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# Evaluation of synthetic sphingolipid analogs as ligands for peroxisome proliferator-activated receptors

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ABSTRACT

In this Letter, we assessed newly synthesized sphingolipid analogs as ligands for peroxisome proliferator-activated receptor (PPAR)α, PPARβ or PPARγ, using a dual-luciferase reporter system. We tested 640 sphingolipid analogs for ligand activity. As a result, seven types: A9, B9, C9, C50, F66, G66 and H66, were found to show agonistic activities for PPARs.



Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily; three subtypes have been identified: PPARα/NR1C1, PPARγ/NR1C3, and PPARβ/PPARδ/NR1C2.[1–3] PPARs are involved in the regulation of lipid and glucose metabolism.[3,4] These receptors form heterodimers with the retinoid X receptor (RXR) and regulate transcription of target genes by binding to their PPAR response elements (PPREs), which are specific direct repeats containing a 1 bp spacer (DR1) in the enhancer site of regulated genes.[5,6] Binding of an agonist to the ligand binding domain (LBD) of a PPAR is necessary for its transcriptional activation.

Many ligands that activate PPARs are known, including fatty acids, eicosanoids, carbaprostacyclin, non-steroid anti-inflammatory drugs, etc.[7] PPARβ is reportedly activated by exogenous synthetic ceramide (Cer).[8] Natural Cer, a fundamental structure of sphingolipids, is a basic construction unit of the cell membrane and a lipid second messenger involved in various cellular processes including proliferation, apoptosis, and cell signaling.[9,10] Therefore, its activation of PPARβ suggests the possibility that sphingolipids are involved in the transcriptional activation of PPARs. However, structural varieties of sphingolipids and activation of PPARs has not been well studied. Recently, we synthesized a series of structurally distinct sphingolipid analogs,[11] schematic structures for seven of these analogs are shown in Graphical abstract. In the present Letter, we assess newly synthesized sphingolipid analogs (640 total) as ligands of PPARα, PPARβ or PPARγ, using a dual-luciferase reporter system. PPARs activities were monitored in NIH3T3 cells, transiently transfected with a PPRE-containing luciferase reporter, in combination with the expression vectors of RXRα and PPARα, PPARβ or PPARγ.

We first constructed a plasmid carrying the mouse RXRα (mRXRα) gene. To clone full-length mRXRα cDNA, an RT-PCR was performed on total RNA from mouse liver (isolated using an RNeasy Mini Kit (QIAGEN, Chatsworth, CA, USA)) using an Omniscript RT Kit (QIAGEN), with oligo (dT) primers, a KOD Plus DNA polymerase (TOYOBO, Osaka, Japan), and the specific primer pair 5′-catggacaccaaacatttcctgccgc-3′ and 5′-gcctaggtggcttgatgtggtgcc tc-3′ (GenBank accession no. NM_011305), for 20 cycles at 94 °C for 15 s, 60 °C for 30 s, and 68 °C for 60 s. The amplified product was separated by agarose gel electrophoresis and purified with a QIAquick gel extraction kit (QIAGEN). For 3′ A-tailing, the purified KOD PCR product (blunt-ended) was incubated at 70 °C for 30 min in a reaction mixture (total volume 10 μl) containing 5 units Takara Ex Taq polymerase (TAKARA Biochemicals, Tokyo, Japan), 1 μl 10× reaction buffer, and 0.8 μl 2.5 mM dATP. The resulting product was cloned into the pGEM-T easy vector (Promega Corporation,

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Madison, WI, USA), according to the manufacturer's instructions. The plasmid was grown in *Escherichia coli* (XL1-Blue strain) and isolated using a Quantum Prep Plasmid <Mini> Prep Kit (BIO-RAD, Hercules, CA, USA). The plasmid was sequenced on an ABI 377 automatic sequencer (Applied Biosystems, Foster City, CA, USA) using a Big-Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). To create the insert fragment, PCR was performed on the plasmids obtained (pGEM-mRXRα) using specific primers incorporating the HindIII. site (underline) 5′-caagcttatgga caccaaacatttcctgccgc-3′ and 5′-caagcttctaggtggcttgatgtggtgcctc-3′, then purification, A-Tailing, and subcloning into the pGEM-T easy vector were achieved as described above. The plasmid obtained (pGEM-mRXRα-HindIII) was digested with HindIII and ligated to HindIII-digested and CIP-treated pCMX, using a ligation mix (TAKARA). The manipulated plasmid (pCMX-mRXRα) was then sequenced as described above and found to be correct. NIH 3T3 mouse fibroblastic cells (NIH3T3 cells) were transfected with pCMX-mRXRα using lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Protein expression of the mRXRα was detected using a rabbit anti RXRα polyclonal antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA). A single band was detected at a predicted molecular weight (54 kDa) corresponding approximately to mRXRα protein (Fig. 1).

For transfectional studies and luciferase reporter assays, NIH3T3 cells were grown in Dulbecco's modified Eagle medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (invitrogen) and antibiotics (100 units/ml penicillin and 0.1 mg/ml streptomycin) (Sigma). The cultures were maintained in a humidified atmosphere of 5% $CO_2$ in air at 37 °C. Cells ($1.0 \times 10^5$ cells/ml) were seeded onto a CulturPlate-96 (PerkinElmer, Boston MA, USA) and incubated for 3 days. The cells were then transfected by incubating in a reaction mixture (total volume 150 μl) containing 0.5 μl lipofectamine 2000 reagent (invitrogen), 100 ng PPREx3-tk-Luc, 100 ng pRL-SV40 control vector, 12.5 ng pCMX-mRXRα, and 12.5 ng pCMX, pCMX-mPPARα, pCMX-mPPARβ or pCMX-mPPARγ in Opti-MEM (GIBCO-BRL, Grand island, NY, USA) for 6 h. The reporter plasmid PPREx3-tk-Luc, carrying three copies of the PPRE from the acyl-CoA promoter upstream of the herpes virus thymidine promoter and the luciferase reporter gene,[5] the receptor expression plasmids encoding cDNA for pCMX-mPPARα, mPPARβ, mPPARγ, and the pCMX plasmid were a kind gift from Dr R. M. Evans (Salk Institute, San Diego, CA, USA).[12] The pRL-SV40 control plasmid, containing the potent simian virus 40 promoter and the enhancer driving renilla luciferase gene (Promega Corporation (Madison, WI, USA), was used as a control for variability in transfection efficiency. After 18 h, the transfection mixture was replaced with phenol red-free DMEM (GIBCO-BRL) containing 10% charcoal-filtered FBS (GIBCO-BRL). Six hundred and forty synthetic sphingolipid analogs (powder) were dissolved in chloroform/methanol (2:1 [vol:vol]), and then were dried under reduced pressure using a vacuum concentrator. The dried compounds were dissolved in DMSO, and used in treatment of the cells. Cells were treated with each synthetic sphingolipid analog (640 total); WY-14643 (WY),[13,14] a selective agonist of PPARα; L-165, 041 (LD),[15] a selective agonist of PPARβ; or Ciglitazone (Cig),[13,14] a selective agonist of PPARγ (Sigma), or vehicle control (DMSO).[15,16] After 24 h, firefly luciferase and renilla luciferase activities were measured by a Dual-Luciferase reporter assay system (Promega), according to the manufacturer's protocol. The levels of firefly luciferase activity were normalized to the levels of renilla luciferase activity in each sample. Results were reported as the mean ± SD for each sample. Statistical analysis was performed using an unpaired Student's *t*-test. Statistical significance was established at the $P < 0.05$ or 0.01 versus vehicle control (DMSO).

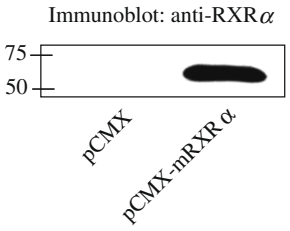


**Figure 1.** The protein expression of mouse RXRα. Plasmids carrying the mouse RXRα (mRXRα) gene were transfected to NIH3T3 cells. Protein expression of mRXRα in NIH3T3 cell transfectants was determined by immunoblotting with a rabbit anti-RXRα polyclonal antibody. Protein from NIH3T3 cells transfected with pCMX was used as a control for nonspecific bands.

We tested 640 sphingolipid analogs for ligand activity. Almost all the sphingolipid analogs were rarely shown to induce the remarkable transactivation of PPARs in comparison with vehicle control (data not shown). Of these, seven analogs, A9, B9, C9, C50, F66, G66 and H66, were found to greatly activate PPARs. The maximal PPARα activities induced by six of these (A9, B9, C9, F66, G66 and H66) were greater than that of the vehicle control at a concentration of $5 \times 10^{-6}$ M (Fig. 2A). The transactivation of PPARα induced by F66, G66 or H66 was greater than or equal to that of WY at concentrations of $1 \times 10^{-5}$–$1 \times 10^{-7}$ M. Dose-dependent transactivation of PPARβ was induced by A9, B9, C9, F66, G66 or H66, yet in each case the transactivation was less than that observed with LD (Fig. 2B). Dose-dependent transactivation of PPARγ was also induced by A9, B9, C9, C50, F66, G66 or H66, and the PPARγ agonistic activities of these analogs were greater than or equal to that of Cig (Fig. 2C).

Structures of all synthetic sphingolipid analogs are phytosphingosine-based ceramides (Graphical abstract).[11] The results in Figure 2 show that phytosphingosine structures, common structures in the seven synthetic sphingolipid analogs, A9, B9, C9, C50, F66, G66 or H66, are partially involved in transactivation of PPARs. And, acyl groups may participate in modulation of PPAR activation. These results might expect the interaction between natural sphingolipids, such as sphingoid bases or long-chain phytoceramides, and PPARs. Veldhove et al. reported that sphingoid bases (sphingenine, sphinganine or phytosphingosine), but not ceramide (*N*-palmitoyl-sphingenine), potentially interacted with PPARα in a solid phase binding assay.[17] Because sphingoid bases and natural ceramides are considered to differ from each other in the hydrophobic natures, it is difficult to examine exact ligand functions for PPARs by a luciferase reporter system. Future research on the function of natural sphingolipids will be required.

PPAR subtypes are known to bind to a comparative wide range of structurally diverse compounds, such as unsaturated fatty acids (UFA).[18,19] The eicosanoids, such as leukotriene B4 or 15-deoxy-δ-12,14-prostaglandin J2, are reported as endogenous ligands for PPARα or PPARγ, respectively.[18,19] In this Letter, the seven synthetic sphingolipid analogs, A9, B9, C9, C50, F66, G66 or H66, had no selective transactivation of PPAR subtypes (Fig. 2), and showed a similar tendency to UFA in the induction of PPAR activations.

In a previous report from our lab, Cer was found to participate in keratinocyte apoptosis and skin barrier function.[20] In addition, PPARβ was shown to up-regulate the gene expression of ceramide kinase, an enzyme that converts Cer to ceramide 1-phosphate, and to enhance keratinocyte survival.[21] Tan et al. determined that exogenous Cer enhanced transcription activation, by using a fusion protein comprising the GAL4-DNA-binding domain and the PPARβ-ligand binding domain (LBD) to monitor the activation of PPARβ-LBD in a keratinocyte cell line.[8] Furthermore, the gene expression of the ATP-binding cassette sub-family A member 12 (ABCA12), which has been reported to function as a glucosylceramide transporter, was shown to be induced by the activation of PPARβ or PPARγ in cultured human keratinocytes.[22,23] Considering all this