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Computer-aided discovery, validation and mechanistic characterisation of novel neolignan activators of PPARγ

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Running title: PPARγ and neolignans

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List of abbreviations: BADGE, bisphenol A diglycidyl ether; CC, column chromatography; CHM, 3D natural product database of Chinese herbal medicine; DIOS, 3D natural product database based on Dioscorides *De materia medica*; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EGFP, enhanced green fluorescent protein; GST, glutathione-*S*-transferase; *Ki*, inhibition constant; LBD, ligand binding domain; NMR, nuclear magnetic resonance; PDB, Protein Data Bank; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR response elements; RXR, retinoid X receptor; TR-FRET, time-resolved fluorescence resonance energy transfer; TZD, thiazolidinedione.

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Abstract

Peroxisome proliferator-activated receptor gamma (PPARγ) agonists are used for the treatment of type 2 diabetes and metabolic syndrome. However, the currently used PPARγ agonists display serious side effects leading to a great interest in the discovery of novel ligands with favourable properties. Aim of our study was to identify new PPARγ agonists by a PPARγ pharmacophore–based virtual screening of 3D natural product libraries. This *in silico* approach led to the identification of several neolignans predicted to bind the receptor ligand binding domain (LBD). To confirm this prediction, the neolignans dieugenol, tetrahydrodieugenol, and magnolol were isolated from the respective natural source or synthesized and subsequently tested for PPARγ receptor binding. The neolignans bound to the PPARγ LBD with EC_{50}s in the nanomolar range, exhibiting a binding pattern highly similar to the clinically used agonist pioglitazone. In intact cells, dieugenol and tetrahydrodieugenol selectively activated hPPARγ–, but not hPPARα– or hPPARβ/δ–mediated luciferase reporter expression, with a pattern suggesting partial PPARγ agonism. The coactivator recruitment study also demonstrated partial agonism of the tested neolignans. Dieugenol, tetrahydrodieugenol, and magnolol but not the structurally related eugenol induced 3T3-L1 preadipocyte differentiation confirming effectiveness in a cell model with endogenous PPARγ expression. In conclusion, we identified neolignans as novel ligands for PPARγ, which exhibited interesting activation profiles, recommending them as potential pharmaceutical leads or dietary supplements.

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Introduction

Western lifestyle with a high intake of simple sugars, saturated fat, and physical inactivity promotes pathologic conditions such as type 2 diabetes, obesity and metabolic syndrome, which are currently taking a devastating epidemical spread worldwide. Compounds that are activating PPARγ may help to fight these pathological conditions (Cho and Momose, 2008).

PPARs are ligand-activated transcription factors belonging to the nuclear receptor superfamily, and their main function relates to the regulation of genes involved in glucose and lipid metabolism (Desvergne et al., 2006; Tenenbaum et al., 2003). Three isoforms of this nuclear receptor have been identified so far: PPARα, PPARβ/δ, and PPARγ. PPARα is highly expressed in skeletal muscle, liver, kidney, heart, and the vascular wall and it was shown to be mainly involved in the regulation of lipid catabolism (Fruchart, 2009). PPARγ is predominantly expressed in adipose tissue and its activation promotes adipogenesis and increases insulin sensitivity (Anghel and Wahli, 2007). More recently, PPARγ has been shown to be involved in the regulation of genes contributing to inflammation, hypertension, and atherosclerosis (Gurnell, 2007). PPARβ/δ has a broader expression pattern and is involved in the regulation of lipid metabolism and energy expenditure (Bedu et al., 2005; Luquet et al., 2005).

Once activated by their ligands, the PPARs translocate into the nucleus, form heterodimers with the retinoid X receptor (RXR), and subsequently bind to PPAR response elements (PPREs) that are located in the promoter regions of PPAR-responsive target genes (Bardot et al., 1993). Binding of the PPAR-RXR heterodimers to the PPREs triggers further recruitment of diverse nuclear receptor coactivators (SRC-1, TRAP220, CBP, p300, PGS-1, and/or others), contributing to the transcriptional regulation of the target genes (Yu and Reddy, 2007).

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PPARγ activators are currently used as insulin sensitizers to combat type 2 diabetes and metabolic syndrome (Cho and Momose, 2008). However, the PPARγ agonists in clinical use, represented by thiazolidinediones (TZDs), have serious side effects such as weight gain, increased bone fracture, fluid retention, and heart failure (Rizos et al., 2009). Therefore, the discovery and optimization of new PPARγ agonists that would display reduced side effects is of great interest. TZDs are full PPARγ agonists inducing maximal receptor activation. Of note, partial PPARγ agonists recently came into focus as a possible new generation of promising PPARγ ligands. Partial agonists induce alternative receptor conformations and thus recruit different coactivators resulting in distinct transcriptional effects compared to TZDs. There are firm indications that such partial agonists might retain the needed effectiveness while having reduced side effects (Chang et al., 2007; Yumuk, 2006).

Natural products are an important and promising source for drug discovery (Newman and Cragg, 2007). Aim of our study was, therefore, to identify natural products that activate human PPARγ (hPPARγ) acting possibly as partial agonists. To achieve this objective, we used an *in silico* approach making use of a pharmacophore model for hPPARγ developed previously (Markt et al., 2008; Markt et al., 2007) and 3D databases of natural products. The conducted virtual screen utilizing two 3D databases and the pharmacophore model led to the identification of neolignans. The three neolignans dieugenol, tetrahydrodieugenol, and magnolol were isolated from the respective natural source or synthesized and characterized in several PPARγ-specific *in vitro* models or intact cells.

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Materials and Methods

Chemicals, cell culture reagents, and plasmids

Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose was purchased from Lonza (Basel, Switzerland). The fetal bovine serum was from Gibco (Invitrogen, Lofer, Austria). GW7647, GW0742 and BADGE were purchased from Cayman Europe. Pioglitazone was purchased from Molekula Ltd (Shaftesbury, UK). All other chemicals were obtained from Sigma–Aldrich (Vienna, Austria). The test compounds were dissolved in DMSO, aliquoted and kept frozen until use. In all test models a solvent vehicle control was always included to assure that DMSO does not interfere with the respective model. For all cell-based assays the final concentration of DMSO was kept 0.1% or lower. The PPAR luciferase reporter construct (tk-PPREx3-luc) and the expression plasmid for murine PPARγ (pCMX-mPPARγ) were a kind gift from Prof. Ronald M. Evans (Howard Hughes Medical Institute, California), the plasmid encoding enhanced green fluorescent protein (pEGFP-N1) was obtained from Clontech, and the expression plasmids for the three human PPAR subtypes (pSG5-PL-hPPAR-alpha, pSG5-hPPAR-beta, pSG5-PL-hPPAR-gamma1) were a kind gift from Prof. Walter Wahli and Prof. Beatrice Desvergne (Center for Integrative Genomics, University of Lausanne, Switzerland).

Pharmacophore-based Virtual Screening

The pharmacophore model used for virtual screening was taken from the model collection reported previously (Markt et al., 2008; Markt et al., 2007). Data mining of the natural product databases was performed using Catalyst 4.11. For virtual screening, the fast flexible search algorithm of Catalyst was used.

Virtual natural product databases

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The two virtual 3D compound databases used in this study have been generated previously. The DIOS database contains 9676 individual small-molecular weight natural products found in ancient herbal medicines described in *De materia medica*, by Pedanius Dioscorides (1st cent. AD) (Rollinger et al., 2008). The Chinese Herbal Medicine (CHM) database contains 10216 compounds which are reported to be contained in medicinal preparations used in traditional Chinese medicine. Both 3D databases were generated within Catalyst. 3D structures of the compounds were built and consequently energetically minimized using the structure editor of Catalyst. The catConf algorithm was applied to create conformational models for the compounds using the following settings: maximum number of conformers = 100, generation type = fast quality, and energy range = 20 kcal/mol above the calculated lowest energy conformation.

Isolation

Magnolol **(3)** was isolated from the bark of *Magnolia officinalis* Rehd. & Wils. The plant material provided by Plantasia (Oberndorf, Austria) corresponded to the quality described at the Chinese Pharmacopoeia. 880 g powdered bark of *Magnolia officinalis* (Plantasia, Oberndorf, Austria; Ch.Nr. 710786) were exhaustively macerated with dichloromethane (8.0 l, 12 times, at room temperature) yielding 96.7 g crude extract. 80.0 g of the obtained extract were separated by flash silica gel column chromatography (CC) (400 g silica gel 60, 40-63 µm, Merck, VWR, Darmstadt, Germany; 41 x 3.5 cm) using a petroleum ether-acetone gradient with an increasing amount of acetone resulting in 18 fractions (A1-A18). Fraction A5 (17.29 g) was further separated by means of vacuum liquid chromatography (10 x 5 cm) with LiChroprep® RP-18 material (100.0 g; 40-63 µm; Merck, Darmstadt, Germany) using an acetonitrile-water gradient with an increasing amount of acetonitrile. Fractions eluted with 45% to 60% acetonitrile were combined (4.19 g) and further separated by Sephadex® LH-20 (Pharmacia Biotech, Sweden) CC using a

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dichloromethane-acetone mixture (85+15, v/v) as mobile phase (subfractions B1-B17). Fraction B13 (3.67 g) was recrystallized from dichloromethane resulting in 2.32 g of **3** as colourless crystals. The compound was identified by mass spectrometry and NMR-spectroscopy (H-NMR purity > 98%). NMR and MS data are provided as online supplementary information.

Synthesis of compounds

Synthesis of dieugenol (**1**) was performed by oxidative dimerization of eugenol (**4**) (Sigma-Aldrich, Germany) as described by Ogata et al. (Ogata et al., 2000) and Marque et al. (Marque et al., 1998). After recrystallization from 2-propanol, isolated **1** was analysed by NMR (H-NMR purity > 99%). Tetrahydrodieugenol (**2**) was synthesized by hydrogenation of **1** in a Parr apparatus at 40 psi as described by Ogata et al. (Ogata et al., 2000). After recrystallization from 2-propanol, compound **2** was analysed by NMR (H-NMR purity > 97%). NMR and MS data of **1** and **2** validating the identification of the two compounds are provided as online supplementary information.

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 were incubated together with the hPPARγ LBD tagged with GST, terbium-labelled anti-GST antibody and fluorescently labelled PPARγ ligand (Fluormone™ Pan-PPAR Green, Invitrogen). In this assay, the fluorescently labelled ligand is binding to the hPPARγ LBD which brings it in a close spatial proximity to the terbium-labelled anti-GST antibody. Excitation of the terbium at 340 nm results in energy transfer (FRET) and partial excitation

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of the fluorescently labelled ligand, followed by emission at 520 nm. Test-compounds binding to the hPPARγ LBD are competing with the fluorescently labelled ligand and displacing it, resulting in a decrease of the FRET signal. The 520 nm signals were normalized to the signals obtained from the terbium emission at 495 nm and therefore the decrease in the 520 nm/495 nm ratios was used as a measure for the ability of the tested compounds to bind to the hPPARγ LBD. All measurements were performed with a GeniosPro plate reader (Tecan, Austria).

PPAR luciferase reporter gene transactivation

HEK-293 cells (ATCC, USA) were grown in DMEM with phenol red supplemented with 584 mg/ml glutamine, 100 U/ml benzylpenicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum. Cells were maintained in 75 cm^2 flasks with 10 ml medium at 37°C and 5% CO_2. For transient transfection, 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 the respective PPAR receptor expression plasmid, 4 µg reporter plasmid (tk-PPREx3-luc), and 2 µg green fluorescent protein plasmid (pEGFP-N1) as internal control. The total DNA and the ratio tk-PPREx3-luc:PPAR:EGFP were kept 10 µg and 2:2:1, respectively. Six hours after the transfection, cells were harvested and re-seeded in 96-well plates (5×10^4 cells/well) in DMEM without phenol red, supplemented with 584 mg/ml glutamine, 100 U/ml benzylpenicillin, 100 µg/ml streptomycin, and 5% charcoal stripped fetal bovine serum. Then cells were treated with the respective compounds 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, Austria). The luminescence signals were normalized to the EGFP-derived fluorescence, to account for differences in cell number and/or transfection efficiency.

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PPARγ coactivator recruitment

The LanthaScreen™ TR-FRET PPARγ coactivator assay (Invitrogen, Lofer, Austria) was performed according to the manufacturers' protocol. The test compounds dissolved in DMSO or solvent vehicle were incubated together with fluorescein-labelled TRAP220/DRIP-2 coactivator peptide (Rachez et al., 2000), hPPARγ LBD tagged with GST, and terbium-labelled anti-GST antibody. In this assay the binding of an agonist to hPPARγ LBD results in a conformational change leading to recruitment of the coactivator TRAP220/DRIP-2 peptide. This recruitment brings the fluorescein attached to the coactivator peptide and the terbium attached to the GST antibody in close spatial proximity, and excitation of the terbium at 340 nm results in a FRET and a consequent partial excitation of the fluorescein that is monitored at 520 nm. The 520 nm signals were normalized to the signals obtained from the terbium emission at 495 nm and thus the 520 nm/495 nm ratios were used as a measure for the TRAP220/DRIP-2 coactivator recruitment potential of the tested compounds. All quantifications were performed with a GeniosPro plate reader (Tecan, Austria).

Adipocyte differentiation

3T3-L1 preadipocytes (ATCC, USA) were propagated in DMEM supplemented with 10% calf serum. For differentiation, the preadipocytes were grown to confluence (day -2) and kept for two more days before medium was changed to DMEM supplemented with 10 % fetal calf serum, 1 µg/ml insulin, and potential PPARγ activators (day 0). In case we wanted to cross-check PPAR dependency of our observations, the PPARγ antagonist BADGE was added one hour prior to the addition of the potential agonists. Medium was renewed every two days until day 7 or 8. For an estimate of accumulated lipids and thus for the adipogenic potential of the test compounds, Oil Red O staining was performed. For

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this, cells were fixed in 10% formaldehyde for 1 h and stained with Oil Red O for 10 minutes. After washing off the excessive dye, photos were taken, and bound dye was solubilized with 100% isopropanol and photometrically quantified at 550 nm.

Molecular Docking

The molecular docking was performed using the GOLD Suite software package (CCDC, 2008). Extraction and preparation of the human PPARγ ligand binding pocket for the docking of two molecules of dieugenol (**1**), tetrahydrodieugenol (**2**), and magnolol (**3**) simultaneously, was done within this software package. To find a suitable ligand binding pocket, we examined the Brookhaven Protein Data Bank (PDB) (Berman et al., 2000) for crystal structures of a PPARγ-ligand complexes. The PDB entry 2vsr provides the X-ray data with the best resolution among all PPARγ-ligand complexes including two copies of the ligand binding simultaneously to their ligand binding pocket. Thus, we used the ligand binding pocket of this PDB complex for molecular docking. For ligand preparation, we applied Corina 3.00 (Molecular_Networks, 2009) and the ilib framework (Wolber and Langer, 2001) to generate 3D structures, and to calculate the protonation states of the neolignans at physiological pH, respectively. The best docking poses were selected based on their GOLDScores and their plausibility. Thus, if a docking pose represented PPARγ-ligand interactions well known from literature, the pose was determined to be more realistic than a higher scored docking pose including unknown and implausible protein-ligand interactions. Finally, the docking pose and the interactions with the binding site were visualized using the LigandScout software 2.0. (Wolber et al., 2006; Wolber and Langer, 2005).

Statistical methods and data analysis

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Statistical analysis and non linear regression (with settings for sigmoidal dose response and variable slope) were done using GraphPad Prism software version 4.03 (GraphPad Software Inc, USA). One-way Analysis of Variance (ANOVA) with Bonferroni post test was used to calculate the statistical significance. For comparison of just two experimental conditions, two-tailed paired t-test was applied. Results with $p < 0.05$ were considered significant. *Ki* values of competitor compounds were also calculated with the GraphPad Prism software version 4.03 utilizing Cheng-Prusoff equation (Ki) = $IC_{50}/(1 + L/KD)$).

Results

Pharmacophore-based virtual screening

To identify new natural product-derived PPARγ ligands we used a pharmacophore-based virtual screening approach. The generation and experimental validation of the pharmacophore models were described previously (Markt et al., 2008; Markt et al., 2007). For our study, the best pharmacophore model for PPARγ partial agonists based on the PDB entry 2g0g (Lu et al., 2006) was selected. The generated model consists of three hydrophobic features, one aromatic ring, one hydrogen bond acceptor, and exclusion volume spheres lining the ligand binding domain of PPARγ. Virtual screening of the 3D multi-conformational natural product databases DIOS and CHM resulted in 34 (0.4%) and 27 (0.3%) hits, respectively. Highly scored virtual hits were obtained from the chemical class of neolignans. The small molecular weight compounds dieugenol (**1**) and tetrahydrodieugenol (**2**), both representing dimers of the abundant natural phenylpropanoid eugenol (**4**), were selected from the hit list. A further neolignan with highly similar structure, magnolol (**3**), which is a prominent constituent of the traditional Chinese herbal remedy magnolia bark (hòu pò), has also been selected for pharmacological evaluation (Fig. 1).

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Isolation and synthesis of compounds

The synthesis of **1** was performed by oxidative dimerization of **4** as previously described. After recrystallization from 2-propanol the isolated **1** was analyzed by NMR. The melting point was found at 101-104 °C (cf. Crossfire BRN 2061706 m.p. between 93 and 108 °C), and the NMR data are in accordance with the published data (Marque et al., 1998; Ogata et al., 2000). Synthesis of **2** was performed by hydrogenation of **1** as described by Ogata et al. (Ogata et al., 2000). After recrystallization from 2-propanol the melting point (147-149 °C) and the NMR data are in accordance with the published data (Ogata et al., 2000).

The isolation of **3** from the bark of *Magnolia officinalis* Rehd. & Wils using different chromatographic methods yielded 0.26%. The compound was identified by mass spectrometry and NMR-spectroscopy and had physical and spectroscopic properties consistent with the literature (Yahara et al., 1991).

PPARγ ligand binding

To validate the predicted hits from the virtual screening approach, we first studied the ability of the neolignans to bind to the purified hPPARγ LBD, assessed by a LanthaScreen™ TR-FRET PPARγ competitive binding assay. Dose-response studies were performed with **1-4** (Fig. 2). Stronger binding of the tested compound to the PPARγ LBD in this assay results in a stronger displacement of the fluorescently labelled ligand (Fluormone™ Pan-PPAR Green, Invitrogen) leading to a decrease of the FRET signal. Pioglitazone (Actos®), a selective PPARγ agonist in clinical use, was used as positive control. **1-3** showed binding properties similar to pioglitazone, while **4** did not bind to the receptor at all (at test concentrations up to 100 µM). Interestingly, compounds **1** and **2** were binding to the hPPARγ LBD with even higher affinity (*Ki* values of 0.24 µM and

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0.32 μM, respectively) than pioglitazone (*Ki* = 1.19 μM), whereas **3** was binding with a slightly lower affinity (*Ki* = 2.04 μM).

PPARγ luciferase reporter gene transactivation

To assess whether the neolignans are able to act as functional PPARγ agonists in intact cells, we next performed PPARγ luciferase reporter gene assays. HEK-293 cells were cotransfected with a hPPARγ expression plasmid, a PPAR luciferase reporter plasmid (tk-PPREx3-luc), and EGFP as an internal control. Compounds **1-3** induced a dose-dependent activation of PPARγ in a concentration range similar to pioglitazone (Fig. 3). The maximal activation in response to **1-2** and pioglitazone was achieved with similar concentrations (about 1 μM from the respective compound to reach saturation response), indicating again similar binding affinities to PPARγ. The maximal fold activation by **1-2**, however, was several folds lower than by the full agonist pioglitazone, indicating a partial agonism of the neolignans in this test system.

PPARγ coactivator recruitment

The binding of ligands to the PPARγ LBD results in a conformational change of the receptor and subsequent recruitment of coactivator proteins (TRAP220, SRC-1, CBP, p300, PGS-1, and/or others), that contribute to the transcriptional regulation of the different PPAR-responsive promoters (Yu and Reddy, 2007). To assess whether the lower maximal activation achieved by the neolignans in the luciferase reporter gene assay might be due to differences in the coactivator recruitment potential of the formed ligand-receptor complex, we next performed a TRAP220/DRIP-2 coactivator recruitment assay.

As shown in Figure 4, indeed compounds **1-3** induced just a partial recruitment of the fluorescein-labelled TRAP220/DRIP-2 coactivator peptide to the PPARγ LBD, whereas

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the known full PPARγ agonist pioglitazone induced a several fold stronger activation. As expected, compound **4** again was not active. In agreement with the result from the luciferase reporter gene assay, the concentration range needed for a saturation response was highly similar for compounds **1-3** and pioglitazone. However, the maximal activation induced with the full agonist pioglitazone was again several times higher.

Taken together, the data obtained from the receptor binding, the luciferase reporter gene transactivation, and the coactivator recruitment are indicating that the neolignans **1-3** are binding to the PPARγ LBD with a high affinity, in a concentration range similar to that of the clinically used agonist pioglitazone (Actos®). The conformation of the ligand-receptor complex formed with **1-3** is, however, different than the one induced with the full agonist pioglitazone and as a consequence the neolignans exhibit partial agonism with respect to TRAP220/DRIP-2 coactivator recruitment and tk-PPREx3 promoter activation.

Molecular docking

To get a deeper mechanistic understanding of the binding of **1-3** to the PPARγ ligand binding pocket, we examined the putative binding modes of these ligands *in silico* by docking them into the hPPARγ binding pocket. The initial docking of **1-3** to the PPARγ binding pocket showed that these small PPARγ ligands occupy only a minor part of the large ligand binding pocket, and thus leave space and hydrogen bond possibilities for a second ligand. Recently, Itoh et al. (Itoh et al., 2008) crystallized a PPARγ-ligand complex containing a fatty acid bound to the ligand binding pocket in a dimeric way. Based on this new information, we have assumed for our docking studies that the agonistic activity of **1-3** is caused by the simultaneous binding of two copies of this ligand to the binding site. From docking compound **1** into the PPARγ ligand binding pocket twice, several hydrogen bonds between the two dieugenol ligands and the binding site can be observed (Fig. 5A).

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Ser289 and Cys285 form a hydrogen bond network with one 2-methoxyphenol moiety of the molecule of **1** that is located next to arm I, as visualized in Fig 5A. The second part of the ligand dimer establishes several hydrogen bonds between residues Ser342 and Glu343 and one of its 2-methoxyphenol moieties. The other 2-methoxyphenol group of this copy of **1** is involved in a hydrogen bond network formed between both ligands. In addition, the vinyl, phenyl, and methoxy moieties of both molecules established hydrophobic contacts to the binding pocket.

Next, two molecules of **2** were docked to the ligand binding pocket, which resulted in the prediction of a binding mode including similar protein-ligand interactions as described for **1** (Fig. 5B).

The best docking pose for compound **3** consisted of one copy of **3** located between arm I and arm III and the other part of the ligand dimer situated between arm II and arm III (Fig. 5C). The molecule of **3** oriented towards arm I forms hydrogen bonds between one hydroxyl group and residues Ser289 and Cys285. Both hydroxyl groups of this copy of **3** are involved in hydrogen bonding with one hydroxyl group of the second part of the dimer. The remaining hydroxyl group of the latter ligand formed another hydrogen bond to residue Gly284. In addition, both molecules establish several hydrophobic interactions with the three arms of the binding pocket.

Similar docking poses determined for **1** and **2** show that the putative binding modes for both compounds include more interactions with the PPARγ binding pocket than the predicted binding mode for compound **3**. This could be an explanation for the higher affinity of **1** and **2** compared to **3**.

Selectivity of action

We next studied whether the neolignans are activating PPARγ selectively over the other two PPAR subtypes. The subtype specificity of the assay was achieved by replacing the

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expression plasmid for hPPARγ with an expression plasmid for hPPARα and hPPARβ/δ, respectively (Table 1). Mouse PPARγ (mPPARγ) was also tested to exclude species differences that could modulate the effectiveness of neolignans in PPARγ experimental models from rodent origin, such as the 3T3-L1 cells that we used (Fig. 6). The specificity of the assays was verified with known selective agonists of PPARα (GW7647), PPARβ/δ (GW0742), and PPARγ (pioglitazone). **1**-**3** activated PPARγ 3.58-fold (with an EC_{50} of 0.62 μM), 3.34-fold (with an EC_{50} of 0.33 μM) and 3.03-fold (with an EC_{50} of 1.62 μM), respectively. Highly similar activation was seen with mPPARγ, suggesting that the neolignans have very comparable potency of action also with the rodent receptor. Compound **4** had no effect on any of the PPAR subtypes tested. The positive control for PPARγ, pioglitazone, induced an 8.05-fold activation (EC_{50} of 0.26 μM) with hPPARγ and a 6.80-fold activation (EC_{50} of 0.22 μM) with mPPARγ. Interestingly, **1** and **2** activated PPARγ selectively with no effect on the other two PPAR subtypes. Compound **3** was not equally specific, since it was activating also hPPARβ/δ at higher concentrations.

Adipocyte differentiation

We next aimed to confirm the effectiveness of the neolignans in a functionally relevant cell model with endogenous expression of PPARγ. Since it is known that PPARγ is an essential player in adipocyte differentiation (Rosen et al., 1999) we examined the adipogenic potential of **1-4** in 3T3-L1 preadipocytes. As positive control we chose rosiglitazone (1 μM) the most often used control TZD in this model (Fig. 6A, 6B) (Wright et al., 2000). As evident by the accumulation of lipid droplets and subsequent Oil Red staining, the treatment with 10 μM of **1-3** resulted in the differentiation to adipocytes, whereas **4** had no adipogenic activity (Fig. 6A). Furthermore, the PPARγ antagonist BADGE (Wright et al.,

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2000) significantly reduced the adipogenic potential of **1-3** and the positive control rosiglitazone, demonstrating PPARγ dependency of the observed effect (Fig. 6B).

Discussion

Here we show the identification of several neolignans that act as partial PPARγ agonists using an *in silico* approach including a pharmacophore-based virtual screening of the natural product databases DIOS and CHM (Rollinger et al., 2008).

The affinity of the virtual hits for the hPPARγ LBD was experimentally confirmed in a PPARγ competitive ligand binding assay (Fig. 2). Compounds **1-3** potently bind to the receptor LBD in a concentration range similar to that of the clinically used PPARγ agonist pioglitazone (Actos®). Based on these promising *in vitro* results we further verified the ability of these neolignans to activate PPARγ also in a cellular model. Indeed, **1-3** dose-dependently activated hPPARγ in a HEK-293 cell-based luciferase reporter gene transactivation model (Fig. 3). The concentrations needed to reach a saturation response by compounds **1-3** were similar to that of pioglitazone indicating again a similar affinity to the PPARγ receptor binding pocket. The maximal activation reached by pioglitazone, however, was several fold higher indicating that the neolignans are acting as partial PPARγ agonists in this model. It is well known, that different PPAR ligands might form ligand-receptor complexes with different coactivator recruitment potentials and thus different transactivation properties. We, therefore, studied the TRAP220/DRIP-2 coactivator recruitment properties of the PPARγ-ligand complexes induced by the neolignans in comparison to the known full agonist pioglitazone (Fig. 4). The neolignans **1-3** again acted as partial agonists, since the maximal activation was several fold lower than the activation induced by the full TZD agonist. Thus, compared to pioglitazone, the neolignans **1-3** possess a similar affinity to PPARγ but apparently induce a receptor-ligand complex with a

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different conformation leading to partial agonism. TZDs that are currently clinically used as PPARγ activators are potent full agonists of PPARγ. To avoid the undesired side effects of TZDs, the development of novel partial PPARγ agonists was suggested as a highly promising approach (Chang et al., 2007; Yumuk, 2006). Thus, MBX-102 (metaglidasen), a selective partial PPARγ agonist, exhibiting a weaker transactivation activity and a reduced coactivator recruitment potential, was recently reported to retain antidiabetic properties in the absence of weight gain and edema (Gregoire et al., 2009). The activation pattern of the neolignans makes them, therefore, a highly interesting class of PPARγ activators.

In all systems used, **1** and **2** had the highest potency among the tested neolignans, closely followed by **3**, whereas **4** had no activity. To get a deeper insight into the binding mode of the neolignans to the hPPARγ LBD, we utilized molecular docking (Fig. 5). The docking studies assume the neolignans to bind as dimers to the receptor binding pocket, and reveal **1** and **2** to make more interactions with the PPARγ binding pocket than **3**, underlining the higher activity observed with these compounds.

1 and **2** selectively activated hPPARγ but not hPPARβ/δ or hPPARα (Table 1). This is another favourable profile of action since all PPARγ agonists that are currently approved on the market are isoform specific PPARγ activators. There are though some experimental indications that PPAR dual-agonists or pan-agonists might also provide advantages (Chang et al., 2007).

Since the PPAR luciferase reporter gene assay represents an artificial cell model with transient overexpression of PPARγ, we examined all neolignans for their ability to differentiate 3T3-L1 adipocytes, which is a functionally relevant cell model making use of endogenously expressed PPARγ. In line with the results from all other models, **1-3** induced adipocyte differentiation and their activity was abolished by the PPAR antagonist BADGE.

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Compound **4** was, however, not active, again confirming the results from all previous experimental models as well as the predictions from the molecular docking studies.

In addition to our findings shown here, the compounds dieugenol (**1**) and tetrahydrodieugenol (**2**)**,** have been reported previously to act as antioxidants (Ogata et al., 2000), antimutagenics (Miyazawa and Hisama, 2003), and to exert anti-inflammatory activity (Murakami et al., 2003). Magnolol (**3**) is a prominent constituent of the traditional Chinese herbal remedy *magnolia bark* (hòu pò) (Bensky et al., 2004). From a western perspective *Magnolia bark* was suggested to be among the herbal drugs effective in combating metabolic syndrome (Banos et al., 2008). In a recent study, treatment with magnolol decreased fasting blood glucose and plasma insulin levels, and was able to prevent or retard the pathological complications in type 2 diabetic Goto-Kakizaki rats (Sohn et al., 2007). A recent report also showed that magnolol enhances adipocyte differentiation in 3T3-L1 cells and C3H10T1/2 cells, and suggested that these effects might be due to PPARγ modulation (Choi et al., 2009). Here we show that magnolol indeed activates PPARγ in several *in vitro* or cell-based models, but distinct from **1** and **2**, it acts as a dual agonist activating also PPARβ/δ at higher concentrations (Table 1). Although magnolol was not the most potent and specifically acting neolignan in our study, its PPAR activating potential is of interest, since it fits well to the traditional use of *magnolia bark* as a herbal drug combating metabolic disorders.

In summary, we describe the computer-aided discovery of several neolignans as novel ligands of PPARγ. In receptor binding assays, dieugenol (**1**) and tetrahydrodieugenol (**2**) exhibited a higher affinity for PPARγ than the clinically used agonist pioglitazone (Actos®). Furthermore, **1** and **2** were identified as selective activators of PPARγ, but not of PPARα or PPARβ/δ. In comparison to the TZD pioglitazone, **1** and **2** displayed a partial agonism with respect to PPARγ luciferase reporter gene transactivation and

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TRAP220/DRIP-2 coactivator recruitment. In addition, they induced adipocyte differentiation in 3T3-L1 cells PPARγ-dependently. The activation pattern exhibited from **1** and **2** makes them highly interesting novel PPARγ agonists, having the potential to be further explored as leads for the development of novel pharmaceuticals or dietary supplements.

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Footnotes

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Numbered footnotes:

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Legends to figures

Figure 1. Chemical structures of the compounds selected for pharmacological investigation.

Figure 2. PPARγ ligand binding potential of neolignans. Serial dilutions of the tested compounds were prepared in DMSO and then mixed with a buffer solution containing the hPPARγ LBD tagged with GST, terbium-labelled anti-GST antibody, and fluorescently-labelled PPARγ agonist. After 1 h of incubation, the ability of the test compounds to bind to the PPARγ LBD and thus displace the fluorescently labelled ligand was estimated from the decrease of the emission ratio 520 nm/495 nm upon excitation at 340 nm. Each data point represents the mean ± SD from three independent experiments performed in duplicate.

Figure 3. Influence of the neolignans on the hPPARγ-mediated reporter gene transactivation. HEK-293 cells, transiently cotransfected with a plasmid encoding full-length hPPARγ, a reporter plasmid containing PPRE coupled to a luciferase reporter and EGFP as internal control, were stimulated with the indicated concentrations of the respective compounds for 18 h. Luciferase activity was normalized by the EGFP-derived fluorescence, and the result was expressed as a fold induction compared to the negative control (DMSO vehicle treatment). The data shown are means ± SD of three independent experiments each performed in quadruplet.

Figure 4. Influence of neolignans on PPARγ coactivator recruitment. The ability of the hPPARγ-ligand complex formed with the test compounds to recruit the TRAP220/DRIP-2

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coactivator peptide was measured as described in detail in the *Materials and Methods* section. Serial dilutions of the tested compounds were prepared in DMSO and then mixed with a buffer solution containing the hPPARγ LBD tagged with GST, terbium-labelled anti-GST antibody, and fluorescein-labelled TRAP220/DRIP-2 coactivator peptide. After incubation for 1 h, the emission at 520 nm and 495 nm after excitation at 340 nm was measured, and the 520 nm/495 nm ratio was used as a measure for the TRAP220/DRIP-2 coactivator recruitment potential of the respective compounds. Each data point represents the mean ± SD from three independent experiments performed in duplicate.

Figure 5. Putative interactions between the hPPARγ binding pocket and the neolignans 1 (A), 2 (B), and 3 (C). The docking results were visualized using the LigandScout software with the following colour code: hydrogen bond acceptor (red arrow), hydrogen bond donor (green arrow), hydrophobic interaction (yellow sphere) and aromatic interaction (blue rings). The ligand binding pocket was depicted as surface coloured based on the hydrophilicity/lipophilicity.

Figure 6. Adipogenic activity of compounds 1-4. (A) 3T3-L1 preadipocytes were differentiated to adipocytes as described in the *Materials and Methods* section. After 7-8 days of differentiation with the indicated test compounds (1 µM rosiglitazone, 50 µM BADGE, and 10 µM of the neolignans, respectively), Oil Red O staining was performed in order to clearly visualize the accumulated lipids. Representative photos of one experiment out of three with consistent results are depicted. **(B)** In order to get a quantitative measure, the dye accumulated in the cells (treated as described under A) was solubilized by 100% isopropanol and photometrically quantified at 550 nm. The data shown are means ± SD from three independent experiments. * $p < 0.05$ and *** $p < 0.001$, as estimated by two-tailed paired t-test.

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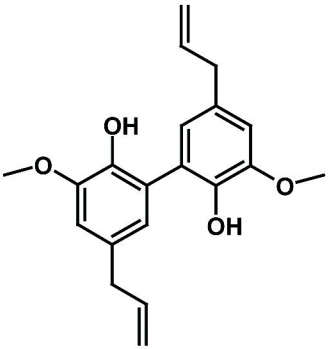
Tables

Table 1. Selectivity of the neolignans towards PPAR subtype (-α, -β/δ, -γ)-driven luciferase reporter transactivation.

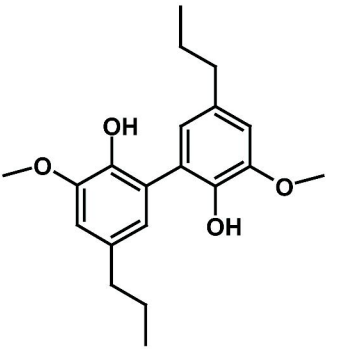
HEK-293 cells were transiently cotransfected with an expression plasmid for the respective PPAR subtype, a reporter plasmid containing PPRE coupled to the luciferase reporter, and EGFP as internal control. Cells were stimulated with the indicated concentrations of the respective compounds for 18 h. Luciferase activity was normalized by the EGFP-derived fluorescence, and the result was expressed as fold induction compared to the negative control (DMSO vehicle treatment). The selective agonists for PPARα (GW7647), PPARβ/δ (GW0742), and PPARγ (pioglitazone), were used to verify the specificity of the respective assays. EC_{50} and maximal fold activation were determined by the GraphPad Prism software version 4.03 (GraphPad Software Inc, USA) using settings for non linear regression with sigmoidal dose response and variable slope. The data shown represent means of three to five independent experiments each in quadruplet. ANOVA analysis showed statistical significance with $p < 0.001$ for the presented effects.

	hPPARα		hPPARβ/δ		hPPARγ		mPPARγ	
	EC_{50} (μM)	maximal fold activation	EC_{50} (μM)	maximal fold activation	EC_{50} (μM)	maximal fold activation	EC_{50} (μM)	maximal fold activation
GW7647	**0.0016**	**3.09**	**-**	**-**	**-**	**-**	**-**	**-**
GW0742	**-**	**-**	**0.0015**	**22.47**	**-**	**-**	**-**	**-**
Pioglitazone	**-**	**-**	**-**	**-**	**0.26**	**8.05**	**0.22**	**6.80**
Dieugenol (1)	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**0.62**	**3.58**	**0.93**	**2.93**
Tetrahydrodieugenol (2)	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**0.33**	**3.34**	**0.38**	**2.98**
Magnolol (3)	**n.d.**	**n.d.**	**11.41**	**2.45**	**1.62**	**3.03**	**1.14**	**2.81**
Eugenol (4)	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**	**n.d.**

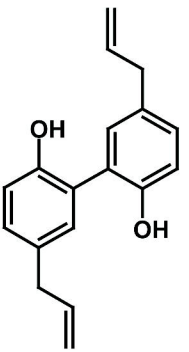
n.d. not detected up to 100 μM



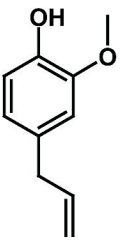
Dieugenol (1)



Tetrahydrodieugenol (2)



Magnolol (3)



Eugenol (4)

Figure 1

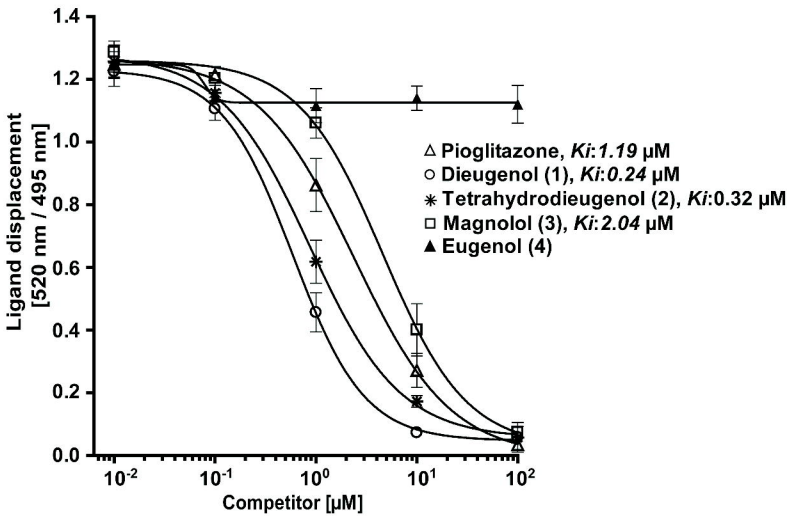

Ligand displacement
[520 nm / 495 nm]
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10-2
10-1
100
101
102
Competitor [μM]
△ Pioglitazone, Ki:1.19 μM
○ Dieugenol (1), Ki:0.24 μM
✳ Tetrahydrodieugenol (2), Ki:0.32 μM
□ Magnolol (3), Ki:2.04 μM
▲ Eugenol (4)

Figure 2

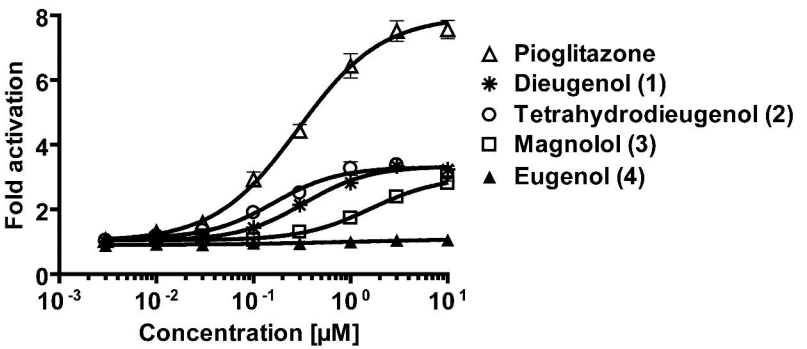

Fold activation
8
6
4
2
0
10 -3
10 -2
10 -1
10 0
10 1
Concentration [µM]
Pioglitazone
Dieugenol (1)
Tetrahydrodieugenol (2)
Magnolol (3)
Eugenol (4)

Figure 3

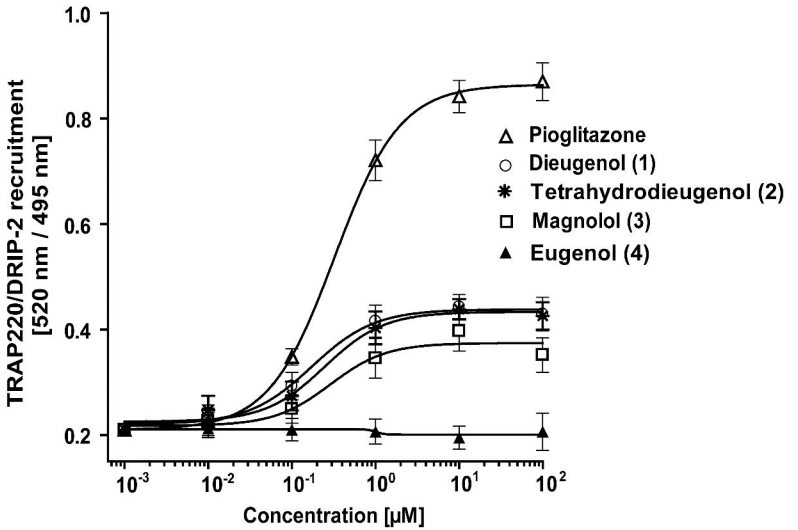

TRAP220/DRIP-2 recruitment
[520 nm / 495 nm]
1.0
0.8
0.6
0.4
0.2
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
Concentration [µM]
Pioglitazone
Dieugenol (1)
Tetrahydrodieugenol (2)
Magnolol (3)
Eugenol (4)

Figure 4

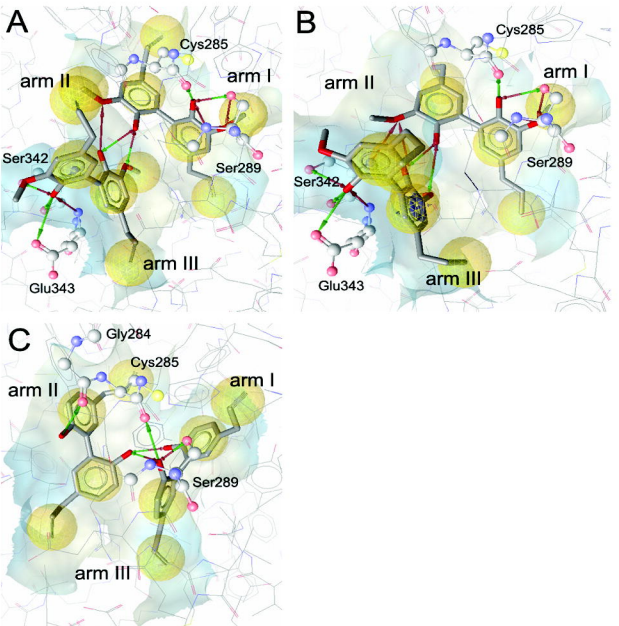

A
Cys285
arm II
arm I
Ser342
Ser289
arm III
Glu343
B
Cys285
arm II
arm I
Ser289
Ser342
Glu343
arm III
C
Gly284
Cys285
arm II
arm I
Ser289
arm III

Figure 5

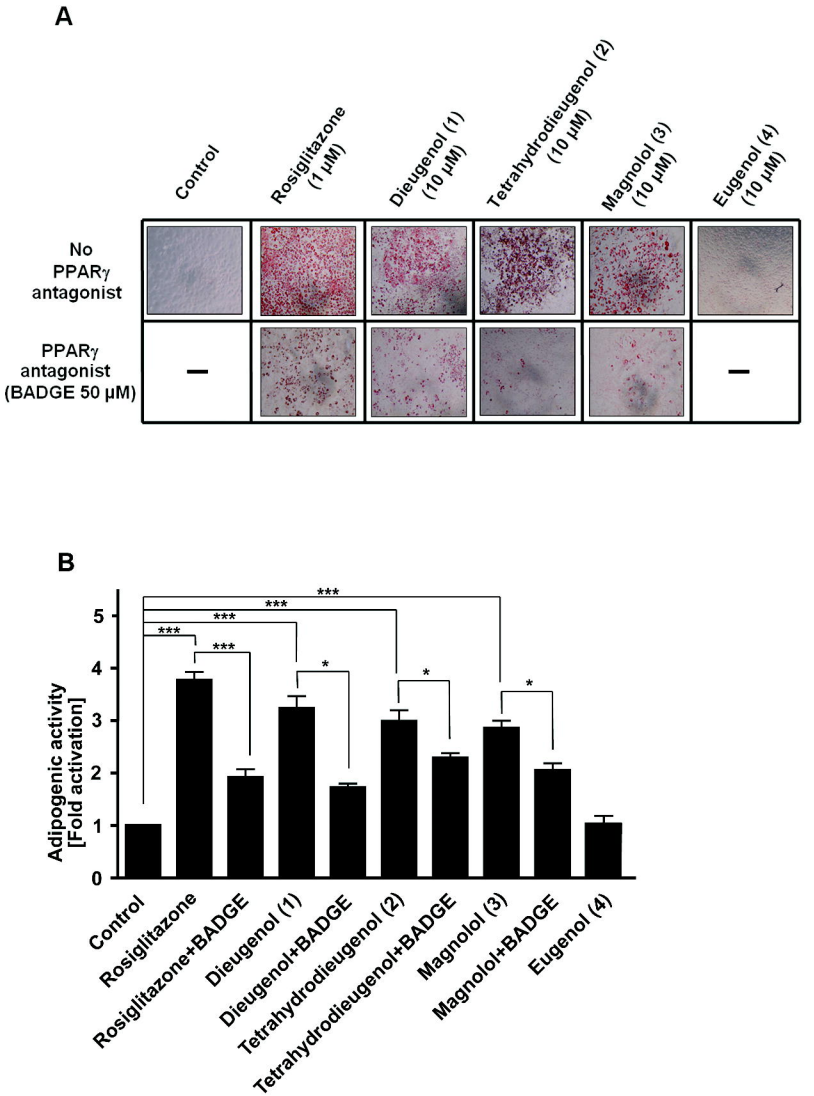

A
Control
Rosiglitazone (1 μM)
Dieugenol (1) (10 μM)
Tetrahydrodieugenol (2) (10 μM)
Magnolol (3) (10 μM)
Eugenol (4) (10 μM)
No PPARγ antagonist
PPARγ antagonist (BADGE 50 μM)
B
Adipogenic activity [Fold activation]
0
1
2
3
4
5
***
***
***
***
*
*
*
Control
Rosiglitazone
Rosiglitazone+BADGE
Dieugenol (1)
Dieugenol+BADGE
Tetrahydrodieugenol (2)
Tetrahydrodieugenol+BADGE
Magnolol (3)
Magnolol+BADGE
Eugenol (4)

Figure 6