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Honokiol: A non-adipogenic PPARγ agonist from nature ☆

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ABSTRACT

Background: Peroxisome proliferator-activated receptor gamma (PPARγ) agonists are clinically used to counteract hyperglycemia. However, so far experienced unwanted side effects, such as weight gain, promote the search for new PPARγ activators.
Methods: We used a combination of in silico, in vitro, cell-based and in vivo models to identify and validate natural products as promising leads for partial novel PPARγ agonists.
Results: The natural product honokiol from the traditional Chinese herbal drug Magnolia bark was in silico predicted to bind into the PPARγ ligand binding pocket as dimer. Honokiol indeed directly bound to purified PPARγ ligand-binding domain (LBD) and acted as partial agonist in a PPARγ-mediated luciferase reporter assay. Honokiol was then directly compared to the clinically used full agonist pioglitazone with regard to stimulation of glucose uptake in adipocytes as well as adipogenic differentiation in 3T3-L1 pre-adipocytes and mouse embryonic fibroblasts. While honokiol stimulated basal glucose uptake to a similar extent as pioglitazone, it did not induce adipogenesis in contrast to pioglitazone. In diabetic KKAy mice oral application of honokiol prevented hyperglycemia and suppressed weight gain.
Conclusion: We identified honokiol as a partial non-adipogenic PPARγ agonist in vitro which prevented hyperglycemia and weight gain in vivo.
General significance: This observed activity profile suggests honokiol as promising new pharmaceutical lead or dietary supplement to combat metabolic disease, and provides a molecular explanation for the use of Magnolia in traditional medicine.

1. Introduction

Sedentary lifestyle with low physical activity and high caloric intake promotes obesity, the metabolic syndrome, and type 2 diabetes, which pose a major risk for the individual's quality of life and a burden to the health care systems of industrialized societies. Peroxisome proliferator activated receptor gamma (PPARγ) agonists are clinically used to combat hyperglycemia common to these pathological conditions and to alleviate related comorbidities [1–3]. Generally, PPARs are nuclear receptors and ligand-dependent transcription factors which control lipid and glucose metabolism [4–7]. Upon ligand binding, PPARs form heterodimers with the retinoid X receptor (RXR), another nuclear receptor, and bind to response elements located in the promoter region of their target genes [8]. After recruitment of nuclear receptor coactivators [9,10] further chromatin rearrangement transcription is initiated [11]. From the three known subtypes of PPAR (α, β/δ, and γ), PPARγ is the best studied. It is expressed in adipose tissue, lung, large intestine, kidney, liver, heart, and macrophages

Abbreviations: AMPK, AMP-activated kinase; ANOVA, analysis of variance; ATCC, American type culture collection; BADGE, bisphenol A diglycidyl ether; BMP, bone morphogenic protein 4; BSA, bovine serum albumin; CMCNa, sodium carboxymethyl cellulose; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EC_{50}, effective concentration 50%; EGFP, enhanced green fluorescent protein; FCS, fetal calf serum; GST, glutathione-S-transferase; HPLC, high-performance liquid chromatography; IBMX, 3-isobutyl-1-methylxanthine; mTOR, mammalian target of rapamycin; NBS, newborn bovine serum; NF-κB, nuclear factor κB; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; PPARγ, peroxisome proliferator-activated receptor gamma; LBD, ligand-binding domain; MEF, mouse embryonic fibroblasts; NOX, NADPH-dependent oxidase; RXR, retinoic X receptor; SEM, standard error of the mean; SPF, specific pathogen free; SREBP, sterol regulatory element binding protein; TCM, traditional Chinese medicine; TLC, thin layer chromatography; TR-FRET, time-resolved fluorescence resonance energy transfer

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[12]. The well-established important role in the regulation of glucose and lipid metabolism renders PPARγ a valid pharmacological target for combating metabolic diseases [13,14]. Currently available full PPARγ agonists represented by thiazolidinediones (e.g. pioglitazone) are clinically effective, but they have serious side- and off-target effects (e.g. weight gain or edema formation), urging the retrieval of new PPARγ agonists. Ligands acting as partial agonists induce submaximal receptor activation and have been demonstrated to retain beneficial anti-diabetic properties with reduced side effects. Despite lower activation of the receptor, they are still able to induce PPARγ target genes responsible for the anti-hyperglycemic and insulin sensitizing, but not for the unwanted PPARγ actions [15,16]. The reason for this is not entirely understood. However, it is conceivable that partial agonists may elicit a different conformation of the receptor–ligand complex that leads—by altered recruitment of transcriptional co-activators and repressors—to a more restricted set of expressed target genes compared to full agonists.

Using a computer-aided approach we have recently identified neolignans as a novel class of partial agonists occupying the PPARγ ligand-binding domain as dimers [17]. The hereby generated and optimized in silico tools now enabled us to identify and further characterize the neolignan honokiol, a major bioactive constituent of the traditional Chinese herbal drug Magnolia bark, as novel non-adipogenic partial PPARγ activator. The previously identified neolignans exhibit PPARγ-dependent adipogenic properties similar to pioglitazone [17]. In contrast, we show in this work that the newly identified PPARγ partial agonist honokiol does not trigger adipogenesis in two in vitro cell systems and prevents weight gain in the murine KKAy in vivo diabetes model, while retaining anti-hyperglycemic activity in vitro and in vivo.

2. Materials and methods

2.1. Chemicals, cell culture reagents, and plasmids

Fetal calf serum (FCS), newborn bovine serum (NBS) and Dulbecco's modified Eagle's medium (DMEM) were from Lonza (Basel, Switzerland). Pioglitazone used for all experiments except in vivo tests was from Molekula Ltd. (Shaftesbury, UK). All other chemicals were from Sigma-Aldrich (Vienna, Austria). The test compounds were dissolved in dimethyl sulfoxide (DMSO), divided into aliquots and kept frozen until use. In all experiments, DMSO was used as solvent control. For in vitro and cell-based assays, the final concentration of DMSO was kept ≤0.2%. The PPAR luciferase reporter plasmid (tk-PPREx3-luc) was a gift from Prof. Ronald M. Evans (Howard Hughes Medical Institute, La Jolla, CA) [18], the plasmid encoding enhanced green fluorescent protein (pEGFP-N1) was from Clontech (Mountain View, CA), and the plasmid encoding human PPARγ (pSG5-PL-hPPAR-γ1) was a gift from Prof. Walter Wahli and Prof. Beatrice Desvergne (Center for Integrative Genomics, University of Lausanne, Switzerland) [19].

2.2. Isolation of honokiol

880 g powdered bark of *Magnolia officinalis* (Plantasia, Oberndorf, Austria; lot: 710786) was exhaustively macerated with dichloromethane (8.0 l, 12 times) at room temperature, yielding 96.8 g crude extract. 80.0 g extract was separated by flash silica gel column chromatography (400 g silica gel 60, 40–63 μm, Merck, VWR, Darmstadt, Germany; 41 × 3.5 cm) using a petroleum ether–acetone gradient with an increasing amount of acetone. The eluate was collected in portions of 20 ml and analyzed by TLC. Comparable portions were combined to 18 fractions (A1–A18). Fraction A8 (5.761 g) was further separated by Sephadex LH-20-column chromatography (CC) using methanol as mobile phase. Fractions were monitored by TLC and combined according to their composition to 15 fractions (B1–B15). Fraction B8 (810 mg) was further purified by HSCCC with a mixture of PE–EtOAc–MeOH–H_20 1 + 0.5 + 1 + 0.5 (v/v/v/v) using the upper layer as mobile phase. HSCCC was set to the tail to head mode with 800 rpm and a flow rate of 1 ml/min. The eluate was collected in portions of 5 ml and combined to 18 fractions (C1–C18). Fraction C11 (eluted at 495–580 ml; 249 mg) was further purified by Sephadex LH-20-CC using a dichloromethane–acetone mixture (85 + 15, v/v) as mobile phase to yield 217.54 mg of honokiol in a purity (HPLC) of >98%. Identity of the obtained compound was proven by comparison of 1- and 2D NMR-spectra with literature [20] and by LC–MS analysis. The obtained NMR- and LC–MS-data are provided as online supplement (supplemental Table S1 and supplemental Figs. S1–S4). The isolated honokiol was used for all in vitro bioassays.

2.3. PPARγ luciferase reporter gene transactivation

HEK-293 cells (ATCC, Manassas, VA) were grown in DMEM supplemented with 2 mM glutamine, 100 U/ml benzylpenicillin, 100 μg/ml streptomycin, and 10% FBS. The cells were seeded in 10-cm dishes at a density of 6×10^6 cells/dish for 18 h, and then transfected by the calcium phosphate precipitation method with 4 μg of PPARγ expression plasmid, 4 μg of reporter plasmid (tk-PPREx3-luc), and 2 μg of pEGFP-N1 as internal control. Six hours later, the cells were reseeded in 96-well plates (5×10^4 cells/well) in DMEM without phenol red with 5% charcoal-stripped FBS, glutamine and antibiotics. The cells were treated as indicated and incubated for 18 h. After cell lysis, the luminescence of the firefly luciferase and the fluorescence of EGFP were quantified on a GeniosPro plate reader (Tecan, Grödig, Austria). The luminescence signals were normalized to the EGFP-derived fluorescence, to account for differences in cell number or transfection efficiency. Neither pioglitazone nor honokiol interfered with the luciferase assay background determined upon transfection of the cells with tk-PPREx3-luc in the absence of PPARγ.

2.4. PPARγ competitive ligand binding

The LanthaScreen® time-resolved fluorescence resonance energy transfer (TR-FRET) PPARγ competitive binding assay (Invitrogen, Lofer, Austria) was performed using the manufacturer's protocol. The test compounds dissolved in DMSO or solvent vehicle (DMSO) alone were incubated together with the human PPARγ LBD tagged with GST, terbium-labeled anti-GST antibody and fluorescently labeled PPAR ligand (Fluormone Pan-PPAR Green). In this assay, the fluorescently labeled ligand is binding to the human PPARγ LBD, which brings it in close spatial proximity to the terbium-labeled anti-GST antibody. Excitation of the terbium at 340 nm results in energy transfer (FRET) and partial excitation of the fluorescent PPAR ligand, followed by emission at 520 nm. Test-compounds binding to the human PPARγ LBD are competing with the fluorescently labeled ligand and displacing it, resulting in a decrease of the FRET signal. The signal obtained at 520 nm is normalized to the signal obtained from the terbium emission at 495 nm; therefore, the decrease in the 520 nm/495 nm ratio is used as a measure for the ability of the tested compounds to bind to the human PPARγ LBD. Neither pioglitazone nor honokiol interfered with the background 520 nm/495 nm fluorescence in the absence of PPARγ LBD.

2.5. Induction of adipogenic differentiation

3T3-L1 preadipocytes were subcultivated in DMEM (containing 2 mM glutamine, 100 U/ml benzylpenicillin, 100 μg/ml streptomycin) plus 10% NBS. For differentiation experiments they were grown till confluency in 12-well plates. Two days later cells were treated with DMEM/10% FCS (negative control), DMEM/10% FCS/1 μg/ml insulin