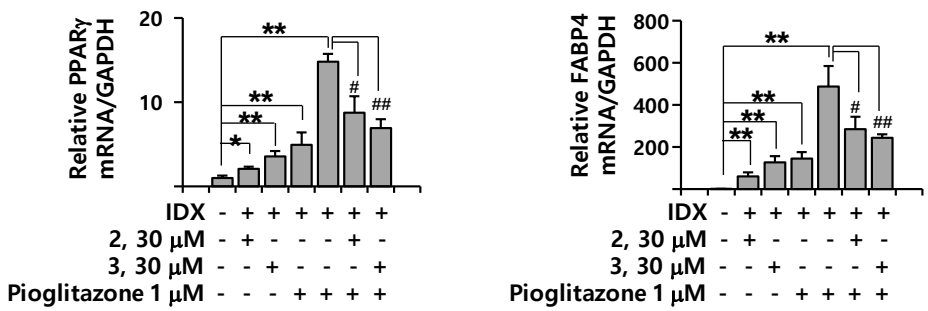


Figure 6. Experimental validation of the PPARγ partial agonism of phenylethylchromone compounds 2 and 3. hBM-MSCs were differentiated in IDX media, and incubated with phenylethylchromone compounds 2 (A) and 3 (B) in the presence of pioglitazone. On the third day of the induction process, adipogenic media containing the compounds were replaced. On the fifth day,

cell culture supernatants were harvested and adiponectin levels in supernatants were measured by ELISA. Additionally, on day 3, total RNA was extracted and q-RT-PCR was performed for *PPARγ* and *FABP4* (C); *GAPDH* was used as an internal control. Values represent means ± SD (n = 3); * $p \leq 0.05$ and ** $p \leq 0.01$.

IV. Discussion

The purpose of this study was to investigate the possible mechanism of adiponectin secretion as a mechanism of action that could explain various pharmacological and physiological activities of agarwood. Agarwood is a part of the wood in which the plant secreted at the bark to restore physical damage to plants of *Aquilaria Malaccensis*. *A. malaccensis*-derived agarwood has been used in conventional medicine to alleviate diverse disease conditions, although the pharmacological mechanisms for its therapeutic effects have not yet been fully elucidated[49]. Adiponectin secreted from adipose tissue is known to be involved with the regulation of biometal homeostasis and antiinflammatory responses. Serum adiponectin levels are relatively low in patients with metabolic diseases, in diverse human diseases, including diabetes, atherosclerosis, and cancers.[50] Therefore, adiponectin is considered to be one of the diagnostic biomarkers of metabolic disease, and the substances promoting adiponectin secretion have been studied as candidates for the treatment of metabolic diseases. In this study, we found that *A. malaccensis*-derived agarwood methanol extract induced adiponectin expression and secretion during adipocyte differentiation in hBM-MSCs. This finding

indicates that the diverse therapeutic potentials of traditional medicine agarwood can be partially explained by an adiponectin-associated mechanism.

In the bioactivity-guided isolation of adiponectin secretagogues, phenylethylchromones were identified as active compounds in *A. malaccensis*-derived agarwood extract. Among chromone compounds, 6-methoxy-2-(2-phenylethyl)chromone (**2**) and 7-methoxy-2-(2-phenylethyl)chromone (**3**) were potent as adiponectin secretion-promoting agents and were identified as PPARγ partial agonists. Chromones are widely found in various natural products and are well-known to exhibit numerous pharmacological activities, such as anti-microbial, anti-inflammatory, anti-oxidant, immunomodulatory, wound healing, and anti-cancer effects.[51-53] In the field of medicinal chemistry, chromones are regarded as a privileged scaffold because the form diverse associations with many approved drugs, such as the anti-allergic cromolyn and anti-asthmatic prankalukast.[52] This study demonstrated that phenylethylchromones enhanced adiponectin expression and secretion via PPARγ partial agonism. Phenylethylchromones are present in notable quantities in *A. malaccensis*-derived agarwood, suggesting that the effects of

agarwood on metabolic diseases are associated with a PPARγ-related mechanism. Therapeutic advantages of PPARγ partial agonists have been proposed from the standpoint of limiting adverse effects compared to those of full PPARγ agonists, such as rosiglitazone and pioglitazone.[47, 48] Therefore, the adiponectin secretion-promoting phenylethylchromones found in *A. malaccensis*-derived agarwood provide important drug discovery insights in to the design of novel PPARγ partial agonists.

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국문요약(국문초록)

침향의 페닐에틸크로몬 유도체의 아디포넥틴 분비 촉진 활성 기전 규명

침향(Agarwood)은 말라켄시스 종(*Aquilaria malaccensis*)의 식물이 물리적 손상을 복구하기 위해 체내에서 분비한 수지가 침착된 목재 부분이다. 침향은 동아시아에서 민간요법으로 소화불량, 기관지천식, 염증, 고열, 알러지 질환 등의 다양한 질병 치료제로서 사용되었지만, 약물학적 기전은 밝혀지지 않은 천연물이다. 지방조직에서 분비되는 아디포넥틴은 생체대사 항상성 조절 및 항염증 반응에 관여하며, 대사성 질환의 진단 바이오 마커 중 하나로 여겨지고 있다. 비만, 제2형 당뇨병, 지방간염, 암 등 대사성 질환을 가진 환자에서 혈중 아디포넥틴 농도는 정상인보다 상대적으로 낮다고 알려져 있으며, 아디포넥틴 분비를 촉진하는 물질이 대사성 질환의 치료 후보물질로 연구되고 있다. 본 연구는 침향의 다양한 약물학적 생리활성을 설명할 수 있는 작용기전으로 아디포넥틴 분비

조절제로서의 가능성을 규명한 것이다. 골수유래 중간엽줄기세포의 지방분화 과정을 이용한 아디포넥틴 생합성 측정 모델에서 침향의 메탄올 추출물이 대조군에 비해 아디포넥틴 분비 촉진 활성이 있음을 확인하였다. 침향 추출물로부터 효능이 있는 단일 화합물을 찾기 위한 생리활성유도분획법(bioactivity-guided fractionation) 연구를 통해 크로몬 계열의 페닐에틸크로몬, 6-메톡시-2-(2-페닐에틸)크로몬, 그리고 7-메톡시-2-(2-페닐에틸)크로몬이 중간엽줄기세포 지방분화 과정 중, 아디포넥틴 분비 촉진 효과가 있음을 규명하였다. 페닐에틸크로몬 유도체의 아디포넥틴 분비 촉진 효과와 관련된 분자 타겟을 탐색한 결과, 페닐에틸크로몬 유도체들이 페록시솜증식체활성화수용체-감마(PPARγ)에 직접 결합하는 것을 규명하였다. PPARγ 효능약인 피오글리타존과의 경쟁적 약물 반응 실험을 통해 페닐에틸크로몬 유도체들은 PPARγ 부분효능약으로 작용함을 증명하였다. 본 연구는 침향의 다양한 생리활성을 설명할 수 있는 작용기전으로서 아디포넥틴 분비 촉진 효과가 연관되어 있고, 이를 설명할 수 있는 분자기전으로 페닐에틸크로몬 유도체들이 PPARγ에 부분효능약으로 작용함을 증명하였다. 본 연구에서 발견한 침향 유래 화합물들은 저아디포넥틴혈증(hypoadiponectinemia)과 연관된 다양한 대사성질환의 증상을 개선하는 치료제로서의 가능성이 기대된다.

주요어: 아디포넥틴, 지방분화, 침향, 사람의 중간엽줄기세포, 페록시솜증식체활성화수용체, 표현형기반평가

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