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Pharmacology and *in Vitro* Profiling of a Novel Peroxisome Proliferator-Activated Receptor γ Ligand, Cerco-A

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Peroxisome proliferator-activated receptor γ (PPAR γ ; NR1C3) is known as a key regulator of adipocytogenesis and the molecular target of thiazolidinediones (TZDs), also known as antidiabetic agents. Despite the clinical benefits of TZDs, their use is often associated with adverse effects including peripheral edema, congestive heart failure, and weight gain. Here we report the identification and characterization of a non-thiazolidinedione PPAR γ partial agonist, Cerco-A, which is a derivative of the natural product, (–)-cercosporamide. Cerco-A was found to be a binder of the PPAR γ ligand-binding domain in a ligand competitive binding assay and showed a unique cofactor recruitment profile compared to rosiglitazone. A crystal structure analysis revealed that Cerco-A binds to PPAR γ without direct hydrogen bonding to helix12. In PPAR γ transcriptional activation assay and an adipocyte differentiation assay, Cerco-A was a potent partial agonist of PPAR γ . After a 14-day oral administration, once per day of Cerco-A in Zucker diabetic fatty (ZDF) rats, an apparent decrease of plasma glucose and triglyceride was observed, as with pioglitazone. To evaluate drug safety, Cerco-A was administered for 13 days orally in non-diabetic Zucker fatty (ZF) rats. Each of the hemodilution parameters (hematocrit, red blood cells number, and hemoglobin), which are considered as undesirable effects of TZDs, was improved significantly compared to pioglitazone. While Cerco-A showed body weight gain, as with pioglitazone, Cerco-A had significantly lower effects on heart and white adipose tissues weight gain. The results suggest that Cerco-A offers beneficial effects on glycemic control with attenuated undesirable side effects.

Key words peroxisome proliferator-activated receptor γ ; diabetes; partial agonist; modulator

The peroxisome proliferator-activated receptor γ (PPAR γ), which belongs to the family of nuclear receptors,¹⁾ plays an important role in regulating the expression of a large number of genes involved in lipid metabolism and energy balance.^{2,3)} PPAR γ is also known as a master transcriptional regulator of adipocyte differentiation and is most abundantly expressed in adipose tissue.⁴⁾ However, PPAR γ is also detected in human skeletal and cardiac muscle, liver, kidney, small intestine, bladder, and spleen. In addition, important functions of PPAR γ in macrophages, liver, and muscle were reported.⁵⁾ These reports suggest that PPAR γ plays an essential role in adipogenesis, glucose and lipid homeostasis, and inflammatory responses.

PPAR γ is considered to be a ligand-activated nuclear receptor. Synthetic PPAR γ agonists for the treatment of type 2 diabetes have been proven successful for glucose control, as in the case of the marketed drugs pioglitazone (ActosTM, Takeda Pharmaceuticals) and rosiglitazone (AvandiaTM, GlaxoSmithKline).⁶⁾ They belong to the class of thiazolidinedione (TZD) antidiabetic agents that control hyperglycemia in type 2 diabetes by an insulin sensitizing mechanism. Despite the beneficial effects of TZDs, chronic use of these PPAR γ agonists often causes undesirable side effects in clinical and preclinical studies such as peripheral edema, cardiac hypertrophy, anemia, and hemodilution due to increased plasma volume.^{7–12)} Due to the frequently observed side effects with TZDs, there is a need to develop PPAR γ ligands that can effectively control hyperglycemia in type 2 diabetes patients without such side effects.¹³⁾ Many pharmaceutical

companies are attempting to develop safer PPAR γ ligands, including selective PPAR γ partial agonists, and some of these were reported previously.^{14–26)} The potency and safety of most of those compounds have not been well characterized *in vivo* compared to the currently marketed PPAR γ agonist.

To find a novel insulin sensitizer, we screened the in-house natural product libraries in a functional adipocytogenesis assay based on the utilization of ¹³C-acetic acid in 3T3-L1 (ATCC, No. CL-173) cells in our early years. (–)-Cercosporamide, which partially promoted the adipocyte function, was originally obtained from a fungal broth. Some (–)-cercosporamide derivatives lowered the plasma glucose of hyperglycemic KK/Ta mice in our previous report,²⁷⁾ though at that time we had not yet determined the mechanism. Using a binding assay and a reporter assay, we revealed that *N*-benzylcarboxamide derivative 4 was a partial agonist of PPAR γ . After that, we started a structure–activity relationship (SAR) investigation, and some results were previously reported.²⁸⁾ A SAR study of the substituents on carboxamide nitrogen afforded the *N*-(1-naphthyl)methylcarboxamide derivative 23 as the most potent selective PPAR γ modulator.²⁸⁾ Finally, we found Cerco-A through subsequent derivatization from derivative 23 and by screening *in vitro* and *in vivo*.

Here we describe a novel, potent, and selective PPAR γ ligand, Cerco-A, which does not have a chemical structure of thiazolidinediones. In our biochemical and structural studies, Cerco-A performs unique properties of binding to PPAR γ and a cofactor recruitment pattern *via* PPAR γ . In our cell-based assay systems, Cerco-A performed as a partial agonist

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of PPAR γ and also partially antagonized a TZD-induced biological response. In *in vivo* studies using diabetic rodent models, chronic oral administration with Cerco-A improved hyperglycemia and hyperlipidemia in the same way as pioglitazone. In our safety assessment study in non-diabetic rodents, Cerco-A attenuated the induction of hemodilution, anemia, cardiomegaly, and adipose tissues weight gain.

MATERIALS AND METHODS

Chemical Compounds Cerco-A, in-house TZD compound (TZD-X), fluorescein-labeled PPAR γ ligand (F-Ligand), rosiglitazone, and pioglitazone (Fig. 1) were synthesized at Daiichi Sankyo Co., Ltd., Tokyo, Japan. Cerco-A: (9a*S*)-8-acetyl-*N*-[(2-ethylnaphthalen-1-yl)methyl]-1,7-dihydroxy-3-methoxy-9a-methyl-9-oxo-9,9a-dihydrodibenzo[*b,d*]furan-4-carboxamide. F-ligand: A mixture of (2*S*)-2-(4-*tert*-butylphenoxy)-3-{4-[2-({[4'-(2-{[6-(fluorescein-5-carboxamide)hexanoyl]amino}ethyl)biphenyl-4-yl]carbonyl}amino)ethoxy]phenyl}propanoic acid and (2*S*)-2-(4-*tert*-butylphenoxy)-3-{4-[2-({[4'-(2-{[6-(fluorescein-6-carboxamide)hexanoyl]amino}ethyl)biphenyl-4-yl]carbonyl}amino)ethoxy]phenyl}propanoic acid. TZD-X: 5-(4-{[6-(4-hydroxy-3,5-dimethylphenoxy)-1-methyl-1*H*-benzimidazol-2-yl]methoxy}benzyl)-1,3-thiazolidine-2,4-dione. Other materials, reagents, and solvents were purchased from commercial suppliers and used as supplied unless otherwise stated.

Expression and Purification of PPAR γ Ligand Binding Domain DNA encoding of the ligand-binding domain (LBD) of human PPAR γ at codons for amino acids (a.a.) 206–477 (Accession No. X90563) was amplified by PCR

and was inserted into the *SalI* site and *BamHI* site of the expression vector pQE30 (QIAGEN, Valencia, CA, U.S.A.) with an N-terminal His-tag. *Escherichia coli* SG13009/pREP4 (QIAGEN) cells carrying plasmid pQE30-hPPAR γ -LBD were grown at 37 °C in LB medium (Invitrogen, Carlsbad, CA, U.S.A.) containing 100 μ g/ml of ampicillin and 25 μ g/ml of kanamycin. The expression of His-PPAR γ -LBD was induced by the addition of isopropyl β -D-thiogalactoside to a final concentration of 0.1 mM and grown for an additional 6 h at 30 °C. Cells were resuspended in 50 mM Tris-HCl (pH 8.0), 0.2 M NaCl, 10 mM imidazole, and protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). Cells were lysed by sonication and pelleted by centrifugation. The supernatant was loaded onto a HiTrap-chelating-Ni²⁺ affinity column (GE Healthcare, Buckinghamshire, U.K.). His-PPAR γ -LBD was eluted with an imidazole gradient (0.01–0.5 M). Fractions containing His-PPAR γ -LBD were pooled and dialyzed to the dialysis buffer containing 20 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.5), 10 mM dithiothreitol (DTT), and 1 mM ethylenediaminetetraacetic acid (EDTA). The dialyzed fraction was applied to a HiTrap SP HP column (GE Healthcare) and eluted with NaCl gradient (0–1 M). The eluted fraction was loaded onto a HiLoad Superdex 75 column (GE Healthcare). The purity of the protein was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The purified protein was applied to a fluorescence polarization (FP)-based binding assay and crystallized.

Fluorescence Polarization-Based PPAR γ Binding Assay Fluorescein-labeled PPAR γ ligand (F-ligand) was used in this competitive binding assay. F-ligand is a derivative from an in-house, non-TZD compound which binds to His-

Fig. 1. Chemical Structure of Cerco-A and PPAR γ Ligands

(A) Cerco-A, (B) TZD-X, (C) rosiglitazone, (D) pioglitazone, (E) a fluorescent-labeled PPAR γ ligand (F-ligand).

PPAR γ -LBD. The FP-based binding assay employed 1 nM of F-ligand in binding buffer (pH 7.0) containing 10 mM HEPES, 10 mM DTT, 1 mM EDTA, 1 mg/ml bovine gamma globulin (Sigma-Aldrich, St. Louis, MO, U.S.A.), and 10.3 nM His-PPAR γ -LBD. After overnight incubation at 4 °C, the competition data with Cerco-A, pioglitazone, or rosiglitazone was obtained with an Analyst (Molecular Devices, Sunnyvale, CA, U.S.A.). Values of K_i were calculated by nonlinear curve fitting using binding models from GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, U.S.A.).

Cofactor Recruitment Assay All cofactor recruitment assays were carried out and reported in Phenex Pharmaceuticals AG, Ludwigshafen, Germany. All measured raw data were provided by Phenex. Briefly, the coactivator recruitment assay and the corepressor release assay were designed based on homogenous time-resolved fluorescence (HTRF) using glutathione *S*-transferase (GST)-tagged PPAR γ -LBD and on each biotinylated peptide derived from cofactors. GST-PPAR γ LBD (185–477 a.a.) was expressed in an *E. coli* expression system and purified using glutathione-agarose affinity purification. The HTRF experiments were performed at a total volume of 25 μ l containing 20 mM Tris-HCl (pH 7.9), 60 mM KCl, 5 mM MgCl₂, 8 μ g/ml bovine serum albumin, and 8 μ g streptavidin conjugated with allophycocyanin (S-APC), 2 μ g/ml GST-PPAR γ -LBD. For peptide fingerprinting analysis, 0.625 μ M of biotin-peptides, CBP1 (58–80 a.a.), NCOA3 (671–695 a.a.), PGC-1 α (130–154 a.a.), RAP250 (837–897 a.a.), RIP140 (805–829 a.a.), RIP140 (922–946 a.a.), SHP (104–128 a.a.), SMRT (2332–2356 a.a.), SRC-1 (676–700 a.a.), TIF-2 (731–755 a.a.), TRAP220 (590–614 a.a.), and TRAP220 (631–655 a.a.) were used. In all experiments, 510 nM of rosiglitazone and 103 nM of Cerco-A were used except in SMRT assay (2.4 μ M rosiglitazone and 118 nM Cerco-A). In compound gradient mode assays for SRC-1 and SMRT, 0.6 μ M of biotin-peptides was used. Detection of proximity was performed using an anti-GST-Eu-chelate complex and Streptavidin-APC by determining the ratio of 665/615 nm upon UV irradiation using a Victor 5 instrument (PerkinElmer, Norwalk, CT, U.S.A.). Each parameter was determined by nonlinear curve fitting using GraphPad Prism 4.0.

Cells and Culture Human osteosarcoma MG-63 cells (ATCC. No. CRL-1427) were cultured in minimum essential medium eagle, alpha modification (α -MEM, Invitrogen) including 10% fetal bovine serum (FBS) purchased from Moregate Biotech (Bulimba, Australia), 100 units/ml penicillin G, and 100 mg/ml streptomycin sulfate. The rat primary subcutaneous preadipocytes, the growth medium, and the differentiation medium were purchased from Primary Cell Co., Ltd. (Hokkaido, Japan). Briefly, the growth medium used was Dulbecco's modified Eagle's medium containing 10% FBS, 10 units/ml penicillin G, 10 μ g/ml streptomycin sulfate, 17 μ M pantothenic acid, 33 μ M (+)-biotin, 100 μ M ascorbic acid, 1 μ M octanoic acid, and 50 nM triiodothyronine. The differentiation medium used was the growth medium supplemented with 10 μ g/ml insulin and 2.5 μ M dexamethasone. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂.

PPAR γ Reporter Gene Assay The expression vector of full-length human PPAR γ (pSG5-hPPAR γ) was constructed by inserting DNA encoding human PPAR γ 2 amino acids into

the *Bam*HI site of pSG5 (Invitrogen). The peroxisome proliferator-activated receptor response element (PPRE) reporter vector (pGV-P2-PPRE) was constructed by inserting synthesized oligomer DNA (5'-CTA GAG GGG ACC AGG ACA AAG GTC ACG TTC GGG GAC CAG GAC AAA AGT CAC GTT CGG GA-3' and 5'-TCG ATC CCG AAC GTG ACC TTT GTC CTG GTC CCC GAA CGT GAC CTT TGT CCT GGT CCC CT-3'), which is derived from the 5'-flanking region of rat acyl-CoA oxidase,²⁹ into the *Nhe*I site and *Xho*I site of pGV-P2 (TOYO INK, Tokyo, Japan). For GAL4-LBD reporter assay, pM vector (Clontech, Mountain View, CA, U.S.A.) inserted with hPPAR γ 2-LBD (177–477 a.a.) was used. pUAS-TK-Luc was generated by the elimination of SV40 promoter from pGL3 promoter-luc vector (Promega, Madison, WI, U.S.A.) and by the insertion of TK promoter from pRL-Luc (Promega) and synthesized UAS oligomer DNA.

All luciferase reporter assays were carried out following transient transfection of MG-63 using LipofectamineTM (Invitrogen) reagent according to the manufacturer's protocol. In full length reporter assay, the transfection mixture for each 500 cm²-cell tray (Sumitomo Bakelite, Tokyo, Japan) containing 0.0486 mg of pSG5-hPPAR γ , 0.0486 mg of pGV-P2-PPRE, 0.0108 mg of pRL-TK (Promega), and LipofectAMINE reagent at a ratio of 1.4 (ml of reagent/mg of DNA) was exposed for 4 h in serum-, antibiotics-free medium. In GAL4-LBD reporter assay, the transfection mixture for each 500 cm²-cell tray containing 0.015 mg of pM-hPPAR γ -LBD, 0.093 mg of pUAS-TK-Luc, and LipofectAMINE reagent at a ratio of 1.4 (ml of reagent/mg of DNA) was exposed for 4 h in serum-, antibiotics-free medium. The cells were trypsinized and replated onto 96-well plates at 40000 cells/well. After 24 h incubation and the replacement of the medium, various concentrations of the test compound in the presence or absence of 3.3 nM of an in-house TZD compound (TZD-X) were added to the culture medium of the transfected cells for 20 h. TZD-X (Fig. 1B), one of strongest classes of PPAR γ full agonist in our TZD-library, was used as a control full agonist of PPAR γ . Luciferase assay using Dual-Glo[®] Luciferase Assay (Promega) or PicaGene LT2.0 (TOYO INK). The luciferase activity was measured with a luminometer Analyst (Molecular Devices).

Effective concentration, 50% (EC₅₀), and IC₅₀ values of a partial agonist were defined as follows. The transcriptional activity in the presence of 3.3 nM TZD-X was defined as 100%, while that in vehicle alone was defined as zero. The maximum transcriptional activity of the test compound alone was defined as the maximum efficacy ($E_{\max}$, %), while the maximum inhibition of the transcriptional activity in the presence of 3.3 nM TZD-X was defined as $I_{\max}$ (%). The concentration of the test compound indicating a half value of $E_{\max}$ was defined as the EC₅₀ value in the transcriptional activity. Furthermore, the concentration of the test compound indicating a half value of $I_{\max}$ was defined as the IC₅₀ value. These calculated IC₅₀ and EC₅₀ values were used for the evaluation of the modulating activity of the compound. Values of each parameter were determined by nonlinear curve fitting using GraphPad Prism 4.0.

Nuclear Receptor Selectivity Assays MG-63 cells were transiently transfected with a pcDNA3.1 (Invitrogen) expression construct encoding human farnesoid X receptor (hFXR,

Accession No. U68233), liver X receptor α (hLXR α , U22662), or hLXR β (NM_007121), and a reporter plasmid, which is a pGL vector (Promega) containing each responsive element (pGL-3-LXRE; AGTTCACTCAAGTTCA x4, pGL2-FXRE; 1070 bp derived from the mouse bile acid-binding protein (BABP) promoter region,³⁰ -1036—+35 bp). Besides LXRs and FXR, the pM vector inserted the LBD of each hPPAR α , δ , human retinoid X receptor (hRXR α), hRXR β , hRXR γ , glucocorticoid receptor (hGR), estrogen receptor (hER) α , hER β , mineralocorticoid receptor (hMR), and progesterone receptor (hPR), and was used in combination with pUAS-TK-Luc plasmid. The ligand-binding domains of NRs were used as follows: hPPAR α (168—468 a.a., S74349), δ (140—440 a.a., L07592),^{4,5} hRXR α (209—462 a.a., X52773), hRXR β (279—533 a.a., X63522), hRXR γ (213—463 a.a., U38480), hGR (494—777 a.a., AY436590), hER α (259—595 a.a., NM_000125), hER β (222—530 a.a., AB006590), hMR (677—984 a.a., NM_000901), hPR (641—933 a.a., NM_000926). These selectivity assays were carried out by using PicaGene LT2.0 (TOYO INK).

Adipocyte Differentiation Assay After the arrival of the purchased cells, all media for transportation were discarded and replaced with a fresh 5 ml/25 cm²-flask of proliferation medium and were cultured for 1 d. Then, the cell suspension was prepared with proliferation medium at 83000 cells/ml, and was plated into a type I collagen-coated 96-well microplate (BD Biosciences, Franklin Lakes, NJ, U.S.A.) at 5000 cells/well (60 μ l/well). For the blank, the proliferation medium that did not contain the cells was plated into the well (blank well) of each plate. The next day, the proliferation medium was discarded and replaced with the differentiation-inducing medium. After the exchange of medium, differentiation was induced with compounds with or without 3.3 nM of TZD-X to provide the differentiation medium. After culturing for 5 d, all differentiation-inducing medium was discarded and replaced with 60 μ l of 10% (v/v) formaldehyde solution for fixation in each well, and the cells were left stationary for 20 min at room temperature. Afterwards, the fixative solution was discarded and replaced with 60 μ l of 0.2% (v/v) Triton X-100 (Sigma-Aldrich) solution in each well and left stationary for 5 min at room temperature. Then, all Triton X-100 solutions were discarded and replaced with 60 μ l of 60% (v/v) isopropanol solution containing dissolved Oil Red O (Sigma-Aldrich) so as to be 0.3%, which was the adipose tissue staining solution. Then, the solutions were left stationary for 10 min at room temperature. After the adipose tissue staining solution was discarded, the well was washed twice with 60 μ l of 60% isopropanol solution. Then, 100 μ l of the dimethyl sulfoxide (DMSO) solution was added to each well and was stirred for 5 min at room temperature. The absorbance was measured at 550 nm (ABS₅₅₀) with the multiplate reader (BioTek Instruments, Winooski, VT, U.S.A.), and the stained amount with Oil Red O was determined. The mean of the measured level at ABS₅₅₀ in the TZD-X-treated group was defined as 100%, while the level at ABS₅₅₀ in the vehicle-treated group was defined as 0%. The differentiation rate (%) of the adipocytes treated with the test compound was also calculated. Each of the parameters ($E_{\max}$, EC_{50} , $I_{\max}$, and IC_{50}) in this experiment were determined to be the same as those in the PPAR γ transactivation assay.

Crystallization, Data Collection, Structure Determina-

tion, and Refinement The purified His-PPAR γ -LBD was concentrated to 22 mg/ml and was mixed with Cerco-A and a coactivator peptide derived from PGC-1 α (QEAEPP-SLLKKLLLAPANT). Crystals of His-PPAR γ -LBD complexed with PGC-1 peptide and Cerco-A were obtained using hanging drop vapor diffusion at 22 °C. The well solution contained 24% (w/v) PEG4000, 0.2 M sodium thiocyanate, and 0.1 M Tris-HCl (pH 8.5). The crystal was transferred to a cryoprotectant comprised of the well solution containing an additional 8% PEG400 and was flash frozen. X-Ray diffraction data were collected using in-house X-ray generator FR-E with detector R-Axis VII (RIGAKU, Tokyo, Japan) and processed with HKL2000.³¹ The structure of PPAR γ -LBD²⁸ was used as an initial model. Several rounds of manual rebuilding with O³² followed by refinement with CNX³³ were carried out.

Animals Five-week-old female Zucker diabetic fatty (ZDF) rats and 6-week-old female non-diabetic Zucker fatty (ZF) rats were obtained from Charles River Laboratories Japan, Inc. and Japan SLC, Inc., respectively. The rats were acclimatized for over 1 week and provided with food and water *ad libitum*. Nine-week-old ZDF rats and 7-week-old ZF rats were used for the *in vivo* experiment. All experimental procedures were performed in accordance with the guideline of the Institutional Animal Care and Use Committee of Daiichi Sankyo Co., Ltd.

In Vivo Experiment Seven clinical parameters: body weight (BW), plasma glucose (PG), insulin, hematocrit (HCT), red blood cells number (RBC), mean cells volume (MCV), and hemoglobin (Hb) for ZDF rats and BW, PG, triglyceride (TG), HCT, RBC, MCV, and Hb for ZF rats were used for randomization into treatment groups. ZDF rats ($n=5$ per treatment group) were fed a normal diet (FR-2, Funabashi Farms, Chiba, Japan) *ad libitum* and were treated once daily by oral gavages with vehicle (propylene glycol: Tween 80=4:1 (v/v)), Cerco-A (0.3, 1, 3, 10, 30, 100 mg/kg), or pioglitazone (0.3, 1, 3, 10, 30, 100 mg/kg) for 14 d. ZF rats ($n=5$ per treatment group) were also treated with Cerco-A (1, 3, 10, 30, 100 mg/kg) or pioglitazone (1, 3, 10, 30, 100 mg/kg) for 13 d under the same conditions as ZDF rats. Body weight was recorded daily. Blood was collected from the tail vein into a heparinized capillary tube. In addition, PG (Glucoroder-GXT, Shino-Test, Tokyo, Japan), TG (Triglyceride E-test Wako, Wako Pure Chemical, Osaka, Japan), free fatty acids (FFAs) (NEFA C-test Wako, Wako Pure Chemical), insulin (Rat Insulin RIA kit, SCETI K.K., Tokyo, Japan), and adiponectin (Mouse/Rat Adiponectin RIA kit, SCETI K.K.) were determined with plasma after centrifugation. RBC, Hb, HCT, and MCV were analyzed by an automatic blood cell counter, (KX-21, SYSMEX, Hyogo, Japan) using whole blood collected into a capillary tube with EDTA. Animals were euthanized by decapitation, and the heart, adipose tissue, and liver were dissected and weighed.

Statistical Analysis Values are represented as mean $\pm$ standard error (S.E.M.). Statistical significance from the control was assessed using 1-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison. Statistical significance from 100 mg/kg of pioglitazone was assessed using an unpaired, two-tailed *t*-test. A difference was considered to be statistically significant when the *p*-value was less than 0.05. GraphPad Prism 4.0 (GraphPad software) was used for all

measurements.

RESULTS

In Vitro Ligand Binding and Transactivation Activity

Cerco-A was a potent ligand of PPAR γ and demonstrated $K_i=3.3$ nM in an FP-based competitive binding assay (Fig. 2). Under the same conditions, rosiglitazone and pioglitazone bound the receptor with $K_i=825$ nM and *ca.* 10 μ M, respectively.

Fig. 2. Cerco-A Binds to PPAR γ

Competition binding curves of Cerco-A, rosiglitazone, and pioglitazone generated by the incubation of a fluorescent-labeled PPAR γ ligand with His-PPAR γ -LBD protein. The displacement of F-ligand after incubation in the indicated concentration of each compound overnight at 4°C. Similar results were obtained in at least two independent experiments performed in quadruplicate. Data are presented as mean $\pm$ S.E.M. ($n=4$).

Fig. 3. Cerco-A Is a Partial Agonist of PPAR γ

Dose-response curve of Cerco-A in the presence and absence of 3.3 nM of TZD-X in reporter gene assays and Oil Red O staining assay. Data are presented as mean $\pm$ S.E.M. ($n=4$). (A) Cerco-A showed partial transactivation and partial inhibition against TZD-X induced transactivation. MG-63 cells were transiently co-transfected with the expression vector pSG5-hPPAR, the reporter construct pGV-P2-PPRE, and pRL-TK. After the measurement of firefly (FF) and *Renilla* (RL) luciferase activity, each ratio was normalized by the ratio of luciferase activity of TZD-X and that of vehicle, and then was plotted. Similar results were obtained in at least two independent experiments performed in quadruplicate. (B) Cerco-A showed no transactivation and showed complete inhibition against TZD-X induced transactivation in GAL4-LBD luciferase assay. MG-63 cells were transiently co-transfected with the expression vector pM-hPPAR γ -LBD and the reporter construct pUAS-TK-Luc. (C) Cerco-A partially stimulated adipocytogenesis and partially antagonized against TZD-induced lipid accumulation in rat primary preadipocytes. Data are plotted after normalization, in the same way as the luciferase assay. Similar results were obtained in at least two independent experiments performed in quadruplicate.

In a full-length PPAR γ reporter gene assay, Cerco-A was a partial agonist of human PPAR γ , indicating an $EC_{50}=3.5$ nM and a maximal efficacy of 27% in comparison to the efficacy of 3.3 nM TZD-X (Fig. 3A). Cerco-A partially inhibited the TZD-X-induced-transactivation and showed $IC_{50}=99$ nM. Rosiglitazone and pioglitazone showed full agonistic profiles ($EC_{50}=17$, 52 nM, respectively), as with TZD-X ($EC_{50}=120$ pM). On the other hand, in a GAL4-PPAR γ -DBD reporter assay, Cerco-A almost fully inhibited transactivation induced by TZD-X (Fig. 3B). In selectivity assays, activation and inhibition were not observed *via* hPPAR α , hPPAR δ , hFXR, hLXR α , hLXR β , hRXR α , hRXR β , hRXR γ , hGR, hER α , hER β , hMR, or hPR at concentrations below 3 μ M (data not shown).

Adipocyte Differentiation Assay PPAR γ agonists such as TZDs are known stimulators of adipocyte differentiation in preadipocyte cell lines and primary preadipocytes. Cerco-A also showed a partial agonistic profile in Oil Red O staining photometric assay using rat primary preadipocytes ($EC_{50}=5.3$ nM, $E_{max}=42\%$, $IC_{50}=30$ nM, $I_{max}=54\%$, Fig. 3C). Under the same assay condition, TZD-X, rosiglitazone and pioglitazone showed full agonists ($EC_{50}=550$ pM, 11, 100 nM, respectively).

Cofactor Recruitment Profiling Using HTRF methods, the interaction between PPAR γ -LBD and each short peptide derived from various cofactors was measured. In peptide profiling experiments (Fig. 4A), rosiglitazone recruited the most

Fig. 4. The Properties of Cerco-A in Cofactor Recruitment Assays

(A) HTRF peptide profiling of Cerco-A and rosiglitazone. Data (mean $\pm$ S.E.M., $n=4$) of vehicle, Cerco-A, and rosiglitazone are shown in opened columns, solid black columns, and solid red columns, respectively. The vehicle is shown as DMSO. (B) Dose-response curve of Cerco-A and rosiglitazone in HTRF assay with a SRC-1 peptide or a SMRT ID1 peptide (SMRT1). Data are presented as mean $\pm$ S.E.M. ($n=4$).

of the coactivator peptides, which were derived from CBP1, NCOA3, PGC-1 α , RAP250, RIP140, SHP, SRC-1, TIF-2, and TRAP220, and released a corepressor SMRT peptide compared with the DMSO control. By contrast, Cerco-A showed partial recruitment of CBP1, NCOA3, PGC-1 α , RAP250, and TRAP220 (631–655 a.a.) and did not recruit RIP140, SHP, SRC-1, TIF-2, and TRAP220 (590–614 a.a.) peptides. On the other hand, the corepressor SMRT-derived peptide was released by Cerco-A. Additionally, dose-dependent experiments were carried out using peptides derived from SMRT and SRC1. Although both compounds can release a SMRT peptide, the decrease of Cerco-A was less than that of rosiglitazone. While rosiglitazone apparently increased SRC-1 peptide recruitment, Cerco-A did not recruit SRC-1 peptide at any dose.

Binding Mode of Cerco-A to PPAR γ Ligand-Binding Domain We determined the three-dimensional crystal structure of Cerco-A with PPAR γ -LBD and PGC-1 peptide (Fig. 5). The overall structure of PPAR γ -LBD adapted a similar fold to the previously reported structures.³⁴ The complex structure of rosiglitazone and PPAR γ -LBD was superposed onto the structure (Fig. 5). While Cerco-A binds in the ligand-binding pocket and is partially overlapped with rosiglitazone, it does not occupy the TZD head-located space of rosiglitazone. Therefore, Cerco-A does not directly interact with Tyr473 on helix12 of PPAR γ -LBD, but binds in the same manner as cercosporamide derivative **23** as described in our previous report.²⁸

Pharmacological Effects of Cerco-A on ZDF Rats

Zucker diabetic fatty (ZDF) rats were treated with Cerco-A, pioglitazone, or vehicle for 14 d, and the measured parameters are shown in Fig. 6. Both Cerco-A and pioglitazone dose-dependently lowered the concentrations of non-fasting plasma glucose below 200 mg/dl at over 3 mg/kg compared to 505 mg/dl in the control group (Fig. 6A). Cerco-A significantly decreased the PG at 0.3, 1 mg/kg ($*p<0.05$), 3, 10, 30, and 100 mg/kg ($**p<0.01$). Pioglitazone also significantly decreased the PG level ($**p<0.01$), except at 0.3 mg/kg. Pioglitazone decreased the plasma insulin level with the lowest value of 5.53 ng/ml at 100 mg/kg compared to 11.40 ng/ml of control, but it was not significant. On the other hand, Cerco-A did not change (Fig. 6B). Both compounds dose-dependently decreased plasma TG levels, with the lowest values of 1338.9 mg/dl at 100 mg/kg of Cerco-A and 572.9 mg/dl at 100 mg/kg of pioglitazone compared to 2719.0 mg/dl of control (Fig. 6C). The decrease was significant in 30 and 100 mg/kg ($**p<0.01$) of Cerco-A-treated rats and in 10, 30 mg/kg ($*p<0.05$), and 100 mg/kg ($**p<0.01$) of pioglitazone-treated rats. Pioglitazone decreased FFAs levels at 3, 30, and 100 mg/kg significantly ($**p<0.01$), while Cerco-A did not significantly decrease FFAs levels (Fig. 6D). The lowest values of FFAs were 0.795 mEq/l at 3 mg/kg of Cerco-A and 0.520 mEq/l at 30 mg/kg of pioglitazone compared to 1.155 mEq/l of control. Both compounds dose-dependently increased plasma adiponectin levels with the highest values of 18.0 μ g/ml at 10 mg/kg of Cerco-A and 23.4 μ g/ml at 30 mg/kg of pioglitazone compared to 9.6 μ g/ml of control (Fig. 6E). Significant

Fig. 5. Crystal Structure of PPAR γ -LBD with Cerco-A

(A) Overall crystal structure of Cerco-A (in cyan) in PPAR γ LBD (grey or red ribbon) in the presence of PGC-1 peptide (yellow ribbon). Helix12 is shown as a red ribbon and the side chain of Tyr473 is indicated as a red stick. Rosiglitazone (pink stick representation) is superimposed on the structure. (B) Detailed view of the crystal structure of Cerco-A with PPAR γ LBD. Rosiglitazone (pink stick) is superimposed on the structure.

Fig. 6. Effects of Cerco-A on ZDF Rats

Each group of rats was orally administered (dose indicated in mg/kg/d) with Cerco-A, pioglitazone, or vehicle for 14 d. Data are represented as mean $\pm$ S.E.M. ($n=5$). * $p<0.05$, ** $p<0.01$, as compared with the vehicle group using ANOVA and Dunnett's multiple comparison test.

increases of adiponectin in Cerco-A-treated rats above 3 mg/kg (** $p<0.01$) were observed, while pioglitazone also showed significant increases at 1 mg/kg (* $p<0.05$) and above 3 mg/kg (** $p<0.01$). A dose-dependent increase of body weight gain was observed with the highest values of 107.5 g at 100 mg/kg of Cerco-A and 126.0 g at 100 mg/kg of pioglitazone compared to 37.9 g of control. (Fig. 6F). Both compounds had increased body weight gain above 3 mg/kg significantly (** $p<0.01$).

Safety Evaluation of Cerco-A in ZF Rats As shown in Fig. 7A, as compared to 124 mg/dl of the PG level in control,

the PG levels were 111, 105, 108, 108, and 105 mg/dl for Cerco-A at 1, 3, 10, 30, and 100 mg/kg and those were 102, 109, 102, 99, and 112 mg/dl for pioglitazone at 1, 3, 10, 30, and 100 mg/kg, respectively. The decreases of PG were significant at 1 mg/kg (* $p<0.05$), 3, 10, 30, and 100 mg/kg (** $p<0.01$) of Cerco-A, while at 1, 10, 30 mg/kg (** $p<0.01$) and 3, 100 mg/kg (* $p<0.05$) of pioglitazone. Both Cerco-A and pioglitazone reduced plasma insulin concentration (Fig. 7B). The lowest values for insulin were 5.206 ng/ml at 30 mg/kg of Cerco-A and 3.165 ng/ml at 30 mg/kg of pioglitazone compared to 9.871 ng/ml of control. How-

Fig. 7. Effects of Cerco-A on ZF Rats

Each group of rats was orally administered (dose indicated in mg/kg/d) with Cerco-A, pioglitazone, or vehicle for 13 d. Data are represented as mean $\pm$ S.E.M. ($n=5$). * $p<0.05$, ** $p<0.01$, as compared with the vehicle group using ANOVA and Dunnett's multiple comparison test. ### $p<0.001$, as compared with 100 mg/kg of pioglitazone-treated group using an unpaired, two-tailed t -test were performed.

ever, the insulin-lowering effect of Cerco-A was not significant, while pioglitazone significantly reduced it at 10, 30 mg/kg (** $p<0.01$), and 100 mg/kg (* $p<0.05$). Both Cerco-A and pioglitazone significantly (** $p<0.01$) decreased concentrations of TG and FFAs at all doses (Figs. 7C,D). The lowest values for TG were 81.9 mg/dl at 100 mg/kg of Cerco-A and 55.1 mg/dl at 100 mg/kg of pioglitazone compared to 237.5 mg/dl of control, and those for FFAs were 0.216 mEq/l at 100 mg/kg of Cerco-A and 0.153 mEq/l at 100 mg/kg of pioglitazone compared to 0.465 mEq/l of control.

Pioglitazone treatment resulted in a significant dose-dependent reduction of HCT (Fig. 7E, 1 mg/kg; * $p<0.05$, 3, 10, 30, 100 mg/kg; ** $p<0.01$), RBC (Fig. 7F, 1, 3, 10, 30, 100 mg/kg; ** $p<0.01$), Hb (Fig. 7H, 1 mg/kg; * $p<0.05$, 3, 10, 30, 100 mg/kg; ** $p<0.01$), and an increase of MCV level (Fig. 7G, 10, 30, 100 mg/kg; ** $p<0.01$). On the other hand, those of Cerco-A treatment had no dose dependency of those parameter changes although reduction of HCT (Fig. 7E, 1, 3, 30 mg/kg; * $p<0.05$, 10 mg/kg; ** $p<0.01$), RBC (Fig. 7F, 1 mg/kg; * $p<0.05$, 3, 10, 30 mg/kg; ** $p<0.01$), Hb (Fig. 7H, 10, 30 mg/kg; ** $p<0.01$), and an increase of MCV level (Fig. 7G, 1, 100 mg/kg; * $p<0.05$, 30 mg/kg; ** $p<0.01$) were observed. The values at 100 mg/kg, the highest dose, for HCT were 45.3% of Cerco-A and 40.0% of pioglitazone compared to 46.7% of control.

Those for RBC ($\times 10000$ cells/ml) were 772 of Cerco-A, 658 of pioglitazone compared to 810 of control; those for Hb were 14.1 g/dl of Cerco-A and 12.5 g/dl of pioglitazone compared to 14.5 g/dl of control; and those for MCV were 58.7 fl of Cerco-A and 60.8 fl of pioglitazone compared to 57.6 fl of control. These parameter changes at 100 mg/kg of Cerco-A were significantly less than 100 mg/kg of pioglitazone (### $p<0.001$, Figs. 7E—H).

Both Cerco-A and pioglitazone significantly increased body weight gain at any dose (1 mg/kg of Cerco-A; * $p<0.05$ and the others; ** $p<0.01$) with the highest values of 120.4 g at 30 mg/kg of Cerco-A, and 129.0 g at 10 mg/kg of pioglitazone compared to 94.8 g of control showed in Fig. 7I.

Pioglitazone treatment resulted in a dose-dependent increase of heart weight (HW, 30, 100 mg/kg; ** $p<0.01$, Fig. 8A) and the ratio of heart weight/body weight (HW/BW, 100 mg/kg; * $p<0.05$, Fig. 8B). Cerco-A treatment did not affect HW and HW/BW significantly at any dose. The values at 100 mg/kg, the highest dose, for HW were 0.9284 g of Cerco-A and 1.0505 g of pioglitazone compared to 0.8811 g of control. The values at 100 mg/kg for HW/BW were 0.2469 g of Cerco-A and 0.2767 g of pioglitazone compared to 0.2543 g of control. There was a significant difference (## $p<0.01$) of HW and HW/BW between each compound at the highest dose (Figs. 8A, B).

Both Cerco-A and pioglitazone increased the weight of ex-

Fig. 8. Anatomical Analysis in ZF Rats

Each group of rats was orally administered (dose indicated in mg/kg/d) with Cerco-A, pioglitazone, or vehicle for 13 d, and each body weight was measured. On Day 14, the rats were sacrificed and each organ shown was weighed. Data are represented as mean $\pm$ S.E.M. ($n=5$). * $p<0.05$, ** $p<0.01$, as compared with the vehicle group using ANOVA and Dunnett's multiple comparison test. # $p<0.05$, ## $p<0.01$, as compared with 100 mg/kg of pioglitazone-treated group using an unpaired, two-tailed t -test were performed.

cised epididymal white adipose tissue (Epi WAT) and mesenteric white adipose tissue (Mes WAT) in rats at Day 14 (Figs. 8C, E). The weights of Epi WAT and Mes WAT at 100 mg/kg Cerco-A were less than those at 100 mg/kg pioglitazone (# $p<0.05$ and ## $p<0.01$, respectively). After the body weight correction, Cerco-A did not significantly increase Epi WAT/BW and Mes WAT/BW at any dose, although significant gain of Epi WAT/BW and Mes WAT/BW treated with pioglitazone was observed above 3 mg/kg and 10 mg/kg, respectively (Figs. 8D, F). Comparing the 100 mg/kg that was treated with each compound, Epi WAT/BW and Mes WAT/BW of Cerco-A were less than those of pioglitazone (# $p<0.05$).

Liver weights in both compound-treated rats did not change significantly (Fig. 8G). Corrected for the body weight of each animal, the liver weight (per cent of body weight) decreased at 3 and 10 mg/kg of Cerco-A and was above 3 mg/kg of pioglitazone compared to the control shown in Fig. 8H.

DISCUSSION

In this report, we describe Cerco-A as a structurally novel class of PPAR γ ligand that shows distinct properties from other PPAR γ agonists. Although Cerco-A was originally a derivative from *N*-(1-naphthyl)methylcarboxamide derivative 23 in our report,²⁸ Cerco-A showed stronger PPAR γ partial

agonist potency (Fig. 3A, $EC_{50}=3.5$ nM) than that of derivative 23, showing $EC_{50}=180$ nM.²⁸⁾

We found that Cerco-A bound to PPAR γ -LBD with a 250-fold and a 3000-fold higher affinity compared to rosiglitazone and pioglitazone, respectively (Fig. 2). Despite being a good binder for PPAR γ , Cerco-A partially stimulated PPAR γ -mediated transcriptional activity compared to TZD compounds and partially antagonized a TZD-X driven transcriptional activation in a full-length PPAR γ reporter gene assay (Fig. 3A). Furthermore, the PPAR γ partial agonistic property of Cerco-A was also shown in an adipocyte differentiation assay (Fig. 3C).

Ligand-dependent nuclear receptor transactivation is considered to be regulated by the dissociation of corepressor and recruitment of coactivators.³⁵⁾ To understand why Cerco-A showed partial agonist activity of PPAR γ , we compared the ability of Cerco-A and rosiglitazone to modulate the interaction of cofactor-derived peptides using HTRF assay. In a cofactor recruitment assay, Cerco-A was generally a very poor supporter of cofactor-derived peptide binding in comparison with rosiglitazone (Fig. 4A). Although rosiglitazone apparently recruited the SRC-1-derived peptide, Cerco-A did not recruit it at all (Fig. 4B). Both Cerco-A and pioglitazone could release a corepressor SMRT-derived peptide, but the decrease of Cerco-A was less than that of rosiglitazone (Fig. 4C). These unique cofactor recruitment properties could be one explanation for the partial agonistic profile of Cerco-A.

The maximal efficacy (E_{max}) of Cerco-A in a GAL4-PPAR γ -LBD reporter gene assay was smaller than the E_{max} in a full-length PPAR γ reporter gene assay (Figs. 3A, B). The GAL4-PPAR γ -LBD protein lacks the N-terminal domain (AF-1) and alters the DNA-binding domain compared with the full length PPAR γ protein. In a previous report,³⁶⁾ the AF-1 domain of PPAR γ showed a transcriptional activation independent of the C-terminal activation domain (AF-2). Considering these results, Cerco-A may be an AF-1-dependent PPAR γ activator. Another possible explanation for the partial agonism of Cerco-A could be the AF-1-dependent transactivation *via* PPAR γ .

We have also confirmed through the crystal structure analysis of Cerco-A with PPAR γ -LBD that the binding site for Cerco-A in the PPAR γ -LBD overlapped but differ from that of rosiglitazone. Although previous X-ray crystallographic studies have revealed that PPAR γ ligands such as TZDs bound to helix 12 of PPAR γ and stabilize active conformation,³⁷⁾ Cerco-A does not directly interact with helix 12 of PPAR γ (Fig. 5). This lack of direct interaction between Cerco-A and helix 12 might result in poor stabilization of PPAR γ -LBD in an active conformation and the partial agonist activity of Cerco-A.

In our *in vivo* experiments, we provided evidence that Cerco-A performs effective glucose and triglyceride lowering activity in female ZDF rats in the same way as the marketed PPAR γ agonist, pioglitazone (Figs. 6A, C). In the safety assessment study in ZF rats, Cerco-A and pioglitazone lowered triglyceride concentration effectively (Fig. 7C), which was similar in ZDF rats (Fig. 6C). Both compounds slightly, yet significantly, lowered concentration of non-fasting PG at all doses (Fig. 7A). From these results, we consider that the exposure of Cerco-A and pioglitazone in ZF rats was very similar to ZDF rats. We report that Cerco-A attenuated adverse

effects, and hemodilution and cardiomegaly in female ZF rats differ from pioglitazone (Figs. 7E, F, H; Figs. 8A, D). Cerco-A also could attenuate anemia, as shown by RBC, MCV, and Hb compared to pioglitazone (Figs. 7F—H). These results suggest that Cerco-A is an equivalent drug in glucose-lowering effect compared to the marketed PPAR γ agonist, pioglitazone, but is a safer drug in terms of the cardiovascular risks caused by hemodilution, anemia, and heart weight gain even at high doses.

From further anatomical analysis in ZF rats, we also revealed the unique property of Cerco-A on white adipose tissues. Cerco-A showed a much smaller increase of weight of Mes WAT and Epi WAT than pioglitazone (Figs. 8B, C, E, F), although each compound showed an increase of body weight (Fig. 7I). In adipocytogenesis assay, Cerco-A partially increased lipid accumulation of rat primary preadipocytes compared to PPAR γ agonists (Fig. 2C). From that, it was expected that Cerco-A gained smaller WAT weight than PPAR γ agonists, although further investigation is required to clarify which organs gained weight.

Thiazolidinedione insulin sensitizers, pioglitazone (Actos), and rosiglitazone (Avandia) were used for the treatment of type-2 diabetes patients and were effective in controlling hyperglycemia. However, their use often causes undesirable effects such as body weight gain, edema, anemia, hemodilution,^{8–12)} and bone fracture.³⁸⁾ These negative effects limit the utility of higher dose of TZDs.

Recently a number of PPAR γ modulators were reported (PAR1622,¹⁴⁾ FK614,^{15–20)} SPPARM γ M5,²¹⁾ INT131,^{22,23)} T2384,²⁴⁾ and MBX-102,^{25,26)} etc.). They showed profiles of the PPAR γ partial agonists *in vitro* and demonstrated attenuating side effects *in vivo* compared to the currently marketed PPAR γ agonists. Although Cerco-A has a distinct chemical structure from the other partial agonists, these reports supported that not only Cerco-A, but also other partial agonists could distinguish beneficial effects from adverse effects for the treatment of diabetes. Most recently, detailed analysis data of INT131^{22,23)} and MBX-102^{25,26)} were reported from two independent research groups. INT131 was introduced as a selective, partial PPAR γ agonist with high affinity ($K_i=10$ nM) and it effectively improved glucose tolerance without inducing cardiomegaly, hemodilution, and plasma volume expansion. In detail, INT131 did not release SMRT-derived peptide²²⁾ while Cerco-A did release it (Figs. 4A, C). We consider that it may lead to some differences of physiological effects between INT131 and Cerco-A. As a comparison of a calculated log P (C log P) using ADMET PredictorTM (Simulation Plus, Inc., Lancaster, CA, U.S.A.), INT131 showed a higher lipophilicity with a C log P=7.098 while a C log P of Cerco-A was 4.275. We consider that the lower lipophilicity of Cerco-A is one merit in the aspect of pharmacokinetics compared to INT131. MBX-102 was introduced as a pro-drug of MBX-102 acid. MBX-102 acid was also reported as a selective, partial PPAR γ agonist and MBX-102 lowered glucose in the absence of some of the side effects, such as edema.^{25,26)} Especially MBX-102, did not cause an increase of body weight gain in their report²⁶⁾ while INT-131²²⁾ and Cerco-A increased body weight gain significantly. MBX-102 showed a good profile *in vivo*, but on the other hand, MBX-102 acid seemed to be a weaker ligand of PPAR γ compared to Cerco-A (Fig. 2, $K_i=3.3$ nM, Fig. 3A, $EC_{50}=3.5$ nM). Ac-

cording to their report,²⁶⁾ in a PPAR γ binding assay, MBX-102 acid and rosiglitazone displayed IC₅₀s of approximately 35 μ M and about 0.1 μ M, respectively. In the reporter assay, MBX-102 acid and rosiglitazone showed transactivation with EC₅₀s of about 12 μ M for MBX-102 acid and approximately 1 μ M for rosiglitazone. From these data, MBX-102 acid might need a higher exposure to tissues than Cerco-A for the same degree of anti-diabetic effect.

In conclusion, we have described a novel non-thiazolidinedione selective PPAR γ partial agonist, Cerco-A, related to the fungal product, (–)-cercosporamide. We have provided evidence that Cerco-A shows glucose-lowering effects attenuating undesirable side effects in our preclinical studies in this report.

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