Macakurzin C Derivatives as a Novel Pharmacophore for Pan-Peroxisome Proliferator-Activated Receptor Modulator

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Abstract

The natural flavonoid macakurzin C (1) exhibited adiponectin biosynthesis-inducing activity during adipogenesis in human bone marrow mesenchymal stem cells and its molecular mechanism was directly associated with a pan-peroxisome proliferator-activated receptor (PPAR) modulator affecting all three PPAR subtypes α, γ, and δ. In this study, increases in adiponectin biosynthesis-inducing activity by macakurzin C derivatives (2–7) were studied. The most potent adiponectin biosynthesis-inducing compound 6, macakurzin C 3,5-dimethylether, was elucidated as a dual PPARα/γ modulator. Compound 6 may exhibit the most potent activity because of the antagonistic relationship between PPARα and PPARγ. Docking studies revealed that the O-methylation of macakurzin C to generate compound 6 significantly disrupted PPARδ binding. Compound 6 has therapeutic potential in hypoadiponec tinemia-related metabolic diseases.

Key Words: Macakurzin C derivative, Peroxisome proliferator-activated receptor, Adiponectin, Human bone marrow mesenchymal stem cells, PPARα/γ dual modulator

INTRODUCTION

Hypoadiponectinemia is associated with metabolic diseases such as type 2 diabetes, obesity, cardiovascular diseases, and fatty liver diseases (Hossain et al., 2015; Avgerinos et al., 2019; Boutari and Mantzoros, 2020). Therefore, adiponectin biosynthesis-inducing compounds have been suggested as therapeutic candidates for hypoadiponectinemia-related metabolic diseases (Hossain et al., 2015; Stern et al., 2016). Adiponectin production in mammalian adipocytes is mainly regulated by nuclear hormone receptors, including the peroxisome proliferator-activated receptor (PPAR), glucocorticoid receptor, and retinoid X receptor (Hoppmann et al., 2010; Har- mon et al., 2011; Nakamura et al., 2014). Notably, two PPAR isotypes α and γ promote adiponectin biosynthesis, whereas PPARδ negatively regulates adiponectin production by suppressing PPARγ activity (Zuo et al., 2006; Yu et al., 2017; An et al., 2020a).

Macakurzin C (5,7-dihydroxy-2,2-dimethyl-8-phenyl-2H,6H-pyran-3,2-g-chroman-6-one) (1), a natural flavonoid isolated from the leaves of Macaranga kurzii, was reported to have acetylcholinesterase inhibitory activity (Trinh Thi Thanh et al., 2012). Synthetic macakurzin C derivatives were shown to improve acetylcholinesterase inhibitory activity compared to that of macakurzin C (Lee et al., 2014; Baek et al., 2015). In addition, synthetic macakurzin C derivatives inhibited phorbol 12-myristate 13-acetate- and oxazolone-induced dermal inflammation (Akram et al., 2016). However, the effect of macakurzin C and its derivatives on adipogenesis remains unclear. Notably, the metabolic effects of flavonoid phytochemicals like macakurzin C on mammalian adipocytes have been reported (Shin et al., 2009; Wang et al., 2014). In preliminary screening for adiponectin biosynthesis-inducing activity, macakurzin C (1) significantly increased adiponectin production during adipogenesis in human bone marrow mesenchymal stem cells (hBM-MSCs). Binding assays based on the time-resolved fluorescence resonance energy transfer (TR-FRET) method revealed that compound 1 significantly bound all three
PPAR subtypes \( \alpha, \gamma, \) and \( \delta \). PPAR\( \delta \) overexpression inhibits the activity of PPAR\( \gamma \) in colorectal cancer cells (Zuo et al., 2006). In addition, a PPAR\( \delta \) antagonist potentiated the effect of PPAR\( \gamma \) on adiponectin production during adipogenesis in hBM-MSCs (Yu et al., 2017; An et al., 2020a). Here, we performed a structure-activity relationship study of macakurzin C derivatives (2–7) to elucidate their improved adiponectin biosynthesis-inducing activity.

MATERIALS AND METHODS

Cell culture and differentiation induction

hBM-MSCs were purchased from Lonza (Walkersville, MD, USA) and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, low glucose, 1 g/L) supplemented with 10% fetal bovine serum (FBS), 1% Glutamax\textsuperscript{TM}, and 1% penicillin-streptomycin. To induce adipocyte differentiation, cell culture media was changed to adipogenesis-inducing media consisting of 10 g/mL insulin, 0.5 \( \mu \)M dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine (IDX condition) in DMEM (high glucose, 4.5 g/L) with 10% FBS and 1% penicillin-streptomycin. DMEM, FBS, Glutamax\textsuperscript{TM}, and penicillin-streptomycin were supplied by Invitrogen (Carlsbad, CA, USA). 3-Isobutyl-1-methylxanthine, dexamethasone, and insulin were acquired from Sigma-Aldrich (St. Louis, MO, USA).

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was conducted to quantify adiponectin levels in culture supernatants using a Quantikine\textsuperscript{TM} immunoassay kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer’s instructions.

Oil Red O staining

Oil Red O staining was used to evaluate lipid accumulation during adipogenesis in hBM-MSCs as previously described (Han et al., 2020). The stained lipid droplets were photographed using an Eclipse TS100 inverted microscope (Nikon Co., Tokyo, Japan).

Time-resolved fluorescence resonance energy transfer

ATR-FRET-based competitive receptor binding assay was performed to determine ligand binding to PPAR subtypes \( \alpha, \gamma, \) and \( \delta \) using Lanthascreen\textsuperscript{TM} competitive binding assay kits (Invitrogen; PV4892, PV4894, and PV4893). All assay measurements were performed using CLARIOstar (BMG LABTECH, Ortenberg, Germany) as previously described (An et al., 2020a). 2-[4-[2-[[4-(Cyclohexylamino)carbonyl][4-cyclohexylbutyl]amino]ethyl][phenyl]thio]-2-methylpropanoic acid (GW7647), N-[2-benzoylphenyl]-O-[2-(methyl-2-pyridinyl)amino]ethyl-L-tyrosine (GW1929), and 2-[2-methyl-4-[[4-[2-(trifluoromethyl)phenyl]-5-thiazolyl]methoxy][thio][phenoxo]acetic acid (GW501516) were obtained from Tocris Bioscience (Bristol, UK).

Computational docking analysis

The structures of the PPAR\( \alpha \), PPAR\( \gamma \), and PPAR\( \delta \)-ligand-binding domains (LBDs) complexed with WY-14643, pioglitazone, and GW501516 (PDB ID: 4BCR (Bernardes et al., 2013); SU6L (Rajapaksha et al., 2017); SU46 (Wu et al., 2017)), respectively, were used after preprocessing for computational docking analysis. Preprocessing was conducted as previously described (An et al., 2020b). Briefly, the X-ray crystalllographic structures of the proteins were downloaded from the RCSB Protein Data Bank (https://www.rcsb.org/), and water and the native co-crystallized ligands were removed. The initial three-dimensional coordinates for docking analysis were set to the centroids of those ligands. Docking analysis was performed using AutoDock Vina v1.1.2 software (The Scripps Research Institute, La Jolla, CA, USA). All graphics were visualized using PyMOL v2.4.0 (Schrödinger, LCC, New York, NY, USA).

Statistical analysis

The experimental values represent the mean ± standard deviation (SD) from at least three different experiments. Statistical analyses were conducted with one-way analysis of variance (ANOVA) followed by post-hoc Welch two-sample t-tests using RStudio\textsuperscript{®} for Windows (RStudio Inc., Boston, MA, USA). The threshold of statistical significance was set at \( *p<0.05 \) and \( **p<0.01 \).

RESULTS

Macakurzin C derivatives showed adiponectin biosynthesis-inducing effects

The synthesis of macakurzin C derivatives (2–7) was performed as previously described (Lee et al., 2014; Baek et al., 2015). The effect of compound 1 and its derivatives 2–7 on adiponectin biosynthesis was evaluated during adipogenesis in hBM-MSCs (Fig. 1A). When treated with compounds 1–7 with the adipogenesis-inducing conditions (IDX conditions), compounds 1, 3, 5, 6, and 7 significantly induced adiponectin biosynthesis at 10 \( \mu \)M compared to that of the vehicle control (Fig. 1B). Notably, two 5-methylether derivatives (6 and 7) significantly upregulated adiponectin production by 4.42- and 3.35-fold, respectively, while compound 5 did not. The concentration-effect analysis showed that the half-maximal effective concentration values (EC\textsubscript{50}) of compounds 6 and 7 were 5.0 and 10.2 \( \mu \)M, respectively, when the maximal adiponectin biosynthesis at 10 \( \mu \)M was set as 100% activity (Fig. 1C). The EC\textsubscript{50} value of compound 1 for adiponectin biosynthesis-inducing activity could not be calculated due to the weak activity of less than a 50% response even at the highest tested concentration. In the phenotypic analysis, compounds 1, 6, and 7 showed the increased number and size of lipid droplets compared to those of the vehicle control during adipogenesis in hBM-MSCs (Fig. 1D).

Macakurzin C derivatives are characterized as PPAR ligands

Next, we investigated the molecular targets responsible for the adiponectin biosynthesis-inducing activity. Compound 6, which showed the most potent adiponectin biosynthesis-inducing activity, was selected to evaluate its effects on PPARs and compared with compound 1 (Fig. 2). The TR-FRET-based competitive binding assays revealed that compound 1 competitively replaced the labeled ligand in all three PPAR subtypes, including PPAR\( \alpha \) (55.1%), PPAR\( \gamma \) (64.9%), and PPAR\( \delta \) (82.8%) at 10 \( \mu \)M (Fig. 2A). In contrast, compound 6 significantly bound to PPAR\( \alpha \) and PPAR\( \gamma \) at 51.1 and 87.4%, respectively, without affecting PPAR\( \delta \) significantly at 51.1 and 87.4%, respectively, while compound 5 did not. The concentration-effect analysis showed that the half-maximal effective concentration values (EC\textsubscript{50}) of compounds 6 and 7 were 3.3 and 3.6 \( \mu \)M, respectively, when the maximal adiponectin biosynthesis at 10 \( \mu \)M was set as 100% activity.
Conducted to determine the binding assays. PPARγ-based competitive binding assays. PPAR17–
affinity of compound 1 (3.2 µM) was more potent than that for compounds 1, 6, and 7. (D) Phenotypic
macakurzin C (GLI, 10 µM) and pioglitazone (PIO, 10 µM) were used as positive control drugs. (C) The
for compounds 1, 6, 3.3 M, 0.9 M, PO, 0.002 M, 0.0001 M, and 0.001 M.
compounds 1 and 6 showed comparable binding modes tively, although their efficacy was not as potent as that of the positive control drug, PPARα agonist GW7647 (Fig. 2B). The
atomics for PPARγ; binding showed that the PPARγ binding affinity of compound 6 (K=0.9 µM) was more potent than that of compound 1 (3.2 µM) (Fig. 2C). The K value for the PPARδ binding of compound 1 was 1.3 µM, whereas compound 6 did not bind to PPARδ (Fig. 2D).
Compounds 1 and 6 showed comparable binding modes in PPARα-LBD

The O-methylation of compound 1 to compound 6 resulted in the loss of PPARδ binding while maintaining an affin-
Fig. 3. Comparative docking analysis of compound 6 against PPARα-LBD and PPARγ-LBD. (A) Energy-minimized binding pose of compound 6 (green) in PPARα-LBD (PDB ID: 4BCR) was superimposed with that of PPARα ligand WY-14643 (WY, magenta). (B) Close-up view of second binding site. Detailed protein-ligand interaction profiles of WY (C), compound 1 (D, cyan) and compound 6 (E) in PPARγ-LBD were compared. Binding free energy from the docking analysis was marked in kcal/mol. The closely contacting residues to ligands were labeled. (F) Energy-minimized binding pose of compound 6 (green) in PPARγ-LBD (PDB ID: 5U5L) was superimposed with that of PPARγ ligand pioglitazone (PIO, orange). (G) Close-up view of PPARγ ligand-binding site. Detailed protein-ligand interaction profiles of PIO (H), compound 1 (I) and compound 6 (J) in PPARγ-LBD were compared. Secondary structures are shown as cartoon, and structures of ligands are presented as sticks or spheres. Oxygen and nitrogen atoms are colored in red and blue, respectively. Hydrogen bonds are presented as red dashes.

In the comparative docking analysis, we preprocessed the LBDs of PPARs by removing co-crystallized ligands as previously described (An et al., 2020b). First, the molecular docking model of compound 6 interacting with the PPARα-LBD (PDB ID: 4BCR; Bernardes et al., 2013) was constructed and compared to those of compound 1 and the PPARα agonist WY-14643 (Fig. 3). The LBP of PPARα-LBD is relatively bigger than that of PPARγ and PPARδ, which enables a bipartite binding mechanism (Bernardes et al., 2013). The large binding pocket allows two ligand molecules to interact with PPARα-LBD, where each molecule has been described as being capable of binding to a classical PPAR binding site between H3 and H4 near H12 or a second binding site between H2’ and H3 (Fig. 3A). The energy-minimized binding mode of compound 6 in the PPARα-LBD was located at the second binding site around H2’-H3 (Fig. 3B). In the second binding site, WY-14643 could hydrophobically interact with the residues Leu247, Val255, Leu258, Ile263, Arg271, His274, Cys275 and Cys278, forming hydrogen bonds with Glu251 and Lys266 (Fig. 3C). Notably, the site-directed mutagenesis study suggested that amino acid residues Val255 and Ile263 were key determinants for ligand recognition and sensitivity in the PPARα-LBD (Bernardes et al., 2013). The ligand-protein interaction profiles of compounds 1 and 6 revealed that both could interact with key residues for PPARα activation. They commonly hydrogen-bonded with the carbonyl groups of Arg271 and His274 and shared most of the other hydrophobic interactions as well (Fig. 3D, 3E). The difference in the chemical structures of compounds 1 and 6 did not lead to differences in binding modes inside the PPARα-LBD, which could result in similar PPARα binding affinities.

**Compound 6 was located in partial agonist-binding region of PPARγ-LBD**

Next, we conducted a molecular modeling study for compounds 1 and 6 in the PPARγ-LBD. Docking simulations of the compounds against the PPARγ-LBD were performed using the PPARγ structure co-crystallized with a PPARγ agonist rivotriptan (PDB ID: 5U5L; Rajapaksha et al., 2017). Similar to the PPARα-LBD, the PPARγ-LBD is also known to have multiple binding regions, and full agonists are often described as interacting mainly with H12, whereas partial agonists or antagonists mainly interact with the β-sheet region (Bruning et al., 2007; Capelli et al., 2016; An et al., 2020b). When the energy-minimized binding modes of the PPARγ ligand pioglitazone
and compound 6 were compared, pioglitazone interacted with both Tyr473 of H12 and Ser342 of the β-sheet, whereas 6 preferentially interacted with amino acid residues in the hydrophobic arm of LBD stabilizing the β-sheet structure containing Ser342 (Fig. 3F, 3G). In the detailed analysis of protein-ligand interactions (Fig. 3H, 3I, 3J), pioglitazone formed a tight hydrogen bond network with Ser289, His323, Tyr327, His449, and Tyr473 near H12 and showed lower binding free energy compared to the compounds 1 and 6 (Fig. 3H). The profiles of the close contacting residues of compound 6 were similar to those of compound 1 (Fig. 3I, 3J). They both could form a hydrogen bond with Ser342, which is a signature binding mode of PPARγ partial agonists, suggesting that they may act as partial agonists. Notably, the distance from the oxygen atoms of compounds 1 and 6 to the nitrogen atom of Ser342 was 3.2 Å and 2.7 Å, respectively. In addition, compound 6 had a lower binding free energy (–9.0 kcal/mol) compared to compound 1 (–8.6 kcal/mol) by locating closer to the β-sheet for hydrogen bonding, which explains the improved PPARγ affinity of compound 6 (Fig. 3I, 3J).

O-Methylation disrupted PPARδ-binding of compound 6 by increasing VdW strain

Compounds 1 and 6 showed comparable binding affinities to PPARα and PPARγ, whereas significant differences in binding affinity were observed in PPARδ binding. To investigate the molecular basis of the PPARδ binding affinities, we performed a molecular docking analysis of the PPARδ ligand GW501516 and compounds 1 and 6 against the PPARδ-LBD (PDB ID: 5U46; Fig. 4) (Wu et al., 2017). The Tyr437 residue of H12 and the β-sheet region were also reported to play essential roles in the ligand-dependent activation of PPARδ (Wu et al., 2017; An et al., 2020a). In addition, the site-directed mutagenesis study suggested that residues Val312 and Ile328 confer the specificity for ligand-mediated PPARδ activation. The optimized binding mode of the PPARδ specific agonist GW501516 was in close contact (under 3.5 Å) with amino acid residues Cys249, Thr252, His287, His413, Met417, and Leu433, as well as Val312 and Tyr437 (Fig. 4B, 4C). The energy-minimized binding mode of compound 6 was preferentially located near the β-sheet interacting with Val312, suggesting that it would act as a PPARδ partial agonist or antagonist, unlike the specific agonist GW501516 (Fig. 4B, 4C). The calculated binding free energy of compound 6 was –6.9 kcal/mol, which was higher than that of 1 (–7.6 kcal/mol), partially supporting the decreased PPARδ-binding affinity of compound 6 (Fig. 4D, 4E). As previously described, the overall size of the LBPs across the PPAR subtypes is similar, but that of PPARδ is narrower than that of PPARα or PPARγ (Xu et al., 2001). When ligands are represented as spheres based on the Van der Waals (VdW) radii of their atoms, the spatial occupancy of the ligands can be analyzed (Fig. 4F). Compared to compound 1, compound 6 was too big to locate inside the PPARδ-LBP (Fig. 4G). When the VdW strain was computed between the ligands and the amino acid residues within 5 Å of the ligands in the docked model, the VdW repulsion of compound 6 in the PPARδ-LBP was calculated as 30.5 kcal/mol, which was much higher than that of compound 1 (1.2 kcal/mol). This qualitative and quantitative evidence indicated that the O-methylation of compound 1 to compound 6 significantly disrupted PPARδ binding.

DISCUSSION

In this study, we showed that macakurzin C (1) functions as...
a pan-PPAR modulator and promotes adiponectin biosynthesis during adipogenesis in hBM-MSCs. This result indicated that macakurzin C derivatives have therapeutic potentials in hypoadiponecinemia-associated metabolic diseases (Hos-sain et al., 2015; Averinos et al., 2019; Boutari and Mantzor-os, 2020). Among macakurzin C derivatives (2–7), the ma-cakurzin C 5-methylether derivatives (6 and 7) showed more potent adiponectin biosynthesis-inducing activity than that of compound 1. Since PPARα has been reported to suppress the activity of PPAR (Zuo et al., 2006; Yu et al., 2017; An et al., 2020a), the improved adiponectin biosynthesis-inducing activity of compound 6 compared to that of compound 1 might be associated with the increased PPARγ2 binding affinity and the concurrent loss of PPARα binding affinity. The 3,5-dimethyl-ation of compound 1 to compound 6 resulted in a significant loss of PPARα-binding affinity while retaining the PPARα1 γ dual modulator potential. Since the O-methylation of compound 1 to compound 6 resulted in the different pharmacological profiles, we investigated the structural basis underlying their PPAR-binding. The difference in the chemical structures of compounds 1 and 6 did not lead to differences in binding modes inside the PPARα-LBD, which could result in similar PPARα-LBD binding affinities. On the other hand, compound 6 showed a lower binding free energy compared to compound 1 by locating closer to the β-sheet for hydrogen bonding, which explains the improved PPARγ2 affinity of compound 6. The optimized docking model against PPARα-LBD demonstrated that the dimethylation significantly disrupted PPARγ2-binding by increasing the VdW repulsion in the LBD. These molecular modeling results provide valuable insight into the future design and development of PPARα1 γ dual modulators, and further studies will be directed to investigate their therapeutic activity against hypoadiponecinemia-related metabolic diseases.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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