J. Biomed. Lab. Sci. 10 (2004) 137-142

# Two Synthetic Ligands for Peroxisome Proliferator-Activated Receptor γ

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The peroxisome proliferator-activated receptor γ (PPARγ) is the molecular target for a class of drugs, the antidiabetic thiazolidnediones (TZDs). The heterodimer of PPARγ with retinoid X receptor (RXR) plays a central role in the regulation of adipogenesis and insulin sensitization. We synthesized two chemicals, DANA87 and DANA88, sharing structural characteristics with TZDs. Given this structural similarity, it was hypothesized that DANA87 and DANA88 may act as PPARγ ligands. In transient transfection assays, DANA87 and DANA88 caused slight increases in the endogenous expression of a luciferase reporter gene containing the PPAR responsive element in 3T3-L1 preadipocytes. However, DANA87 and DANA88 significantly inhibited troglitazone-induced reporter gene activation when cells were treated with a combination of DANA87 or DANA 88 and troglitazone, one of the TZDs that activate PPARγ. These results suggest that DANA87 and DANA88 are not only weak agonists of PPARγ transactivation, but also competitively antagonize troglitazone-induced PPARγ reporter activity.

**Key Words:** PPARγ, Synthetic ligand, Transient transfection assay

## INTRODUCTION

The PPARs are members of a large family of ligand-activated transcription factors with essential roles in glucose and lipid metabolism[4,17,21,23]. Three genetically and functionally distinct PPAR isoforms occur in mammals, PPARα, PPARβ/δ, and PPARγ[3,6,11,12]. Ligand-bound PPARs modulate target gene expression by binding to DNA response elements composed of a direct repeat of the hexameric core motif AGGTCA separated by a single base pair after heterodimerization with RXR. PPAR · RXR heterodimers interact with PPAR-response elements (PPREs) in the promoter region of target genes implicated in a number of physiological processes including fatty acid metabolism, inflammation, adipogenesis, carcinogenesis, and development. PPARγ, in particular, exerts regulatory control over the expression of numerous genes encoding proteins involved in adipogenesis and insulin sensitization[5,8,15,20].

Insight into the biology of PPARγ has been gleaned from the discovery that this receptor is the molecular target for the TZD class of antidiabetic drugs[20]. The TZDs were originally developed for the treatment of type II diabetes on the basis of its ability to lower glucose levels in rodent models of insulin resistance. This drug also has the beneficial effect of lowering the plasma levels of fatty acids and triglycerides[2]. The finding that TZDs mediate their therapeutic effects through direct interactions with PPARγ established this receptor as a key regulator of glucose and lipid homeostasis[14]. Given previous reports that the pharmacology of synthetic PPARγ ligands demonstrates the role of this receptor and establishes their utility as molecular targets for the development of drugs for the treatment of diabetes and cardiovascular disease, we postulated that two synthetic chemicals, DANA87 and DANA88, sharing structural characteristics with TZDs may act as PPARγ ligands.

In this report, we describe two potential PPARγ ligands that are partial agonists of PPARγ transactivation and inhibitors of troglitazone-induced PPARγ reporter gene expression.

*Received: May 1, 2004
Accepted after revision: May 27, 2004
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## METHODS

### 1. Compound synthesis

*N*-cyclohexylsuccinamic acid 17-(4-ethyl-1,5-dimethyl-hexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17

-tetradecahydro-1*H*-cyclopenta[a]phenanthren-3-yl ester (DANA87) and *N*-(3-bromophenyl)succinamic acid 17-(4-ethyl-1,5-dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[a]phenanthren-3-yl ester (DANA88) were prepared by the reaction of β-sitosterol and succinic anhydride in toluene with a catalytic amount of DMAP and the subsequent chlorination of the resultant acid compound with $SOCl_2$, and then the direct amination with cyclohexyl amine and aniline, respectively. Satisfactory spectral data of two compounds were obtained and will be presented elsewhere (Y.-H. Song, unpublished data).

### 2. Transient transfection assay

The expression vectors, pSG5-mPPARγ and $PPRE_3$-tk-luc reporter gene constructs were generously provided by Dr. Frank J. Gonzalez (Laboratory of Metabolism, National Cancer Institute, NIH, Bethesda, MD, USA) as described previously[25]. 3T3-L1 preadipocytes were routinely cultured in DMEM containing 10% fetal bovine serum (Gibco-Brl, Grand Island, NY, USA), penicillin G (100 U/ml), streptomycin sulfate (100 μg/ml), amphotericin B (0.25 μg/ml) and 2-mercaptoethanol (50 μM). Cells were seeded in 6-well tissue culture plates ($2\times10^4$ cells/well) 24 h prior to transfection. For all transfections, 200 ng/well of each of the appropriate plasmids were used. Transfections were performed using GeneSHUTTLE-40 (Q · Biogene, Carlsbad, CA, USA) according to the manufacturer's instructions. After 6 h, the culture medium was changed and the test compounds, troglitazone, DANA87 and DANA88 were added. After incubation for 24 h in the presence of the aforementioned chemicals, cells were washed twice with

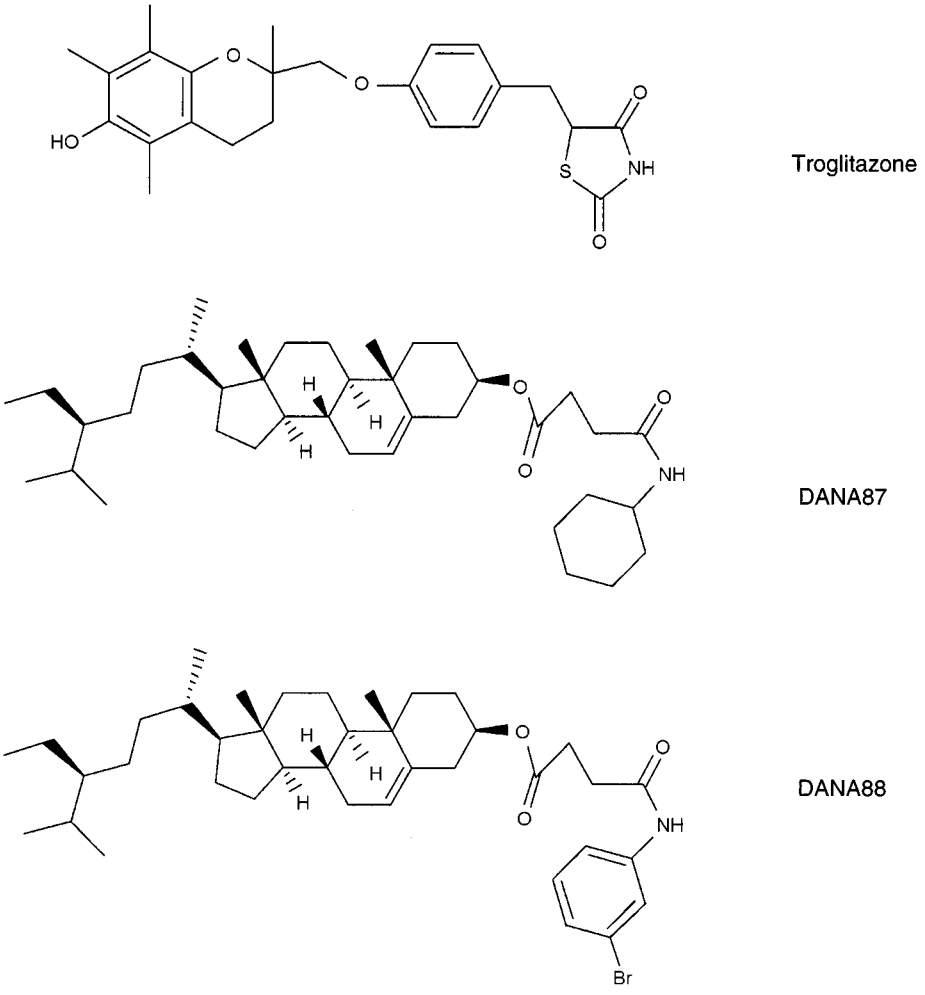



**Fig. 1.** Chemical structures of TZD troglitazone, DANA87 and DANA88.

PBS and assayed for luciferase and β-galactosidase activity using commercial kits according to the manufacturer's instructions (Promega, Madison, WI, USA).

### 3. Statistics

Unless otherwise noted, all values are expressed as mean ± standard deviation (SD). All data were analyzed by ANOVA for statistically significant differences between groups.

## RESULTS

Synthetic PPARγ ligands are used for their potent antidiabetic effects. In the United States, three TZDs, troglitazone, rosiglitazone and pioglitazone are approved for use in type 2 diabetic patients. They bind PPARγ, so it is believed that their hypoglycemic effect is exerted by activating PPARγ. At present, additional compounds of this class are under preclinical evaluation. As shown in Fig. 1, we synthesized two chemicals, DANA87 and DANA88, which are similar to the structure of troglitazone, the first TZD approved for treating type 2 diabetes.

In order to examine the effects of DANA87 and DANA-88 on PPARγ reporter gene expression, 3T3-L1 preadipocytes, on which PPARγ has major effects, were co-transfected with PPARγ and RXRα expression constructs as well as a luciferase reporter construct ($PPRE_3$-tk-luc) containing 3-copies of the PPRE from the rat acyl-CoA oxidase gene[19]. Transfected cells were treated with DANA87 and DANA-88 at doses that did not show any cytotoxic effects as measured by trypan blue exclusion. Treatment of the transfected cells with troglitazone caused a significant increase of luciferase activity compared with vehicle treated cells ($P$<0.001) (Fig. 2). DANA87 and DANA88 also caused increases in reporter gene activation and maximal increases in the magnitude of reporter gene activation were achieved at doses of 0.1 μM for DANA87 ($P$<0.01) and 1 μM for DANA88 ($P$<0.05) although they showed less increases in PPARγ reporter gene activation compared with troglitazone treatment (Fig. 2).

To determine if DANA87 and DANA88 can also regulate troglitazone-mediated PPARγ transactivation, experiments were performed using cells treated with a combination of DANA87/troglitazone or DANA88/troglitazone. Unexpectedly, treatment of PPARγ transfected cells with

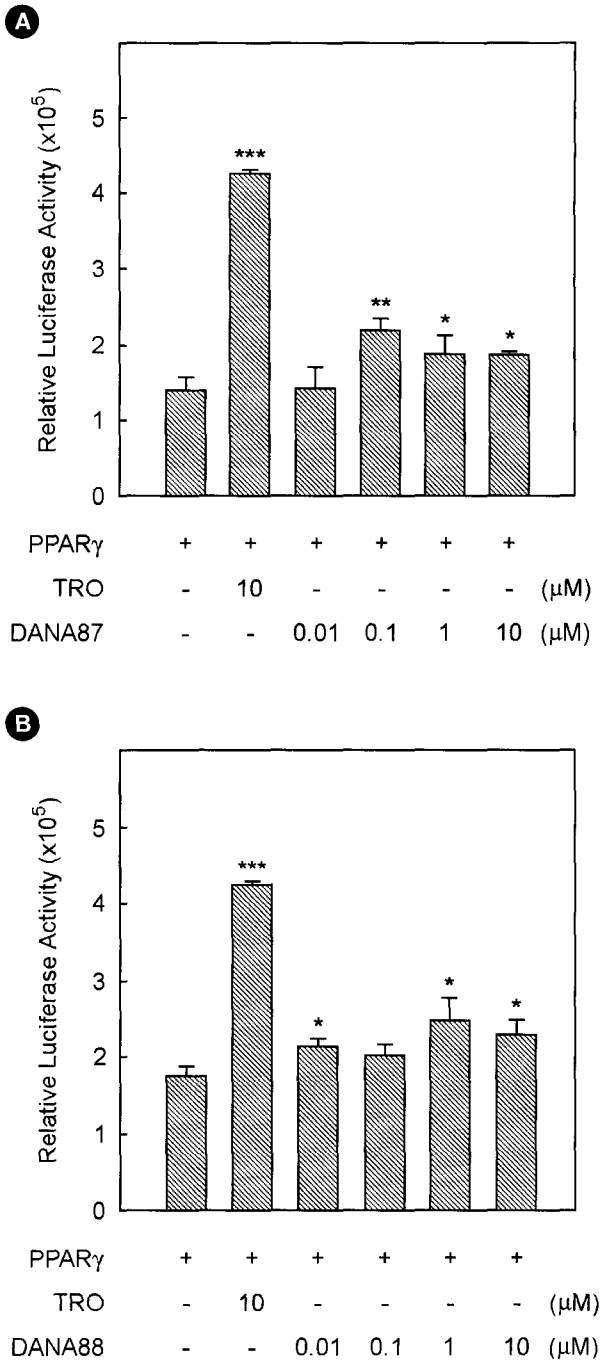
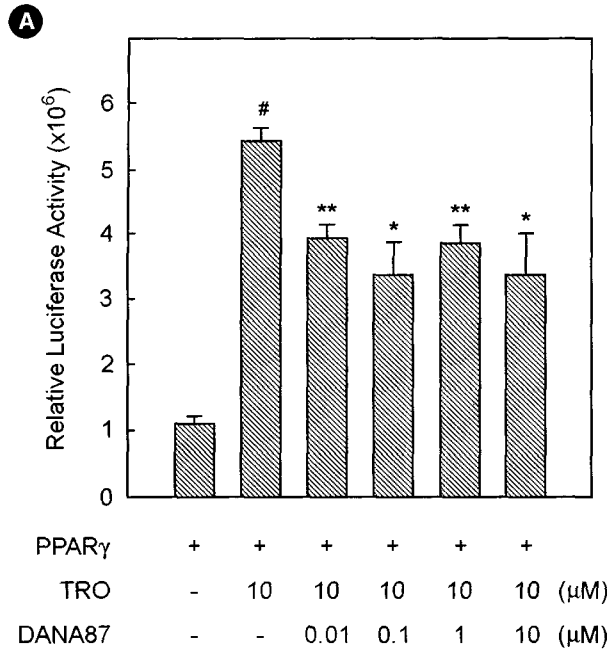



**Fig. 2.** Effects of **(A)** DANA87 and **(B)** DANA88 on PPARγ reporter gene expression. 3T3-L1 cells were transiently transfected with expression plasmids for PPARγ, a luciferase reporter gene construct containing 3 copies of the PPRE and β-galactosidase gene. Cells were treated with various concentrations of DANA87 and DANA88 at the initial time of culture. Following incubation for 24 h, cells were harvested, lysed and were subsequently assayed for luciferase and β-galactosidase activities. All values are expressed as the mean ± SD of relative luciferase units/β-galactosidase activity. Experiments were performed at least three times. Significantly different versus vehicle, *$P$<0.05; **$P$<0.01; ***$P$<0.001. TRO, troglitazone.



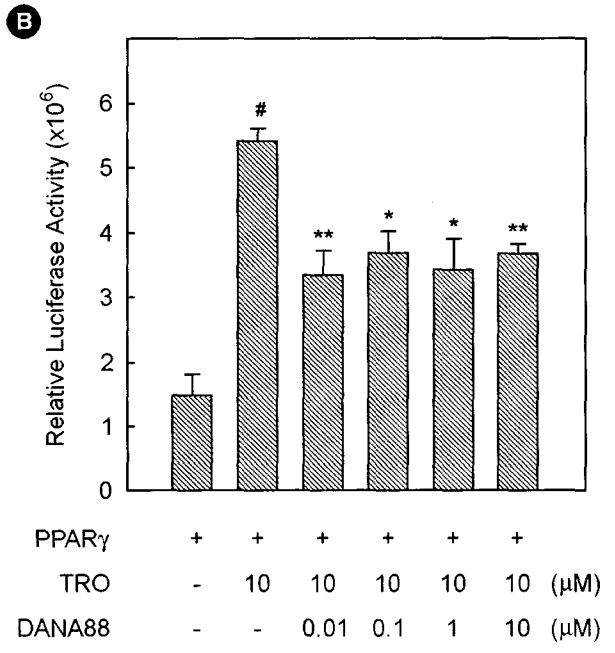




**Fig. 3.** Inhibition of troglitazone-induced PPARγ reporter gene expression by **(A)** DANA87 and **(B)** DANA88. 3T3-L1 cells were transiently transfected with pSG5-mPPARγ, reporter plasmid $PPRE_3$-TK-Luc, β-galactosidase gene and pBSK. Cells were treated with 10 μM troglitazone (TRO) alone or concomitantly treated with TRO and DANA87 or TRO and DANA88. After incubation for 24 h, cells were harvested, lysed and were subsequently assayed for luciferase and β-galactosidase activities. All values are expressed as the mean ± SD of relative luciferase units/β-galactosidase activity. Experiments were performed at least three times. #Significantly different versus vehicle, $P<0.01$. Significantly different versus TRO, $*P<0.05$; $**P<0.01$.

DANA87 and DANA88 at all doses caused significant inhibition of troglitazone-induced reporter gene activation (Fig. 3).

## DISCUSSION

Since PPARγ is known to be a molecular target for a class of TZDs that are potent antidiabetic compounds and to be involved in adipogenesis, immunomodulatory activities and cell proliferation/differentiation pathways, it is important to identify pharmacologically active PPARγ agonists and antagonists. This study was therefore undertaken to determine whether two synthetic compounds, DANA87 and DANA88, with structure common to TZDs act as PPARγ ligands.

Our results demonstrate that DANA87 and DANA88 caused slight increases in the endogenous expression of a luciferase activity in 3T3-L1 preadipocytes, but they significantly inhibited troglitazone-induced reporter gene activation. These results show that DANA87 and DANA88 may be PPARγ ligands that are partial agonists of receptor transactivation and inhibitors of troglitazone-induced PPARγ reporter activity, suggesting that DANA87 and DANA88 may be nuclear receptor ligands that do not interact with C-terminal activation function 2 (AF-2) helix of PPARγ ligand-binding domain and have different mechanism from conventional agonists and antagonists.

Consistent with our data, Oberfiend et al (1999) identified GW0072, a high affinity PPARγ ligand that is a weak partial agonist of PPARγ transactivation, but a potent antagonist of PPARγ-mediated adipocyte differentiation[16]. They reported that GW0072 does not directly affect the positioning of AF-2 or residues involved in coactivator recruitment, although biochemical and structural studies with several nuclear receptors revealed not only that hormone binding induces allosteric changes in the conformation of the ligand-binding domain, which promote recruitment of transcriptional coactivator proteins[9,17], but also that synthetic antagonists physically block the binding of coactivator proteins[1,18]. Instead, GW0072-bound receptor adopted a conformation similar to the unliganded apo-receptor and resulted in either subtle changes in the conformation of the ligand-binding domain or allosteric effects on the heterodimer partner RXR[22].

From a chemical perspective, although agonist and anta-

gonist ligands often are structurally related, the antagonists generally contain an additional chemical appendage that is critical for their inhibitory activities[10]. As a result, these agonist and antagonist ligands use the same binding epitopes within the receptor pocket, but the larger total volume antagonist leads to repositioning of the AF-2 helix[22]. Thus, it is thought that DANA87 and DANA88 may be examples of nuclear receptor ligands that does not interact with AF-2 and be mechanistically distinct from conventional agonists and antagonists.

Because the PPARs are important molecular targets for the development of diabetes, obesity, cardiovascular drugs[24], the identification of partial agonists with modified pharmacological profiles may offer benefit for the treatment of human metabolic diseases[13]. Further studies will be necessary not only to determine the effects of DANA87 and DANA88 on PPARγ function for potential therapeutic implications, but also to investigate the ability of DANA87 and DANA88 to recruit the coactivators or the corepressors.

**Acknowledgements**

This work was supported by a grant No. R05-001099-0 from the Basic Research Program of the Korea Science & Engineering Foundation.

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