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**(54) Title:** METHODS FOR IDENTIFYING LIGANDS FOR NUCLEAR HORMONE RECEPTORS

**(57) Abstract**

Fatty acids (FAs) and their derivatives are essential cellular metabolites whose concentations must be closely regulated. This implies that regulatory circuits exist which can sense changes in FA levels. Indeed, the peroxisome proliferator activated receptor $\alpha$ (PPAR$\alpha$) regulates lipid homeostasis and is transcriptionally activated by a variety of lipid-like compounds. It remains unclear as to how these structurally-diverse compounds can activate a single receptor. In accordance with the present invention, there are provided conformation-based assays which screen activators for their ability to bind to PPARs (i.e., PPAR$\alpha$, PPAR$\delta$ and PPAR$\gamma$) and induce DNA binding. It is shown here that specific FAs, eicosanoids and lipomodulatory agents are ligands for PPAR$\alpha$, PPAR$\delta$ and/or PPAR$\gamma$. Since altered FA levels are associated with obesity, atherosclerosis, hypertension and diabetes, PPARs may serve as molecular sensors which are central to the development and treatment of these metabolic disorders.

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# METHODS FOR IDENTIFYING LIGANDS FOR NUCLEAR HORMONE RECEPTORS

## FIELD OF THE INVENTION

The present invention relates to methods for the modulation of nuclear receptor mediated processes. In a particular aspect, the present invention relates to methods for the identification of compounds useful for modulation of such processes. In another aspect, the present invention relates to methods for the identification of ligands for the PPARs. In yet another aspect, the present invention relates to methods for monitoring fatty acid-containing foodstuffs for the presence of beneficial fatty acids therein. In still another aspect, the present invention relates to the use of a specific class of compounds for the modulation of processes mediated by peroxisome proliferator activated receptor-alpha (PPAR-$\alpha$). In a further aspect, the present invention relates to the use of a specific class of compounds for the modulation of processes mediated by peroxisome proliferator activated receptor-delta (PPAR-$\delta$). In a still further aspect, the present invention relates to the use of a specific class of compounds for the modulation of processes mediated by peroxisome proliferator activated receptor-gamma (PPAR-$\gamma$). In another aspect, the present invention relates to methods to induce fatty acid degradation in a subject.

## BACKGROUND OF THE INVENTION

Fatty acids (FAs) are ubiquitous biological molecules that are utilized as metabolic fuels, as covalent regulators of signaling molecules and as essential components of cellular membranes. It is thus logical that FA levels should be closely regulated. Indeed, some of the most common medical disorders in industrialized societies (e.g., cardiovascular disease, hyperlipidemia, obesity and insulin resistance) are characterized by altered levels of FAs or their metabolites (see, for example, Durrington, in Postgrad Med J **69 Suppl 1**, S18-25; discussion S25-9 (1993) and Reaven, in J Intern Med Suppl **736**, 13-22 (1994)).

The need for precise control of FA levels suggests that organisms possess sensors that can respond to changes in the available levels of FA metabolites. PPARα has been identified as a vertebrate nuclear hormone receptor which regulates genes involved in FA degradation (ß- and ω-oxidation; see Schoonjans et al., in Biochim Biophys Acta **1302**:93-109 (1996)). PPARα is highly expressed in the liver and was originally identified by Green and colleagues as a molecule that mediates the transcriptional effects of drugs that induce peroxisome proliferation in rodents (see Issemann & Green in Nature **347**:645-50 (1990)). Mice lacking functional PPARα are incapable of responding to these agents and fail to induce expression of a variety of genes required for the metabolism of FAs in peroxisomes, mitochondria and other cellular compartments (see Lee et al., in Mol Cell Biol **15**:3012-3022 (1995)). As a result, PPARα-deficient mice inappropriately accumulate lipid in response to pharmacologic stimuli.

PPARα is a member of the nuclear receptor superfamily, which includes receptors for the steroid, thyroid and retinoid hormones (see Mangelsdorf & Evans in Cell **83**:841-50 (1995)). Two other PPARα-related genes (PPARγ and PPARδ) have been identified in mammals. PPARγ is highly enriched in adipocytes, while the δ isoform is ubiquitously expressed (see Schoonjans et al., supra). Like other members of this receptor superfamily, all of the PPAR isoforms contain a central DNA binding domain that recognizes response elements in the promoters of their target genes. PPAR response elements (PPRE) are composed of a directly repeating core-site separated by 1 nucleotide (see Kliewer et al., in Nature **358**:771-4 (1992)). In order to recognize a PPRE, PPARs must heterodimerize with the 9-cis retinoic acid receptor (RXR).

Once bound to a response element, PPARs activate transcription through a conserved C-terminal ligand binding domain. Although no ligand has been identified for PPARα, sequence analysis indicates that its C-terminal region is similar to the ligand binding domains of known members of the nuclear hormone receptor superfamily. This has prompted an intense search for the identification of ligands for the PPARs. Recently, 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ (15d-$J_2$) has been identified as a ligand for PPARγ (see, for example, Forman et al., in Cell **83**:803-12 (1995) and Kliewer et al., in Cell **83**:813-9 (1995)). Activation of PPARγ by 15d-$J_2$ or its synthetic analogs (e.g., thiazolidinediones; see Forman et al., supra) promotes differentiation of pre-adipocytes into mature, triglyceride-containing fat cells. Similarly, thiazolidinediones have been shown to increase body weight in animals (see Zhang et al., in J Biol Chem **271**:9455-9 (1996)), suggesting that 15d-$J_2$ may be utilized as an in vivo signal to store FAs in the form of triglycerides.

In contrast to the γ isoform, PPARα appears to regulate FA oxidation, suggesting that PPARα ligands may represent endogenous signals for FA degradation (see Schoonjans et al., supra). Green and colleagues originally demonstrated that PPARα is activated by fibrates (see Issemann & Green, supra), a group of drugs that induce peroxisome proliferation and FA oxidation in rodents. These drugs are currently being used as serum triglyceride lowering agents. Since fibrates and polyunsaturated FAs (PUFAs) were known to possess similar activities, Gottlicher et al. (Proc Natl Acad Sci USA **89**:4653-7 (1992)) examined the ability of FAs to activate PPARα. These studies and others have uncovered a bewildering array of compounds (see, for example, Fig. 1A) which can activate PPARα (see, for example, Schoonjans et al., supra). However, all attempts to demonstrate that these compounds bind directly to PPARα have failed. This has led to the suggestion that these compounds alter FA metabolism which indirectly leads to the accumulation of an endogenous PPARα ligand (see Gottlicher et al., in Biochem Pharmacol **46**:2177-84 (1993)).

Accordingly, there is a need in the art for new assays which allow the ready identification of ligands for the PPARs. In addition, there is a need in the art for methods to modulate processes mediated by the various PPAR isoforms. These and other needs in the art are addressed by the present invention.

## BRIEF DESCRIPTION OF THE INVENTION

In accordance with the present invention, we have developed a novel ligand binding assay which facilitates the identification of ligands for the PPARs (e.g., PPARα, PPARδ and PPARγ). Contrary to common belief, it has been discovered that fibrates and specific FAs/eicosanoids can bind to these receptors. This indicates that FAs simultaneously serve as intermediary metabolites and as primary regulators of transcriptional networks. In addition, the demonstration of a direct interaction between fibrates and PPARα suggests that this receptor could be utilized as a target for the rapid identification of highly potent and selective hypolipidemic agents.

## BRIEF DESCRIPTION OF THE FIGURES

Figure 1A presents the chemical structures of some compounds that are demonstrated herein to be ligands for PPARα or δ.

Figure 1B presents data demonstrating that fibrates selectively activate PPARα in a cell-based transient transfection assay. Cells were treated with the following concentrations of each compound: 5 μM Wy 14,643, 300 μM ciprofibrate, 300 μM clofibrate and 1 μM BRL 49653.

Figure 1C presents a comparison of the dose response profile of wild-type PPARα (solid circles) with PPARα-G (i.e., a mutant wherein the Glu at position 282 is replaced with Gly; see Hsu et al., in Mol Pharmacol **48**:559-67 (1995); represented in the figure with open circles) in the transient transfection assay (left panel) and the ligand induced complexation (LIC) assay (right panel; see also Example 4). The ligand induced complex was quantified by phosphorimaging analysis. Ligand induced binding represents the amount of complex produced at any concentration of ligand, minus that produced in the absence of ligand. Maximal induced binding was defined to be 100%; binding observed at other concentrations was normalized to this value.

Figure 2A illustrates the activation of PPARα by FAs and fatty alcohols. All compounds were added to a final concentration of 30 μM except for Wy 14,643 which was used at 5 μM.

Figure 2B illustrates the enhancement of PPARα-RXRα heterodimer formation by FAs and fatty alcohols. All compounds were added to a final concentration of 30 μM except for Wy 14,643 which was added to a final concentration of 5 μM. Saturated FAs and alcohols are indicated by their chain length. Unsaturated FAs are as follows:

linoleic acid (cis-$\Delta^{9,12}$-C18:2),
α-linolenic acid (cis-$\Delta^{9,12,15}$-C18:3),
γ-linolenic acid (cis-$\Delta^{6,9,12}$-C18:3),
arachidonic acid (cis-$\Delta^{5,8,11,14}$-C20:4),

erucic acid (cis-$\Delta^{13}$-C22:1) and
nervonic acid (cis-$\Delta^{15}$-C24:1).

Figure 2C illustrates that inhibitors of ß-oxidation both activate (left panel) and bind (right panel) to PPAR$\alpha$. Experiments were performed as described with respect to Figure 1 (see also, Example 6). Triacsin C (10 $\mu$M, left panel; 30 $\mu$M, right panel) was used as an inhibitor of fatty LC-FACS. Inhibitors of carnitine palmitoyltransferase I included LY 171883 (30 $\mu$M), 2-bromopalmitate (2Br-C16; 5 $\mu$M) and tetradecylglycidic acid (TDGA, 5 $\mu$M). Fatty acyl-CoA dehydrogenase was inhibited with octylthioproprionic acid (OTP, 30 $\mu$M), tetradecylthioproprionic acid (TTP, 30 $\mu$M), nonylthioacetic acid (NTA, 30 $\mu$M) and tetradecylthioacetic acid (TTA, 30 $\mu$M). Wy 14,643 (5 $\mu$M) was included as a positive control.

Figure 3A relates to the identification of eicosanoid ligands for PPAR$\alpha$. Thus, carbaprostacyclin (cPGI), iloprost, 8-hydroxyeicosatetraenoic acid (8S-HETE) and 8-hydroxyeicosapentaenoic acid (8S-HEPE) are seen to transactivate (left panel) and bind (right panel) to PPAR$\alpha$. For transfections (left panel), compounds were added to cells at the following concentrations: 5 $\mu$M Wy 14,643; 10 $\mu$M $PGA_1$, $PGA_2$, $PGB_2$, $PGD_2$, $PGE_2$ and $PGF_{2\alpha}$; 3 $\mu$M 15d-$PGJ_2$; 10 $\mu$M $PGI_2$; 1 $\mu$M cPGI and iloprost; 10 $\mu$M cicaprost; 10 $\mu$M ±8-HEPE (±8-hydroxy-$\Delta^{5Z,9E,11Z,14Z,17Z}$-C20:5), ±8-HETE (±8-hydroxy-$\Delta^{5Z,9E,11Z,14Z}$-C20:4), ±8(9)-EpEtrE (±8(9)-epoxy-$\Delta^{5Z,11Z,14Z}$-C20:3) and ±12-HETE (±12-hydroxy-$\Delta^{5Z,8Z,10E,14Z}$-C20:4); 5 $\mu$M 8S- and 8R-HETE; 10 $\mu$M $LTB_4$. For the ligand binding assay (right panel), compounds were added as follows: 10 $\mu$M Wy 14,643, $PGA_1$, $PGA_2$, $PGB_2$, $PGD_2$, $PGE_2$ and $PGF_{2\alpha}$, 15d-$PGJ_2$ and $PGI_2$; 2 $\mu$M cPGI, iloprost and cicaprost; 1 $\mu$M ±8-HEPE, ±8-HETE, ±8(9)-EpEtrE and ±12-HETE; 300 nM 8S-HETE and 8R-HETE; 10 $\mu$M leukotriene $B_4$ ($LTB_4$).

Figure 3B presents dose response curves comparing the potency of 8S-HETE (solid circles), cPGI (triangles) and Wy 14,643 (shaded squares) in the transactivation of (left panel) and binding to (right panel) PPARα.

Figure 4A demonstrates that PPARα, PPARδ and PPARγ display distinct ligand response profiles. Thus, linoleic acid, arachidonic acid, cPGI and iloprost are seen to transactivate (left panel) and bind to (right panel) PPARδ. After transfection (left panel), compounds were added to test cells at the following concentrations: 5 μM Wy 14,643; 100 μM ciprofibrate; 1000 μM clofibrate; 5 μM BRL 49653; 30 μM C12, C16, linoleic acid, α-linoleic, arachidonic, docosahexaenoic (DHA, all-Z-$\Delta^{4,7,10,13,16,19}$-C22:6) and eicosapentaenoic (EPA, all-Z-$\Delta^{5,8,11,14,17}$-C20:5) acids; 5 μM 2Br-C16; 30 μM TTA; 10 μM $PGA_1$, $PGA_2$, $PGB_2$, $PGD_2$, $PGE_2$ and $PGF_{2\alpha}$; 15d-$PGJ_2$ 3 μM; $PGI_2$ 10 μM; 1 μM cPGI and iloprost; 10 μM cicaprost and 3 μM ±8-HETE. For the ligand binding assay (right panel), compounds were added as follows: 5 μM Wy 14,643; 100 μM ciprofibrate; 1000 μM clofibrate; 50 μM BRL 49653; 30 μM C12, C16, linoleic acid, α-linoleic, arachidonic acids, DHA and EPA; 10 μM 2Br-C16, TTA , $PGA_1$, $PGA_2$, $PGB_2$, $PGD_2$, $PGE_2$, $PGF_{2\alpha}$, 15d-$PGJ_2$, $PGI_2$, cPGI, iloprost and cicaprost and 1 μM ±8-HETE.

Figure 4B presents a comparison of the responsiveness of PPARα (solid bars), PPARγ (shaded bars) and PPARδ (striped bars) to various compounds. Control cells (i.e., containing reporter vector only) are designated by spotted bars. After transfection, cells were treated with the following concentrations of compounds: 30 μM C16; 5 μM 2Br-C16; 30 μM TTA, linoleic, arachidonic acids and EPA; 3 μM ±8-HETE; 10 μM $PGA_1$; 3 μM 15d-$PGJ_2$; 1 μM cPGI; 5 μM Wy 14,643 and BRL 49653.

## DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there are provided methods to determine if test compounds are ligands for members of the nuclear receptor superfamily. In one aspect of this embodiment, invention methods comprise:

determining the binding activity of a homodimer or heterodimer containing said member with respect to a hormone response element (HRE) in the presence of said test compound, relative to the binding activity of said homodimer or heterodimer with respect to said HRE in the absence of said test compound.

In another aspect of this embodiment, invention methods comprise:

contacting a homodimer or heterodimer containing said member and a hormone response element (HRE) with said test compound, and

monitoring for the formation of a complex comprising said homodimer or heterodimer, said HRE, and said test compound, wherein formation of said complex indicates that said test compound is a ligand for said member.

In yet another aspect of this embodiment, the invention method to determine if test compounds are ligands for members of the nuclear receptor superfamily comprises:

contacting said member and a dimeric partner therefor with said test compound, and

monitoring for the formation of a complex comprising said member, said dimeric partner therefor and said test compound, wherein formation of said complex indicates that said test compound is a ligand for said member.

As employed herein, the phrase "members of the nuclear receptor superfamily" (also known as "members of the steroid/thyroid superfamily of receptors" or "intracellular receptors") refers to hormone binding proteins that operate as ligand-dependent transcription factors, including identified members of the steroid/thyroid superfamily of receptors for which specific ligands have not yet been identified (referred to hereinafter as "orphan receptors"). These hormone binding proteins have the intrinsic ability to bind to specific DNA sequences. Following binding, the transcriptional activity of target gene (i.e., a gene associated with the specific DNA sequence) is modulated as a function of the ligand bound to the receptor.

The DNA-binding domains of all of these nuclear receptors are related, consisting of 66-68 amino acid residues, and possessing about 20 invariant amino acid residues, including nine cysteines.

A member of the superfamily can be identified as a protein which contains the above-mentioned invariant amino acid residues, which are part of the DNA-binding domain of such known steroid receptors as the human glucocorticoid receptor (amino acids 421-486), the estrogen receptor (amino acids 185-250), the mineralocorticoid receptor (amino acids 603-668), the human retinoic acid receptor (amino acids 88-153). The highly conserved amino acids of the DNA-binding domain of members of the superfamily are as follows:

Cys - X - X - Cys - X - X - Asp* - X -
Ala* - X - Gly* - X - Tyr* - X - X -
X - X - Cys - X - X - Cys - Lys* -
X - Phe - Phe - X - Arg* - X - X - X -
X - X - X - X - X - X - (X - X -) Cys -
X - X - X - X - X - (X - X - X -) Cys -
X - X - X - Lys - X - X - Arg - X - X -
Cys - X - X - Cys - Arg* - X - X -
Lys* - Cys - X - X - X - Gly* - Met
(SEQ ID NO:1);

wherein X designates non-conserved amino acids within the DNA-binding domain; the amino acid residues denoted with an asterisk are residues that are almost universally conserved, but for which variations have been found in some identified hormone receptors; and the residues enclosed in parenthesis are optional residues (thus, the DNA-binding domain is a minimum of 66 amino acids in length, but can contain several additional residues).

Exemplary members of the steroid/thyroid superfamily of receptors include steroid receptors such as glucocorticoid receptor, mineralocorticoid receptor, progesterone receptor, androgen receptor, vitamin $D_3$ receptor, and the like; plus retinoid receptors, such as RAR$\alpha$, RARß, RAR$\gamma$, and the like, plus RXR$\alpha$, RXRß, RXR$\gamma$, and the like; thyroid receptors, such as TR$\alpha$, TRß, and the like; as well as other gene products which, by their structure and properties, are considered to be members of the superfamily, as defined hereinabove. Examples of orphan receptors include the PPARs (e.g., PPAR$\alpha$, PPAR$\gamma$ and PPAR$\delta$), HNF4 [see, for example, Sladek et al., in Genes & Development 4: 2353-2365 (1990)], the COUP family of receptors [see, for example, Miyajima et al., in Nucleic Acids Research 16: 11057-11074 (1988), Wang et al., in Nature 340: 163-166 (1989)], COUP-like receptors and COUP homologs, such as those described by Mlodzik et al., in

Cell 60: 211-224 (1990) and Ladias et al., in Science 251: 561-565 (1991), the ultraspiracle receptor [see, for example, Oro et al., in Nature 347: 298-301 (1990)], and the like.

The retinoic acid receptor (RAR), the thyroid hormone receptor ($T_3R$), the vitamin $D_3$ receptor (VDR) and the fatty acid/peroxisome proliferator activated receptor (PPAR), for example, preferentially bind to DNA as heterodimers with a common partner, the retinoid X (or 9-cis retinoic acid) receptor (RXR; see, for example, Yu et al., in Cell 67:1251-1266 (1991); Bugge et al., in EMBO J. 11:1409-18 (1992); Kliewer et al., in Nature 355:446-449 (1992); Leid et al, in Cell 68:377-395 (1992); Marks et al., in EMBO J. 11:1419-1435 (1992); Zhang et al., in Nature 355:441-446 (1992); and Issemann et al., in Biochimie. 75:251-256 (1993).

Those of skill in the art can readily determine suitable response elements for use in the practice of the present invention, such as, for example, the response elements described in United States Patent No. 5,091,518 and PCT published application no. WO 92/16546, both of which are hereby incorporated by reference herein.

Naturally occurring HREs are composed of direct repeats (i.e., DRs; see Umesono et al., in Cell 65:1255-1266 (1991), inverted repeats (i.e., IRs; see Umesono et al., in Nature 336:262-265 (1988), and Williams et al. in J. Biol. Chem. 266:19636-19644 (1991)), and/or everted repeats (ERs; see Baniahmad et al., in Cell 61:505-514 (1990); Farsetti et al., in J. Biol. Chem. 267:15784-15788 (1992); Raisher et al., in J. Biol. Chem. 267:20264-20269 (1992); or Tini et al., in Genes Dev. 7:295-307 (1993)) of a degenerate $X_n$-AGGTCA core-site.

In direct repeats (DR, head-to-tail arrangement), the $X_n$ sequence also serves as a gap which separates the two core-binding sites. Thus, for example, spacers of 1, 3, 4 and 5 nucleotides serve as preferred response elements for heterodimers of RXR with PPAR, VDR, $T_3R$ and RAR, respectively (see, for example, Naar et al., in Cell 65:1267-1279 (1991); Umesono et al., 1991, supra; Kliewer et al., in Nature 358:771-774 (1992); and Issemann et al., supra). The optimal gap length for each heterodimer is determined by protein-protein contacts which appropriately position the DNA binding domains (DBDs) of RXR and its partner (see, for example, Kurokawa et al., in Genes Dev. 7:1423-1435 (1993); Perlmann et al., in Genes Dev. 7:1411-1422 (1993); Towers et al., in Proc. Natl. Acad. Sci. USA 90:6310-6314 (1993); and Zechel et al., in EMBO J. 13:1414-1424 (1994)).

Direct repeat hormone response elements (HREs) contemplated for use in the practice of the present invention are composed of at least one direct repeat of two or more half sites, optionally separated by one or more spacer nucleotides (with spacers of 1-5 preferred). The spacer nucleotides can be selected from any one of A, C, G or T. Each half site of direct repeat HREs contemplated for use in the practice of the invention comprises the sequence

-RGBNNM-,

wherein

R is selected from A or G;

B is selected from G, C, or T;

each N is independently selected from A, T, C, or G; and

M is selected from A or C;

with the proviso that at least 4 nucleotides of said -RGBNNM- sequence are identical with the nucleotides at corresponding positions of the sequence -AGGTCA-. Response elements employed in the practice of the present invention can optionally be preceded by $N_x$, wherein x falls in the range of 0 up to 5.

PPAR response elements (PPREs) contemplated for use in the practice of the present invention are composed of at least one direct repeat of two or more of the above-described half sites, separated by a spacer of one nucleotide. The spacer nucleotide can be selected from any one of A, C, G or T. Presently preferred PPREs contain at least one copy (with one, two or three copies most common) of the minimal sequence:

AGGACA A AGGTCA (SEQ ID NO:2).

As readily understood by those of skill in the art, a wide variety of compounds can be assayed employing the invention method. Examples of the classes of compounds contemplated for testing herein include lipomodulatory agents (e.g., fibrates), optionally substituted long-chain mono-, di- or polycarboxylic acid containing at least one site of unsaturation (such as, for example, monounsaturated fatty acids (e.g., erucic acid, nervonic acid, and the like), polyunsaturated fatty acids (e.g., linoleic acid, α-linolenic acid, γ-linolenic acid, arachidonic acid, docosahexaenoic acid, eicosapentaenoic acid, and the like), eicosanoids (e.g., arachidonic acid, prostaglandin $A_1$, prostaglandin $A_2$, prostaglandin $B_2$, prostaglandin $J_2$, 8,9-dihydroxy eicosatrienoic acid, 11,14-dihydroxy eicosatrienoic acid, and the like), hydroxy-substituted fatty acids (e.g., 8-hydroxyeicosatetraenoic acid (8S-HETE), 8-hydroxy-eicosapentaenoic acid (8S-HEPE), and the like), epoxy-substituted fatty acids (e.g., ±8(9)-EpEtrE (i.e., ±8(9)-epoxy-$\Delta^{5Z,11Z,14Z}$-C20:3)), α-alkoxy fatty acids, α-alkyl fatty acids, and the like.

In accordance with yet another embodiment of the present invention, there are provided methods to monitor fatty acid-containing foodstuff(s) for the presence of beneficial fatty acids therein. In one aspect, invention methods comprise:

determining the binding activity of a PPARα-containing heterodimer with respect to a PPAR response element (PPRE) in the presence of the fatty acid(s) contained in said foodstuff, relative to the binding activity of said PPARα-containing heterodimer with respect to said PPRE in the absence of said fatty acid(s), wherein binding of said heterodimer to said PPRE is indicative of the presence of beneficial fatty acids in said foodstuff.

In accordance with another aspect of this embodiment, the invention method comprises:

determining the ability of the fatty acid(s) contained in said foodstuff to activate a PPARα-responsive reporter in an assay system comprising:

PPARα,

RXRα, and

a PPARα-responsive reporter comprising a PPAR response element (PPRE) in operative communication with a reporter gene,

wherein expression of the reporter gene product is indicative of the presence of beneficial fatty acids in said foodstuff.

Thus, compounds in food which are capable of activating PPARα-mediated pathways are identified as beneficial in that they are expected to promote metabolism of fatty acids. As can be readily determined by those of skill in the art, the fatty acids in a foodstuff can be separated therefrom using standard techniques, such as, for example, by extraction.

In accordance with still another embodiment of the present invention, there are provided methods to characterize the profile of fatty acids in a fatty acid-containing foodstuff. In one aspect, invention methods comprise:

> determining the quantity of binding of a PPARα-containing heterodimer, a PPARδ-containing heterodimer or a PPARγ-containing heterodimer, to a PPAR response element (PPRE) in the presence of the fatty acid(s) contained in said foodstuff, relative to the quantity of binding of said PPARα-containing heterodimer, said PPARδ-containing heterodimer or said PPARγ-containing heterodimer, respectively, to said PPRE in the absence of the fatty acid(s) contained in said foodstuff.

In another aspect of this embodiment, invention methods comprise:

> determining the ability of the fatty acid(s) contained in said foodstuff to activate a PPARα-responsive reporter, a PPARδ-responsive reporter or a PPARγ-responsive reporter, in an assay system comprising:
>
> PPARα, PPARδ or PPARγ, respectively,
> RXRα, and
> a PPAR-responsive reporter comprising a PPAR response element (PPRE) in operative communication with a reporter gene,
>
> wherein expression of the reporter gene product is indicative of the presence of fatty acids in said foodstuff which modulate a metabolic pathway mediated by PPARα, PPARδ and/or PPARγ.

Thus, in accordance with the present invention, the activity profile of the fatty acid content of food can readily be obtained, so that a given foodstuff can be characterized in terms of its ability to activate PPARα-mediated pathways (e.g., fatty acid metabolism), PPARδ-mediated pathways and/or PPARγ-mediated pathways (e.g., fatty acid storage). Depending on the particular foodstuff, the quantity to be ingested, the condition of the consumer, and the like, various profiles may be indicated for actual consumption.

In accordance with a still further aspect of the present invention, there are provided methods to induce fatty acid degradation in a subject, said methods comprising administering to a subject an effective amount of a PPARα ligand.

The identification of mammalian nuclear receptors with FA and eicosanoid ligands have a number of important implications. First, this establishes an important link between metabolism and transcriptional control. PPARα induces transcription of a number of gene products that contribute to the metabolism of FAs. These include enzymes necessary for the degradation of FAs through ß- and ω-oxidation pathways. It has long been established that metabolic intermediates modulate feedback control by promoting allosteric changes in enzymatic activity. The demonstration that FAs bind to PPARα provides direct evidence that metabolic intermediates can also regulate transcription. This complements the immediate effects of allosteric control by modulating the metabolic capacities of the organism over longer time periods.

Transcriptional control by metabolic intermediates has long been appreciated in bacteria and yeast. For example, the lac and trp repressors coordinately regulate transcription by binding to micromolar concentrations of allolactose and tryptophan, respectively (see Jobe & Bourgeois, in J Mol Biol **69**:397-408 (1972) and He & Matthews, in J Biol Chem **265**:731-737

(1990)). Similarities between the lac operon and PPARα-regulated transcription are particularly striking. In both cases, metabolic precursors (lactose/FAs) are converted to higher affinity inducers (allolactose/8S-HETE) which coordinately regulate the synthesis of enzymes required for the catabolism of the initial metabolites (lactose/FAs). The data presented herein establish that metabolite-controlled intracellular (metacrine) signaling systems are operative in higher organisms. The development of the LIC assay may facilitate the identification of other metacrine signals that function as micromolar ligands for other orphan nuclear receptors.

In accordance with the present invention, it has been shown that PPARα can recognize a broad array of ligands. This is unique among the nuclear receptors and suggests that PPARα senses broad changes in FA status and dietary inputs. In particular, as metabolism may vary from cell-to-cell and tissue-to-tissue, PPARα may act locally to integrate a variety of cell-specific metabolic parameters. In contrast to PPARα (which promotes FA catabolism), PPARγ appears to stimulate the opposing function of FA storage. The results presented herein demonstrate that PPARα ligands are distinct from those of PPARγ. The ability of these receptors to respond to distinct metabolic cues provides a potential mechanism for the animal to maintain a balance between FA breakdown and storage. Although a function for PPARδ remains to be established, it is of interest to note that this receptor recognizes a subset of PPARα ligands, suggesting that it may respond to similar endogenous signals. Thus, the overall balance between FA catabolism and storage may be determined by the relative levels PPARα/δ and PPARγ ligands.

It is demonstrated herein that 8S-HETE is a high affinity ligand for PPARα. The identification of this ligand in the skin (see, for example, Furstenberger et al., in J Biol Chem **266**:15738-15745 (1991) and Hughes & Brash in Biochim Biophys Acta **1081**:347-354 (1991)) suggests that it may play a specialized function in this tissue. In contrast to 8S-HETE, other eicosanoids were found which activate but fail to bind to PPAR (e.g. $PGA_1$ and PPARδ) (see Yu et al., in J Biol Chem **270**:23975-83 (1995) and Figs. 3A and 4A). By analogy to all-trans-retinoic acid, which binds to RXR after conversion to the active ligand (9-cis retinoic acid; see Mangelsdorf & Evans, supra), these eicosanoids may represent precursors to additional PPAR ligands. Thus, it is likely that additional eicosanoid ligands exist and that their production is regulated in a tissue-specific manner.

A previous report suggested that $LTB_4$ binds xenopus PPARα with an affinity of approximately 100 nM (see Devchand et al., in Nature **384**:39-43 (1996)). However, non-specific binding to PPARα was not accounted for and half-maximal displacement required 10-50 μM of unlabeled $LTB_4$. Since neither activation nor binding were detected with 10 μM $LTB_4$ (see Fig. 3A), it is unclear whether $LTB_4$ is a physiologically relevant ligand for mouse PPARα.

The ability to regulate FA pools is essential for normal homeostasis. Indeed, inappropriately high levels of triglycerides and non-esterified FAs are a common component of obesity, insulin resistance, hypertension and hyperlipidemia (see, for example, Durrington, supra and Reaven, supra). These abnormalities often develop in the same individual and are ominous signs of impending coronary heart disease, a major cause of death in industrialized societies. It has been proposed that increased levels of triglycerides and FAs are key factors in the progression of these disorders, suggesting that normalization of these parameters would provide an effective therapy. Indeed, it is well known that dietary PUFAs can be beneficial in this regard (see, for example, Willumsen et al., in Lipids

**28**:683-90 (1993) and Spady et al., in Annu Rev Nutr **13**:355-81 (1993)). This may reflect both activation of PPAR-regulated ß- and ω-oxidation pathways (see Green in Mutat Res **333**:101-9 (1995)) as well as PUFA-dependent suppression of lipogenic and glycolytic enzymes (see Jump et al., in J Lipid Res **35**:1076-84 (1994)).

A negative PUFA response element has been identified in the promoter of the pyruvate kinase gene (see Liimatta et al., in Mol Endocrinol **8**:1147-53 (1994)). This response element binds HNF-4, a constitutively active orphan nuclear receptor whose DNA-binding specificity overlaps that of PPAR-RXR heterodimers. It has previously been shown that PPARα antagonizes HNF-4 by down-regulating its expression in liver and by binding non-productively to HNF-4 response elements (see Hertz et al., in J Biol Chem **271**:218-24 (1996)). These observations, along with the demonstration herein that PUFAs promote the binding of PPARα/δ-RXRα heterodimers suggests that PUFAs may suppress transcription by displacing constitutively active HNF-4 and replacing it with an abortive PPARα/δ-RXRα complex. Thus, in addition to promoting ß- and ω-oxidation, PPARα and PPARδ may also inhibit lipogenesis. Taken together, these observations suggest that PPARα and PPARδ may directly mediate some of the beneficial effects of dietary PUFAs.

In addition to dietary factors, drugs of the fibrate class are also known to regulate transcription of apolipoproteins A-I, A-II and C-III (see Schoonjans et al., supra) and are useful for the treatment of hyperlipidemias. However, the effective doses of the best available drugs are in the high micromolar range. The demonstration herein that fibrates bind directly to PPARα indicates that screening for high affinity PPARα ligands employing the methods described herein provides a rapid approach for the development of more effective treatments for these lipid-related disorders. Since PPAR isoforms have distinct functions, the relative specificity of a drug for each PPAR isoform may be an important factor in evaluating its therapeutic potential.

In conclusion, the results presented herein confirm that PPARs play a central role in a signaling system that controls lipid homeostasis in higher organisms. As the number of orphan receptors continue to grow, it is likely that these proteins will provide important tools for the discovery of additional regulatory signals.

In accordance with yet another embodiment of the present invention, there are provided methods to modulate expression of PPARα-responsive genes in a biological system. Invention methods comprise contacting a biological system with an effective amount of a lipomodulatory agent, an optionally substituted long-chain mono-, di- or polycarboxylic acid containing at least one site of unsaturation, and the like.

As employed herein, the term "modulate" refers to the ability of a modulator for a member of the steroid/thyroid superfamily to either directly (by binding to the receptor as a ligand) or indirectly (as a precursor for a ligand or an inducer which promotes production of ligand from a precursor) induce expression of gene(s) maintained under hormone expression control, or to repress expression of gene(s) maintained under such control.

As employed herein, the phrase "PPARα-responsive genes" refers to genes whose expression products are involved in the biological, physiological, endocrinological, and other bodily processes which are mediated by receptor or receptor combinations which are responsive to the PPARα ligands described herein (e.g., genes involved in fatty acid metabolism in peroxisomes, mitochondria and other cellular compartments (including FA degradation (by ß- and ω-oxidation), and the like). Modulation of such processes can be accomplished <u>in vitro</u> or <u>in vivo</u>. <u>In vivo</u> modulation can be carried out in a wide range of subjects, such as, for example, humans, rodents, sheep, pigs, cows, and the like.

As employed herein, the phrase "biological system" refers to an intact organism or a cell-based system containing the various components required for response to the ligands described herein, e.g., an isoform of PPAR (i.e., PPARα, PPARδ or PPARγ), a silent partner for the PPAR isoform (e.g., RXRα), and a PPAR-responsive reporter (which typically comprises a PPAR response element (PPRE) in operative communication with a reporter gene; suitable reporters include luciferase, chloramphenicol transferase, ß-galactosidase, and the like).

As employed herein, the phrase "effective amount" refers to levels of compound sufficient to provide circulating concentrations high enough to modulate the expression of an isoform of PPAR. Such a concentration typically falls in the range of about 10 nM up to 2 μM; with concentrations in the range of about 100 nM up to 500 nM being preferred. Since the activity of different compounds described herein may vary considerably, and since individual subjects may present a wide variation in severity of symptoms, it is up to the practitioner to determine a subject's response to treatment and vary the dosages accordingly.

The above-described biologically active compounds can be administered in a variety of forms (e.g., in combination with a pharmaceutically acceptable carrier therefor) and by a variety of modes of delivery. Exemplary pharmaceutically acceptable carriers include carriers suitable for oral, intravenous, subcutaneous, intramuscular, intracutaneous, and the like administration.

Administration in the form of creams, lotions, tablets, dispersible powders, granules, syrups, elixirs, sterile aqueous or non-aqueous solutions, suspensions or emulsions, and the like, is contemplated.

For the preparation of oral liquids, suitable carriers include emulsions, solutions, suspensions, syrups, and the like, optionally containing additives such as wetting agents, emulsifying and suspending agents, sweetening, flavoring and perfuming agents, and the like.

For the preparation of fluids for parenteral administration, suitable carriers include sterile aqueous or non-aqueous solutions, suspensions, or emulsions. Examples of non-aqueous solvents or vehicles are propylene glycol, polyethylene glycol, vegetable oils, such as olive oil and corn oil, gelatin, and injectable organic esters such as ethyl oleate. Such dosage forms may also contain adjuvants such as preserving, wetting, emulsifying, and dispersing agents. They may be sterilized, for example, by filtration through a bacteria-retaining filter, by incorporating sterilizing agents into the compositions, by irradiating the compositions, or by heating the compositions. They can also be manufactured in the form of sterile water, or some other sterile injectable medium immediately before use.

In accordance with still another embodiment of the present invention, there are provided methods to modulate expression of PPARδ-responsive genes in a biological system. Invention methods comprise contacting a biological system with an effective amount of a lipomodulatory agent, an optionally substituted long-chain mono-, di- or polycarboxylic acid containing at least one site of unsaturation, and the like.

As employed herein, the phrase "PPARδ-responsive genes" refers to genes whose expression products are involved in the biological, physiological, endocrinological, and other bodily processes which are mediated by receptor or receptor combinations which are responsive to the PPARδ ligands described herein. Modulation of such processes can be accomplished in vitro or in vivo. In vivo modulation can be carried out in a wide range of subjects, such as, for example, humans, rodents, sheep, pigs, cows, and the like.

In accordance with yet another embodiment of the present invention, there are provided methods to modulate expression of PPARγ-responsive genes in a biological system. Invention methods comprise contacting a biological system with an effective amount of a lipomodulatory agent, an optionally substituted long-chain mono-, di- or polycarboxylic acid containing at least one site of unsaturation, and the like.

As employed herein, the phrase "PPARγ-responsive genes" refers to genes whose expression products are involved in the biological, physiological, endocrinological, and other bodily processes which are mediated by receptor or receptor combinations which are responsive to the PPAR-γ ligands described herein (e.g., cell differentiation to produce lipid-accumulating cells, regulation of insulin-sensitivity and blood glucose levels, especially as related to hypoglycemia/hyperinsulinism (resulting, for example, from abnormal pancreatic beta-cell function, insulin-secreting tumors and/or autoimmune hypoglycemia due to autoantibodies to insulin, the insulin receptor or autoantibodies that are stimulatory to pancreatic beta-cells), the formation of macrophages which lead to the development of atherosclerotic plaques, and the like). Modulation of such processes can be accomplished in vitro or in vivo. In vivo modulation can be carried out in a wide range of subjects, such as, for example, humans, rodents, sheep, pigs, cows, and the like.

The invention will now be described in greater detail by reference to the following non-limiting examples.

## Example 1
## Cell Culture and Transfection

CV-1 cells were grown and transfected as described by Forman et al., in Cell **83**:803-12 (1995). The reporter construct, PPREx3 TK-LUC, contained 3 copies of the acyl CoA oxidase PPRE upstream of the Herpes virus thymidine kinase promoter (see Kliewer et al., in Nature **358**:771-4 (1992)). Expression vectors contained the cytomegalovirus IE promoter/enhancer (pCMX) upstream of wild-type mouse PPARα, mouse PPARγ1-ΔN (Met$^{105}$-Tyr$^{475}$), mouse PPARδ-ΔN (Leu$^{69}$-Tyr$^{440}$), mouse PPARα-G (Glu$^{282}$-› Gly) (see Hsu et al., in Mol Pharmacol **48**:559-67 (1995)) or E. coli ß-galactosidase as an internal control. Cells were exposed to the compounds for 24 hours then harvested and assayed for luciferase and ß-galactosidase activity. All points were performed in triplicate and varied by less than 10%. Normalized luciferase activity was determined and plotted as fold-activation relative to untreated cells. Each experiment was repeated three or more times with similar results.

## Example 2
## Electrophoretic Mobility Shift Assays

In vitro translated mouse PPARα (0.2 μl) and human RXRα (0.1 μl) were incubated for 30 minutes at room temperature with 100,000 cpm of Klenow-labeled acyl CoA oxidase PPRE as described by Forman et al., in Cell **81**:687-93 (1995), but with 150 mM KCl.

## Example 3

## Hypolipidemic Drugs are PPARα Ligands

In order to evaluate the selectivity of PPARs toward hypolipidemic drugs, CV-1 cells were transiently transfected with a PPAR responsive reporter, PPAR expression vectors and then treated with various hypolipidemic agents (Fig. 1B). Wy 14,643 and BRL 49653 were included as positive controls since these compounds selectively activate PPAR α and γ, respectively (see Forman et al., in Cell **83**:803-12 (1995), Kliewer et al., in Cell **83**:813-9 (1995) and Kliewer et al., in Proc Natl Acad Sci USA **91**:7355-9 (1994)).

The hypolipidemic fibrates ciprofibrate and clofibrate activated PPARα maximally at 300 μM and exhibited only weak activity on PPARγ (Fig. 1B). Similar results were seen with gemfibrozil. In contrast, at 1 mM (i.e., the effective serum concentration of clofibrate; see Havel & Kane, in Annu Rev Pharmacol **13**:287-308 (1973)), all three drugs displayed significant activity (5-9 fold) on PPARγ. These compounds are ineffective activators of PPARδ (Fig. 1B), suggesting that hypolipidemic activity is mediated by PPARα and perhaps by PPARγ.

## Example 4

## Ligand Induced Complexation (LIC) Assay

It was next sought to determine whether the compounds found to have activity in the assays described in Example 3 are PPARα ligands. In the past, classical ligand binding assays have been used to identify ligands for other nuclear receptors. This approach has not been informative in the case of PPARα because radiolabeled ligands are either not available, or produce unacceptable levels of non-specific binding. To overcome these limitations, an assay was developed that does not utilize a labeled ligand, referred to herein as the "ligand induced complexation" or "LIC" assay. This assay relies on the ability of nuclear receptor ligands to induce conformational changes that promote dimerization and subsequent DNA binding.

Thus, for example, fibrates are observed to selectively promote binding of PPARα-RXRα heterodimers to labeled DNA in an electrophoretic mobility shift assay. Compounds were added at the following concentrations: 5 μM Wy 14,643, 100 μM ciprofibrate, 1000 μM clofibrate, 1 μM BRL 49653 and 1 μM LG268. Where excess receptor is employed, the amounts of PPARα and RXRα are increased to 0.6 μl and 0.5 μl, respectively. When included, 1 μl of antibody is added to the reaction.

Previous mobility shift assays have demonstrated that PPARα-RXR heterodimers bind to PPREs as obligate heterodimers even in the absence of ligand (see Kliewer et al., in Proc Natl Acad Sci USA **91**:7355-9 (1994)). Indeed, using standard conditions in which both receptors are present in excess, PPARα-RXRα heterodimers are readily observed by mobility shift assay. However, when both receptors are limiting, binding activity is minimal. This minimal binding activity is dramatically enhanced by the addition of Wy 14,643, ciprofibric or clofibric acids. This enhancement is unique to PPARα-activators; enhanced binding was not observed with PPARγ-specific ligands such as BRL 49653, pioglitazone and troglitazone or the RXR-specific ligands LG268, LG69 and 9-cis retinoic acid. PPARα and RXRα are verified to be components of the ligand induced complex by the observations that the complex is supershifted by PPARα-specific and RXRα-specific antibodies, but not by pre-immune serum. Similarly, epitope-tagged PPARα is supershifted by an epitope-specific monoclonal antibody (12CA5). Control experiments indicate that PPAR activators do not promote the DNA binding activity of an RXR homodimer, whereas the RXR homodimer is inducible by RXR-specific ligands. These experiments demonstrate that the ligand induced complex (LIC) assay represents a sensitive approach for the identification of novel ligands for orphan nuclear receptors.

To further validate the LIC assay, the dose response profiles of wild-type PPARα were compared to that of a previously characterized point mutant (PPARα-G; see Hsu et al., in Mol Pharmacol **48**:559-67 (1995)) that exhibits a decreased potency for PPARα activators in co-transfection experiments. As expected, the concentration required for half-maximal transcriptional activation by Wy 14,643 was 4-fold greater with the mutant receptor (Fig. 1C, left panel). In the LIC assay, phosphorimaging analysis revealed a similar increase in the amount of Wy 14,643 required for half-maximal ligand induced binding ($LIC_{50}$) with the mutant receptor (Fig. 1C, right panel). Thus, the $LIC_{50}$ for Wy 14,643 (600 nM) appears to provide an effective estimate of the actual dissociation constant. These data both confirm the validity of the LIC assay and provide evidence that hypolipidemic agents such as Wy 14,643, ciprofibrate and clofibrate are direct ligands for PPARα.

## Example 5

## Long-Chain FAs are PPARα Ligands

The LIC assay was utilized to determine which, if any, naturally occurring FAs bind to PPARα at physiologic concentrations. In the fasting state, the total concentration of non-esterified FAs in serum is approximately 700 μM (see Groop et al., in Am J Physiol **263**:E79-84 (1992)). Abundant dietary FAs such as linoleic and arachidonic acid have average concentrations of 25-30 μM and may reach much higher levels. The intracellular concentrations of these compounds are more difficult to determine but can be inferred from the Michaelis constant of long-chain fatty-acyl CoA synthetase (LC-FACS, 20 μM; see Tanaka et al., in Eur J Biochem **98**:165-72 (1979)). Thus, the ability of a variety of FAs to activate PPARα at 30 μM concentrations were examined.

When compared with Wy 14,643 in the co-transfection assay, saturated short chain FAs (< C10) were poor activators of PPARα, while longer chain FAs (C10-C16) possessed weak activity (Fig. 2A). Surprisingly, 30 μM doses of long-chain FAs (≥ C12) induced complex formation in the LIC assay (Fig. 2B). A carboxyl group is required for this activity since the corresponding fatty alcohols neither activated nor induced binding (Fig. 2A-B). These data indicate that long-chain FAs can bind weakly to PPARα.

The ability of PUFAs to bind to PPARα was examined next. It was found that linoleic, α-linolenic, γ-linolenic, arachidonic (Fig. 2A-B, right panel), docosahexaenoic and eicosapentaenoic acids all bind to and activate PPARα. In contrast, very-long-chain unsaturated FAs such as erucic and nervonic acids failed to bind or activate PPARα (Fig. 2A-B, right panel). This structure-activity relationship suggests that PPARα ligands can be broadly defined as long-chain monocarboxylic acids. Optimal binding activity is observed with compounds containing a 16-20 carbon chain length with several double bonds in the chain.

## Example 6

## Dual-Function PPARα Activators

The structural requirements for PPARα binding are reminiscent of the substrate specificity previously defined for LC-FACS (see Tanaka et al, supra), an intracellular enzyme that converts free FAs to their corresponding acyl-CoA thioesters. In addition to long-chain FAs, several hypolipidemic drugs are also converted to their acyl-CoA thioesters (see Bronfman et al., in Biochem J **284**:289-95 (1992), Aarsland & Berge, in Biochem Pharmacol **41**:53-61 (1991), and Wu & Bremer, in Biochim Biophys Acta **1215**:87-92 (1994)). Accordingly, the ligand binding properties of several long-chain FA-CoA thioesters were examined and found to be incapable of inducing binding in the LIC assay. This is consistent with the observation that a free carboxyl group is required for recognition by PPARα (Fig. 2A-B) and suggests that LC-FACS may inactivate PPARα ligands (see Hertz et al., in Eur J Biochem **221**:611-5 (1994)). To test this possibility, the transcriptional activity of PPARα was assayed in cells treated with triacsin C, an inhibitor of LC-FACS (see Tomoda et al., in Biochim Biophys Acta **921**:595-8 (1987)). Surprisingly, it was found that triacsin C itself activated PPARα (Fig. 2C, left panel) but failed to induce PPARα binding in the LIC assay (Fig. 2C, right panel). These observations are consistent with the hypothesis that inhibition of LC-FACS leads to the accumulation of an endogenous PPARα activator.

LC-FACS catalyzes the first step in the mitochondrial ß-oxidation cascade (Fig. 2C, left panel). Several groups have shown that inhibitors of subsequent steps in this pathway lead to activation of PPARα and peroxisome proliferation (see Gottlicher et al., in Biochem Pharmacol **46**:2177-84 (1993), Gulick et al., in Proc Natl Acad Sci USA **91**:11012-6 (1994), and Asiedu et al., in Biochim Biophys Acta **1166**:73-6 (1993)). This has contributed to the "lipid-overload" hypothesis which suggests that these inhibitors activate PPARα by promoting the accumulation of an endogenous ligand. However, since these enzymatic inhibitors are structural analogs of long-chain FAs, the possibility that they might also be PPARα ligands was addressed.

Consistent with previous results, inhibitors of carnitine palmitoyltransferase I (e.g., LY 171883, 2-bromopalmitate (2Br-C16), and tetradecylglycidic acid (TDGA); see Foxworthy & Eacho, in Biochem J **252**:409-14 (1988), Brady et al., in Biochem J **241**:751-7 (1987) and Kiorpes et al., in J Biol Chem **259**:9750-5 (1984)), as well as inhibitors of fatty acyl CoA dehydrogenase (e.g., octylthioproprionic acid (OTP), tetradecylthioproprionic acid (TTP), nonylthioacetic acid (NTA), and tetradecylthioacetic acid (TTA); see Hovik et al., in Biochem J **270**:167-73 (1990)) all activated PPARα (Fig. 2C, left panel). Surprisingly, the transcriptional activity of these peroxisome proliferators correlated with their ability to bind PPARα (Fig. 2C, right panel). Thus, these compounds represent dual-function activators. As ligands they activate PPARα directly; as metabolic inhibitors they may indirectly lead to the accumulation of endogenous FA ligands.

## Example 7

## PPARs are Nuclear Eicosanoid Receptors

The data generated herein employing the LIC assay indicate that long-chain FAs bind to PPARα at physiologic concentrations. Since these intermediary metabolites also serve as precursors to additional regulators, the possibility that downstream metabolites may also serve as PPARα ligands was also investigated. This line of thinking was prompted by the recent demonstration that the arachidonic acid metabolite 15d-$J_2$ is a ligand for the γ isoform of PPAR (see Forman et al., in Cell **83**:803-12 (1995)). Accordingly, studies were undertaken to determine whether other eicosanoids may be high affinity ligands for PPARα (Fig. 3A). Previous studies (see, for example, Yu et al., in J Biol Chem **270**:23975-83 (1995), Hertz et al., in Eur J Biochem **235**:242-247 (1996) and Brun et al., in Genes Dev **10**:974-84 (1996)) have shown that a number of prostanoids can activate PPARα (Fig. 3A, left panel). Importantly, when examined in the LIC assay, $PGI_2$ analogs such as carbaprostacyclin (cPGI) and iloprost act as ligands while cicaprost (a related analog, see Namba et al., in J Biol Chem **269**:9986-92 (1994)), is inactive (Fig. 3A, right panel). Thus, agonists for the cell-surface $PGI_2$ receptor exhibit a distinct pharmacologic hierarchy on PPARα. Furthermore, since CV-1 cells lack detectable levels of the $PGI_2$ receptor (see Hertz et al., in Eur J Biochem **235**:242-247 (1996)), it appears that this cell-surface pathway is not contributing to PPARα activation.

In searching for additional eicosanoid ligands, attention was focused on oxygenated FA derivatives and other products of lipoxygenase metabolism. While leukotriene $B_4$ ($LTB_4$) (see Devchand et al., in Nature **384**:39-43 (1996)) and other lipoxygenase products were poor or ineffective ligands (see Fig. 3A), 8S-hydroxyeicosatetraenoic acid (8S-HETE) was, as previously reported (see Yu et al., in J Biol Chem **270**:23975-83 (1995)), an effective activator of PPARα (Fig. 3A, left panel). Further structure-activity studies revealed that ±8-hydroxyeicosatrienoic acid (±8-HETrE) was significantly less effective whereas ±8-hydroxyeicosapentaenoic acid (±8-HEPE) was a slightly more effective activator (Fig. 3A, left panel). When examined in the LIC assay, ±8-HETE and ±8-HEPE both served as PPARα ligands (Fig. 3A, right panel). The stereochemistry around the 8-position was determined to be crucial since 8R-HETE was a poor ligand and a poor activator of PPARα (Fig. 3A). Dose response studies (Fig. 3B) revealed that 8S-HETE and cPGI activate with half-maximal activity at 200 nM and 2 μM, respectively (Fig. 3B, left panel) and bind PPARα with affinities estimated to be 100 nM and 500 nM, respectively (Fig. 3B, right panel). Thus, the naturally occurring 8S-HETE is the highest affinity ligand yet to be identified for PPARα.

The data in Fig. 3A indicate that certain compounds can activate PPARα without inducing complex formation in vitro. This could occur if these compounds represented inactive precursors which are metabolized to ligands. Alternatively, they could bind to PPARα without inducing a conformation change that promotes DNA binding. To rule out this possibility, PPARα-RXRα heterodimers were formed in the presence of Wy 14,643 and an excess of each compound that failed to induce complex formation. A compound that binds to PPARα without inducing complex formation would be expected to compete with Wy 14,643 thereby decreasing heterodimer formation. All of the compounds tested ($LTB_4$, BRL 49653, $PGA_1$, $PGA_2$, $PGB_2$, $PGD_2$, $PGE_2$, $PGF_{2\alpha}$, $PGI_2$, 15d-$J_2$ and cicaprost) were ineffective inhibitors of enhanced binding of Wy 14,643, suggesting that these compounds are not ligands for PPARα. Thus, PPARα activators such as $PGA_1$, $PGA_2$, $PGB_2$, $PGD_2$ and 15d-$J_2$ may be inactive precursors that are metabolized to PPARα ligands.

## Example 8

## PPARα and δ Possess Overlapping Ligand Specificities

Since ligands have not been discovered for PPARδ, it was decided to investigate whether FAs or eicosanoids may also bind to this receptor. At concentrations that were sufficient for activation of PPARα, a number of hypolipidemic agents, thiazolidinediones and saturated FAs failed to bind or activate PPARδ (see Figs. 1B and 4A). In contrast, several PUFAs and eicosanoids did activate PPARδ (Fig. 4A, left panel) and a subset of these (linoleic acid, arachidonic acid, cPGI and iloprost) acted as ligands in the LIC assay (Fig. 4A, right panel). Taken together, these data indicate that the PPARs comprise a family of nuclear FA and eicosanoid receptors.

Finally, the specificity of different activator classes for each member of the PPAR family was compared (Fig. 4B). Naturally occurring saturated long-chain FAs

(C12-C16) are weak activators of PPARα and even weaker activators of PPARδ. The dual function long-chain FAs (e.g., 2-bromopalmitate (2Br-C16) and tetradecylthioacetic acid (TTA)) preferentially activate PPARα over PPARδ. In contrast, PUFAs are efficient activators of PPARα and PPARδ, but display little activity on PPARγ. Among the eicosanoids, 8S-HETE was specific for PPARα, while $PGA_1$ preferentially activated PPARδ (see Yu et al., in J Biol Chem **270**:23975-83 (1995)). All three PPAR isoforms were responsive to 15d-$J_2$, whereas the synthetic eicosanoid cPGI selectively activated PPARα and PPARδ. These data indicate that PPARα, PPARγ and PPARδ are a family of nuclear receptors that possess distinct, yet overlapping ligand binding specificities.

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.

## SEQUENCE LISTING

SEQ ID NO:1

Cys - X - X - Cys - X - X - Asp* - X - Ala* -
X - Gly* - X - Tyr* - X - X - X - X - Cys - X
- X - Cys - Lys* - X - Phe - Phe - X - Arg* -
X - X - X - X - X - X - X - X - X - (X - X -)
Cys -X - X - X - X - X - (X -X - X -) Cys -X -
X - X - Lys - X - X - Arg - X - X -Cys - X -
X - Cys - Arg* - X - X - Lys* - Cys - X - X -X
- Gly* - Met

SEQ ID NO:2

AGGACA A AGGTCA

That which is claimed is:

1. A method to determine if a test compound is a ligand for a member of the nuclear receptor superfamily, said method comprising:

determining the binding activity of a homodimer or heterodimer containing said member with respect to a hormone response element (HRE) in the presence of said test compound, relative to the binding activity of said homodimer or heterodimer with respect to said HRE in the absence of said test compound.

2. A method according to claim 1 wherein said member of the nuclear receptor superfamily is an isoform of PPAR.

3. A method according to claim 2 wherein said isoform of PPAR is PPAR$\alpha$.

4. A method according to claim 3 wherein said PPAR$\alpha$ is present as a heterodimer comprising PPAR$\alpha$ and RXR$\alpha$.

5. A method according to claim 2 wherein said isoform of PPAR is PPAR$\delta$.

6. A method according to claim 5 wherein said PPAR$\delta$ is present as a heterodimer comprising PPAR$\delta$ and RXR$\alpha$.

7. A method according to claim 2 wherein said isoform of PPAR is PPAR$\gamma$.

8. A method according to claim 7 wherein said PPAR$\gamma$ is present as a heterodimer comprising PPAR$\gamma$ and RXR$\alpha$.

9. A method to determine if a test compound is a ligand for a member of the nuclear receptor superfamily, said method comprising:

contacting a homodimer or heterodimer containing said member and a hormone response element (HRE) with said test compound, and

monitoring for the formation of a complex comprising said homodimer or heterodimer, said HRE, and said test compound, wherein formation of said complex indicates that said test compound is a ligand for said member.

10. A method according to claim 9 wherein said member of the nuclear receptor superfamily is an isoform of PPAR.

11. A method to determine if a test compound is a ligand for a member of the nuclear receptor superfamily, said method comprising:

contacting said member and a dimeric partner therefor with said test compound, and

monitoring for the formation of a complex comprising said member, said dimeric partner therefor and said test compound, wherein formation of said complex indicates that said test compound is a ligand for said member.

12. A method according to claim 11 wherein said member of the nuclear receptor superfamily is an isoform of PPAR.

13. A method according to claim 11 wherein said dimeric partner for said member is RXR$\alpha$.

14. A method to monitor a fatty acid-containing foodstuff for the presence of beneficial fatty acids therein, said method comprising:

determining the binding activity of a PPARα-containing heterodimer with respect to a PPAR response element (PPRE) in the presence of the fatty acid(s) contained in said foodstuff, relative to the binding activity of said PPARα-containing heterodimer with respect to said PPRE in the absence of said fatty acid(s), wherein binding of said heterodimer to said PPRE is indicative of the presence of beneficial fatty acids in said foodstuff.

15. A method to monitor a fatty acid-containing foodstuff for the presence of beneficial fatty acids therein, said method comprising:

determining the ability of the fatty acid(s) contained in said foodstuff to activate a PPARα-responsive reporter in an assay system comprising:

PPARα,

RXRα, and

a PPARα-responsive reporter comprising a PPAR response element (PPRE) in operative communication with a reporter gene,

wherein expression of the reporter gene product is indicative of the presence of beneficial fatty acids in said foodstuff.

16. A method to characterize the profile of fatty acids in a fatty acid-containing foodstuff, said method comprising:

determining the quantity of binding of a PPARα-containing heterodimer, a PPARδ-containing heterodimer or a PPARγ-containing heterodimer, to a PPAR response element (PPRE) in the presence of the fatty acid(s) contained in said foodstuff, relative to the quantity of binding of said PPARα-containing heterodimer, said PPARδ-containing heterodimer or said PPARγ-containing heterodimer, respectively, to said PPRE in the absence of the fatty acid(s) contained in said foodstuff.

17. A method to characterize the profile of fatty acids in a fatty acid-containing foodstuff, said method comprising:

determining the ability of the fatty acid(s) contained in said foodstuff to activate a PPARα-responsive reporter, a PPARδ-responsive reporter or a PPARγ-responsive reporter, in an assay system comprising:

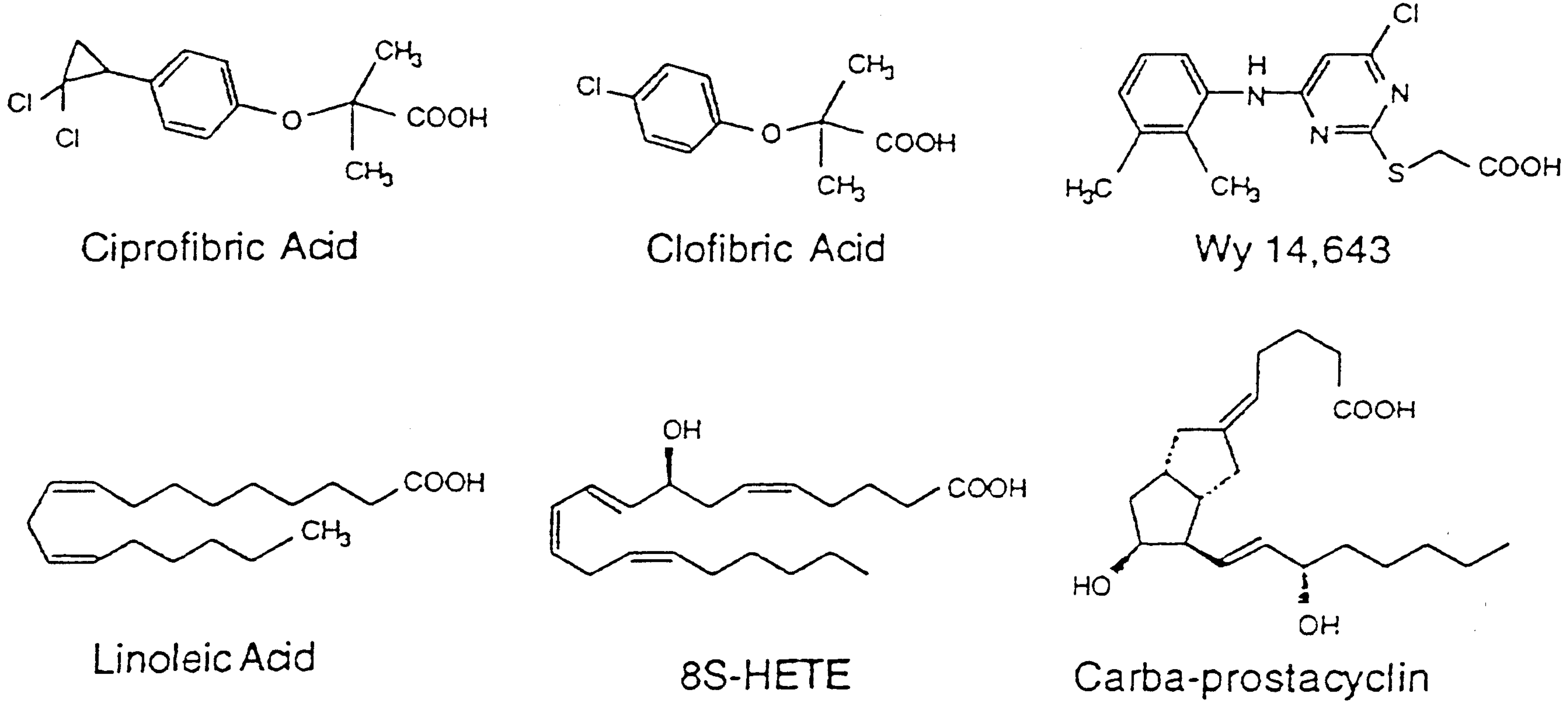
PPARα, PPARδ or PPARγ, respectively,

RXRα, and

a PPAR-responsive reporter comprising a PPAR response element (PPRE) in operative communication with a reporter gene,

wherein expression of the reporter gene product is indicative of the presence of fatty acids in said foodstuff which modulate a metabolic pathway mediated by PPARα, PPARδ and/or PPARγ.

18. A method to induce fatty acid degradation in a subject, said method comprising administering to said subject an effective amount of a PPARα ligand.



Ciprofibric Acid

Clofibric Acid

Wy 14,643

Linoleic Acid

8S-HETE

Carba-prostacyclin

Figure 1A

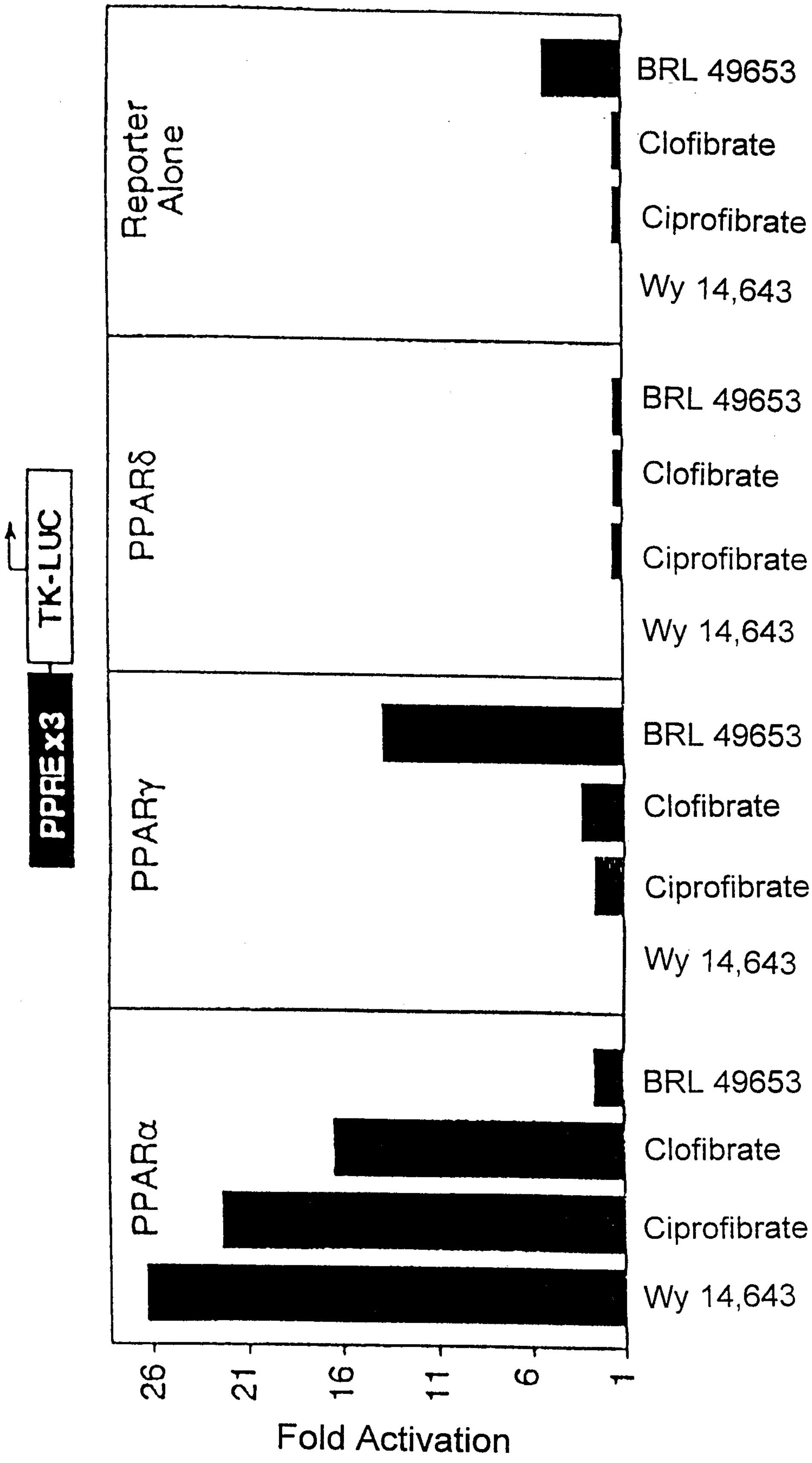

PPREx3
TK-LUC
Reporter Alone
PPARδ
PPARγ
PPARα
BRL 49653
Clofibrate
Ciprofibrate
Wy 14,643
BRL 49653
Clofibrate
Ciprofibrate
Wy 14,643
BRL 49653
Clofibrate
Ciprofibrate
Wy 14,643
BRL 49653
Clofibrate
Ciprofibrate
Wy 14,643
26
21
16
11
6
1
Fold Activation

Figure 1B

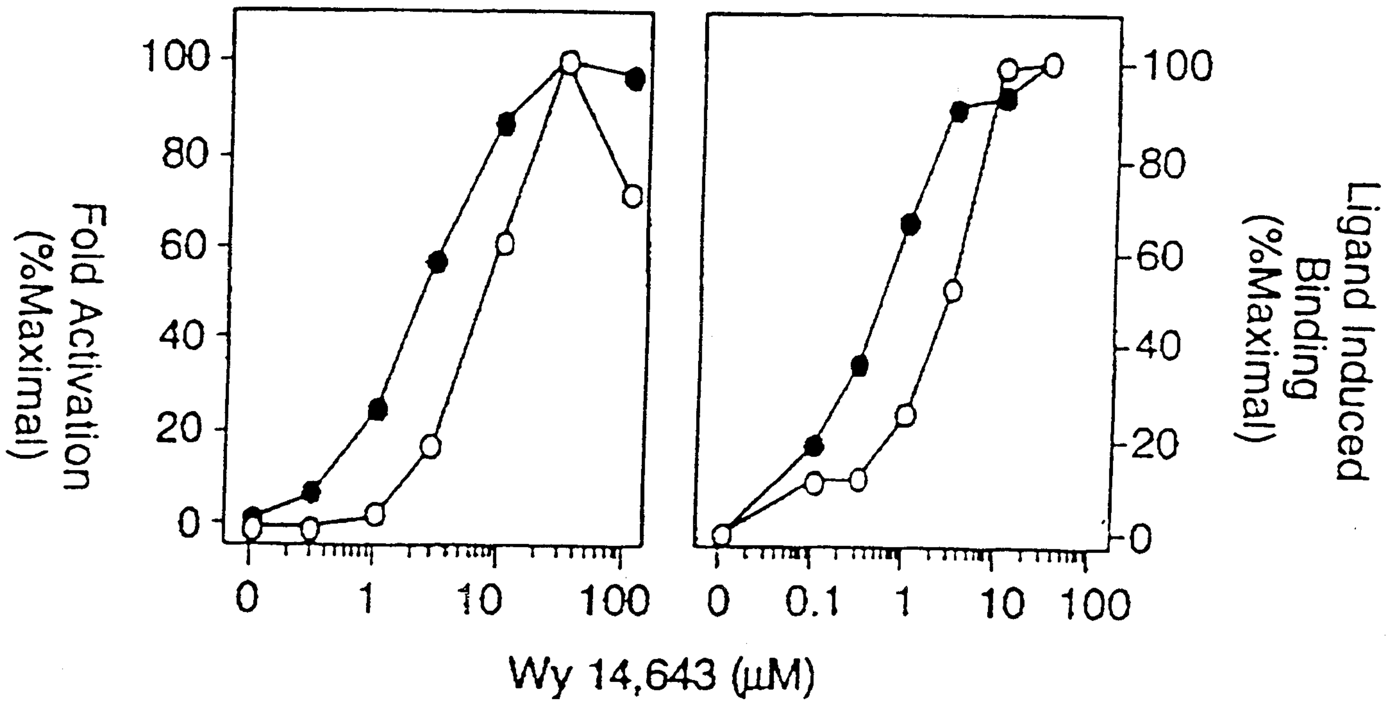

Fold Activation
(%Maximal)
100
80
60
40
20
0
0
1
10
100
0
0.1
1
10
100
Wy 14,643 (μM)
Ligand Induced
Binding
(%Maximal)
100
80
60
40
20
0

Figure 1C

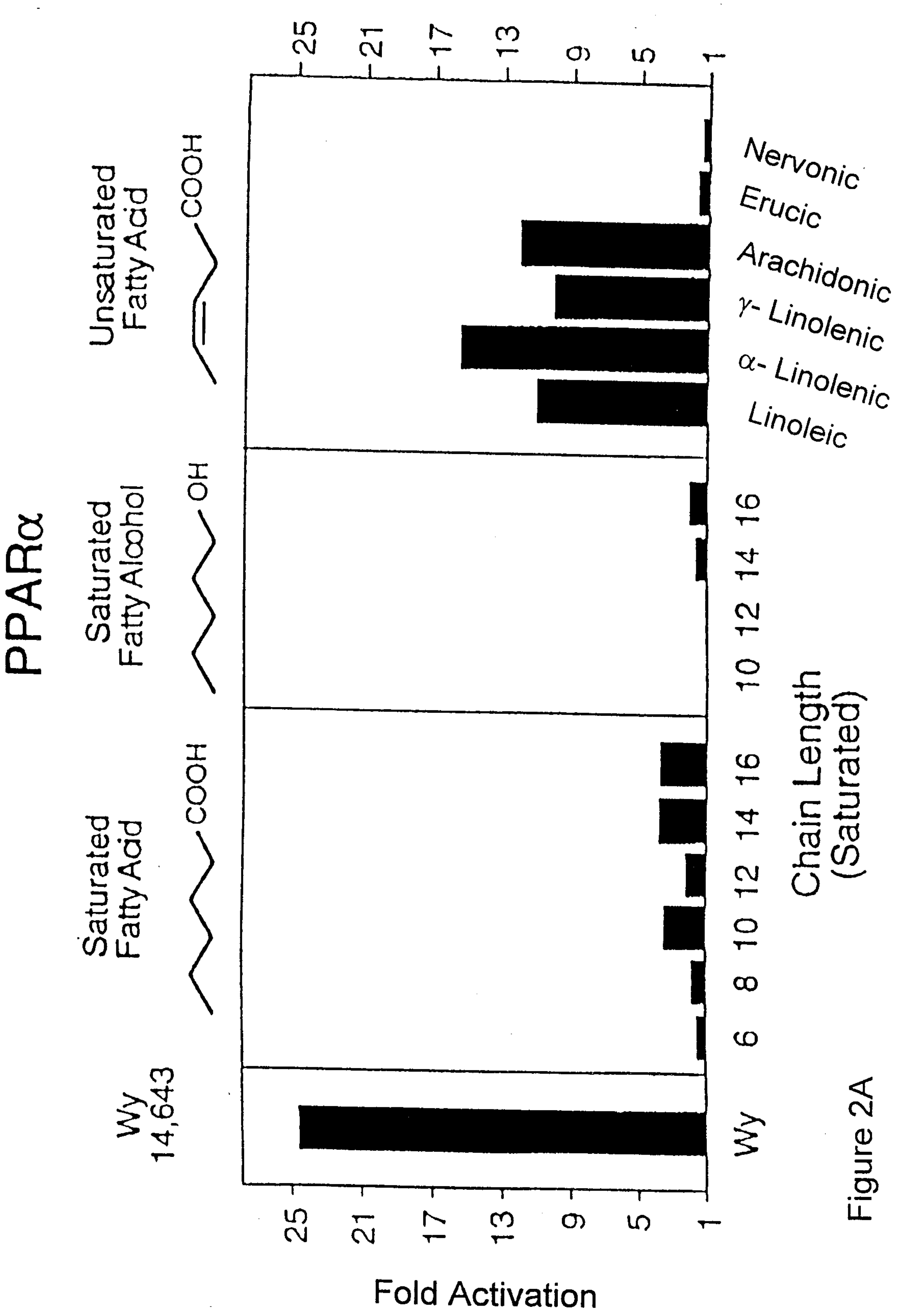

PPARα
Wy 14,643
Saturated Fatty Acid
COOH
Saturated Fatty Alcohol
OH
Unsaturated Fatty Acid
COOH
25
21
17
13
9
5
1
Wy
6
8
10
12
14
16
10
12
14
16
Linoleic
α- Linolenic
γ- Linolenic
Arachidonic
Erucic
Nervonic
Chain Length (Saturated)
Fold Activation

Figure 2A

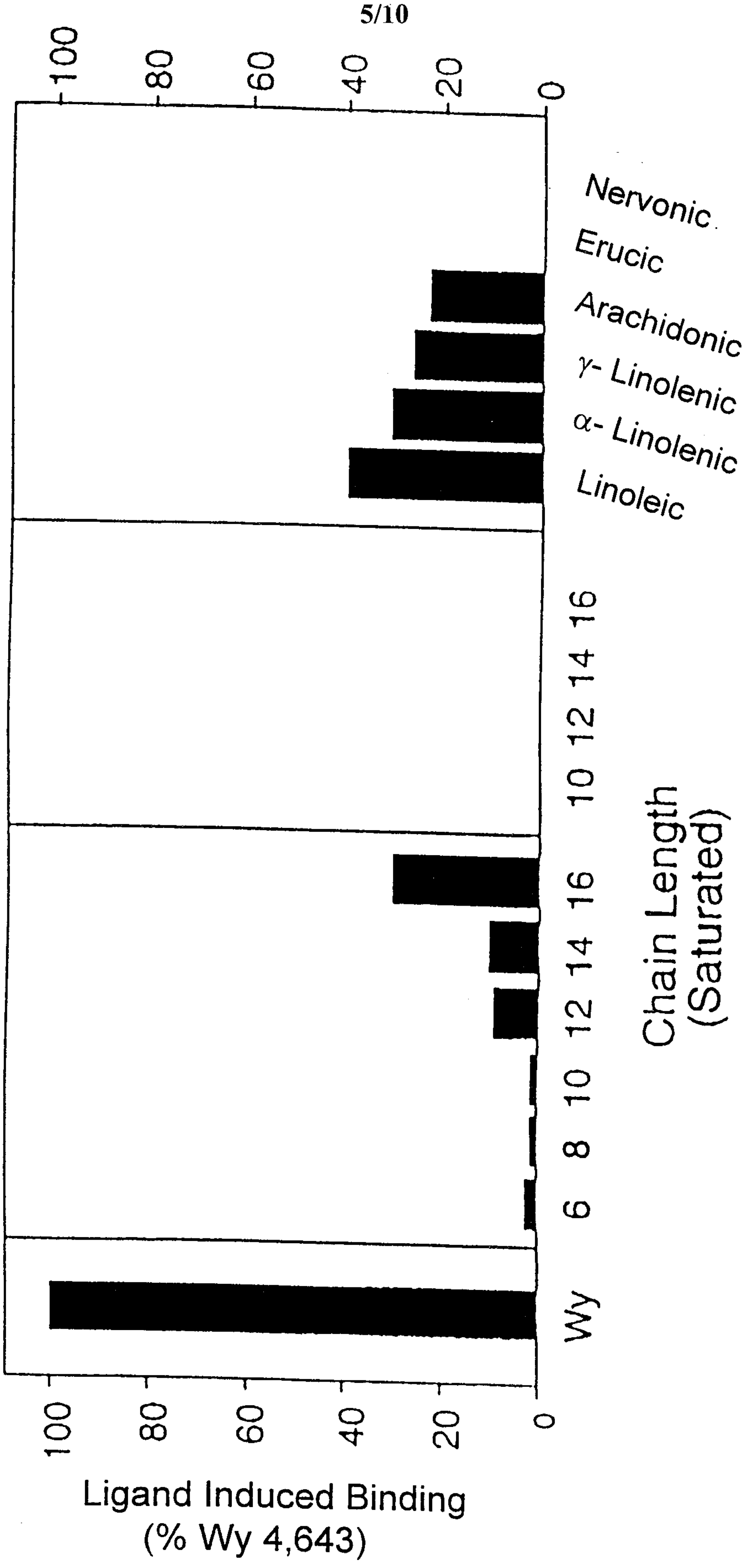

Ligand Induced Binding
(% Wy 4,643)
0
20
40
60
80
100
Wy
6
8
10
12
14
16
Chain Length
(Saturated)
10
12
14
16
Linoleic
α- Linolenic
γ- Linolenic
Arachidonic
Erucic
Nervonic

Figure 2B

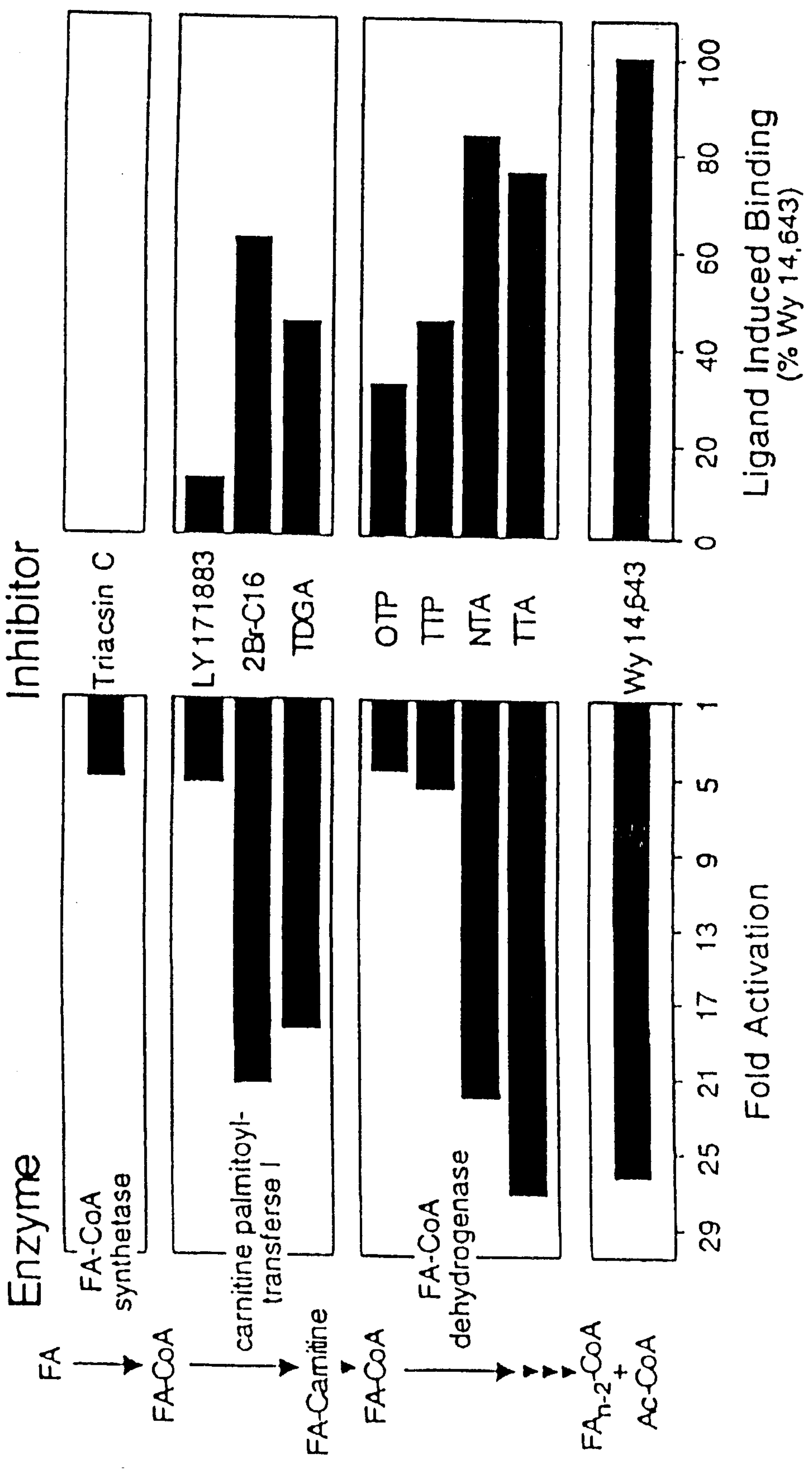

Enzyme
Inhibitor
FA
FA-CoA
FA-Carnitine
FA-CoA
FA$_{n-2}$-CoA
+
Ac-CoA
FA-CoA synthetase
carnitine palmitoyl-transferse I
FA-CoA dehydrogenase
Triacsin C
LY171883
2Br-C16
TDGA
OTP
TTP
NTA
TTA
Wy 14,643
1
5
9
13
17
21
25
29
Fold Activation
0
20
40
60
80
100
Ligand Induced Binding
(% Wy 14,643)

Figure 2C

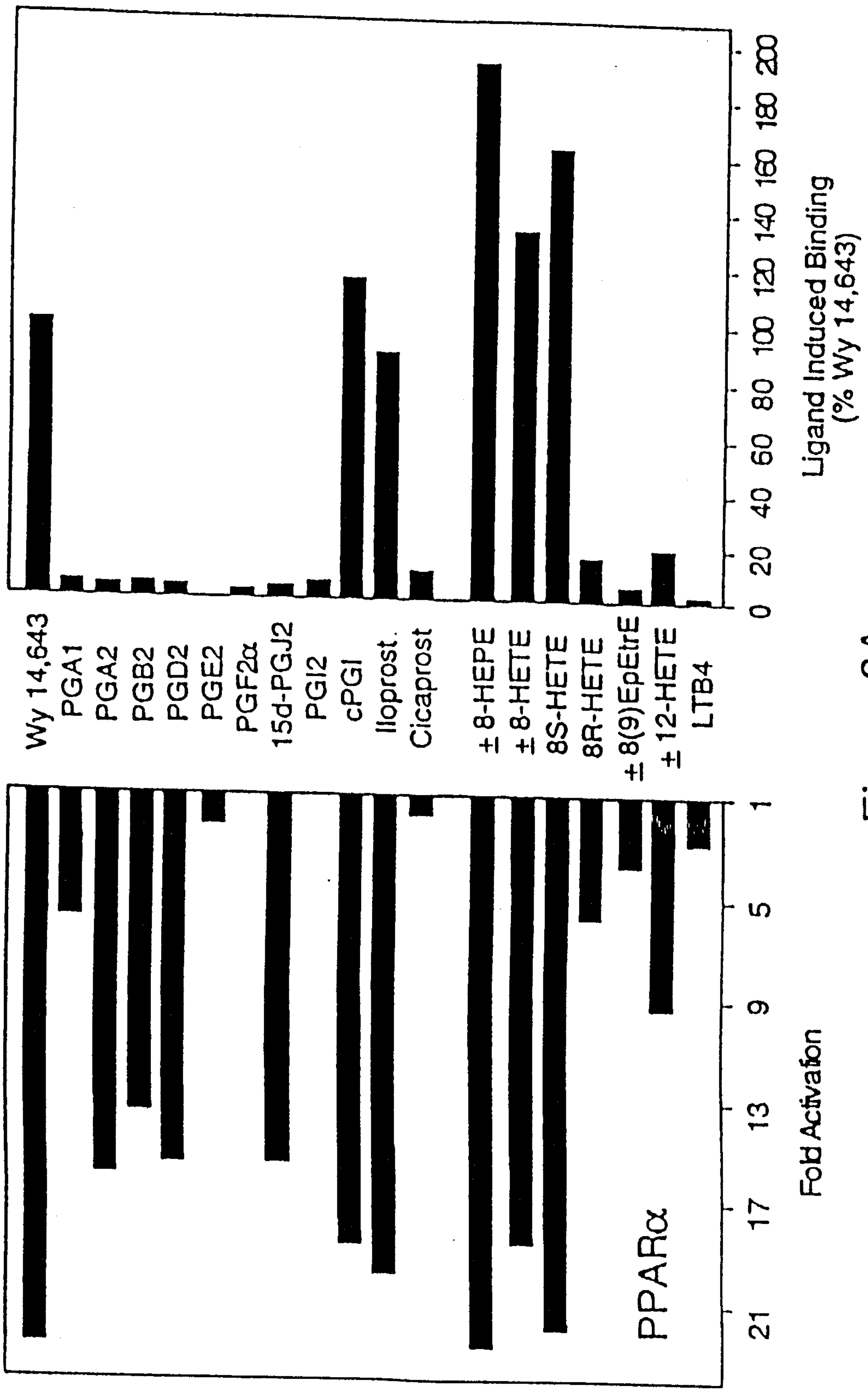

Wy 14,643
PGA1
PGA2
PGB2
PGD2
PGE2
PGF2α
15d-PGJ2
PGI2
cPGI
Iloprost.
Cicaprost
± 8-HEPE
± 8-HETE
8S-HETE
8R-HETE
± 8(9)EpEtrE
± 12-HETE
LTB4
0
20
40
60
80
100
120
140
160
180
200
Ligand Induced Binding
(% Wy 14,643)
PPARα
1
5
9
13
17
21
Fold Activation

Figure 3A

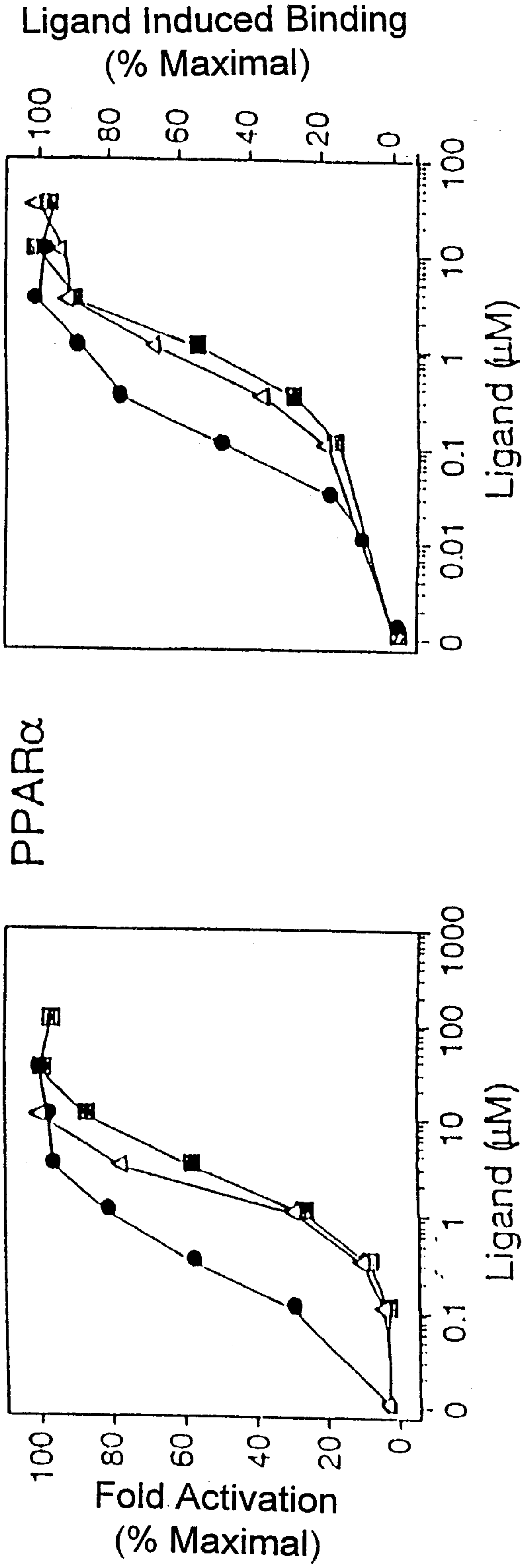

Ligand Induced Binding
(% Maximal)
100
80
60
40
20
0
0
0.01
0.1
1
10
100
Ligand (µM)
PPARα
Fold Activation
(% Maximal)
100
80
60
40
20
0
0
0.1
1
10
100
1000
Ligand (µM)

Figure 3B

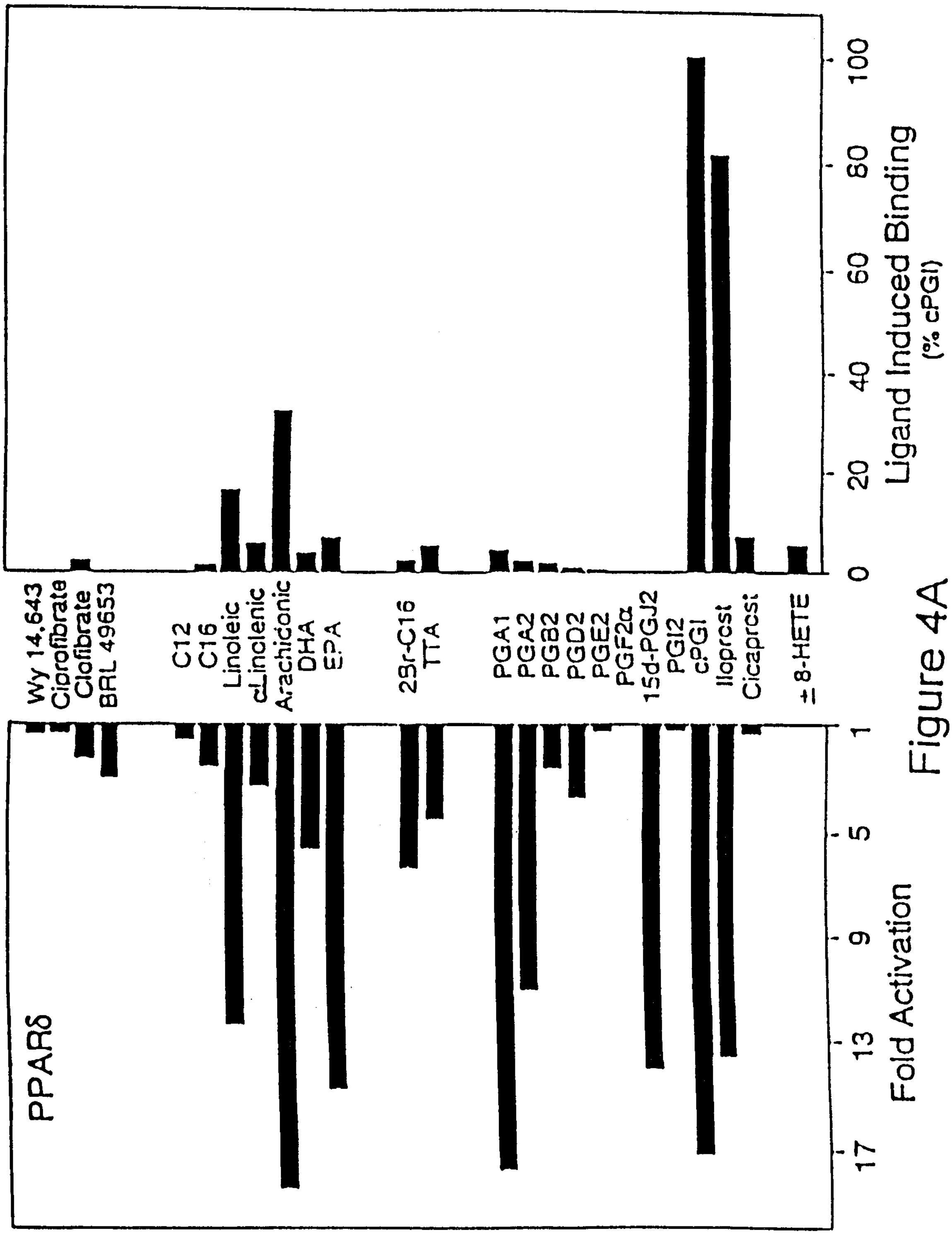

PPARδ
Wy 14,643
Ciprofibrate
Clofibrate
BRL 49653
C12
C16
Linoleic
α-Linolenic
Arachidonic
DHA
EPA
2Br-C16
TTA
PGA1
PGA2
PGB2
PGD2
PGE2
PGF2α
15d-PGJ2
PGI2
cPGI
Iloprost
Cicaprost
± 8-HETE
1
5
9
13
17
Fold Activation
0
20
40
60
80
100
Ligand Induced Binding
(% cPGI)

Figure 4A

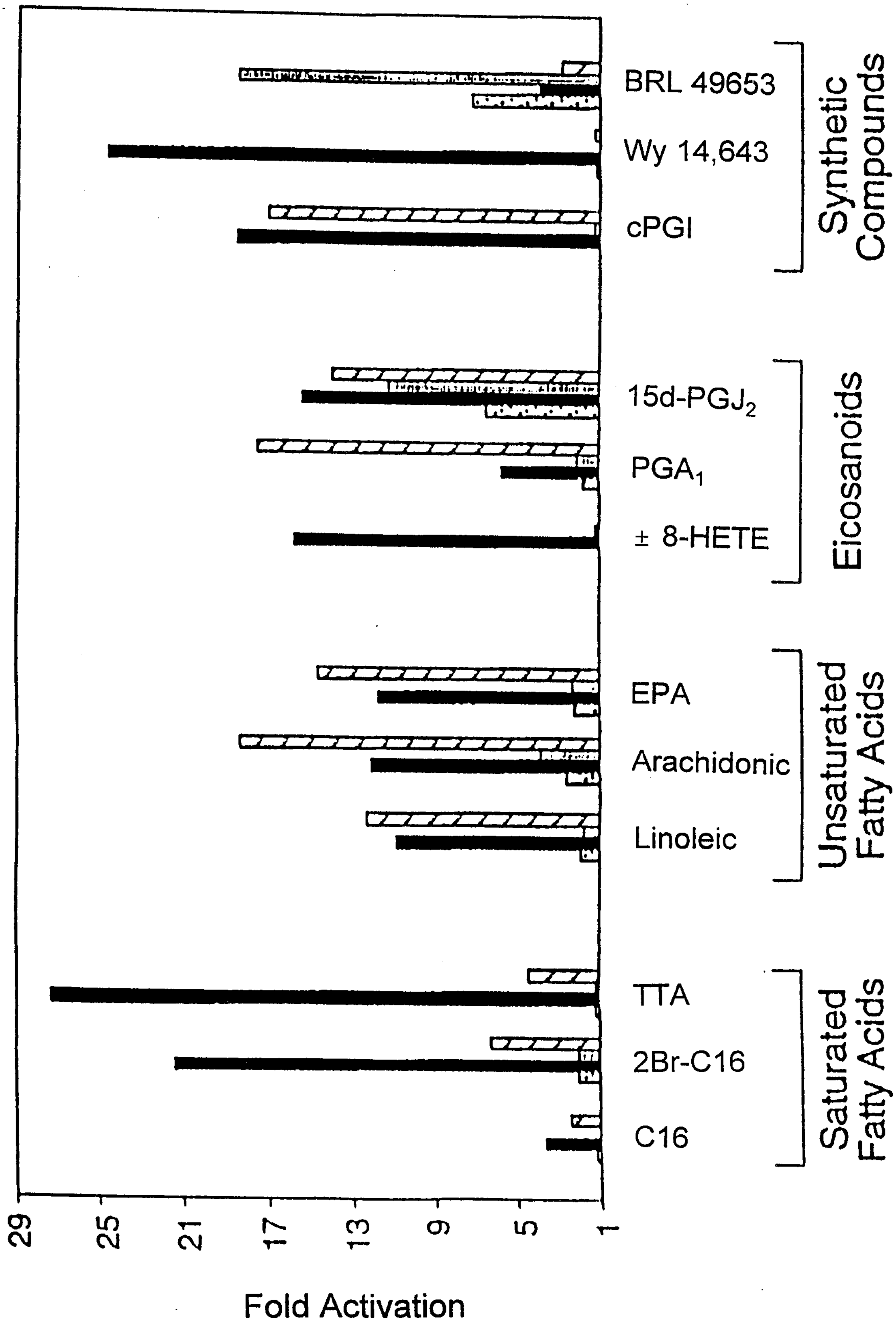

Fold Activation
29
25
21
17
13
9
5
1
BRL 49653
Wy 14,643
cPGI
Synthetic Compounds
15d-PGJ2
PGA1
± 8-HETE
Eicosanoids
EPA
Arachidonic
Linoleic
Unsaturated Fatty Acids
TTA
2Br-C16
C16
Saturated Fatty Acids

Figure 4B

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/06446

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/02, 33/03, 33/53, 33/566
US CL :435/7.8; 436/20
According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.8; 436/20

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br>-<br>Y | KLIEWER et al. Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. Proc. Natl. Acad. Sci. July 1994, Vol. 91, pages 7355-7359, see especially page 7357 and figures 3-4. | 1-13<br>------<br>14-18 |
| Y | SCHOONJANS et al. The peroxisome proliferator activated receptors (PPARs) and their effects on lipid metabolism and adipocyte differentiation. Biochimica et Biophysica Acta. 1996, Vol. 1302, pages 93-109, see especially pages 99 and 102. | 14-18 |

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

| | |
|---|---|
| * | Special categories of cited documents: |
| "A" | document defining the general state of the art which is not considered to be of particular relevance |
| "E" | earlier document published on or after the international filing date |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) |
| "O" | document referring to an oral disclosure, use, exhibition or other means |
| "P" | document published prior to the international filing date but later than the priority date claimed |
| "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "&" | document member of the same patent family |

Date of the actual completion of the international search

09 JUNE 1998

Date of mailing of the international search report

07 AUG 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer
Dorothea Lawrence for
MICHAEL D. PAK

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | SPADY et al. Regulation of plasma LDL-cholesterol levels by dietary cholesterol and fatty acids. Annu. Rev. Nutr. 1993, Vol. 13, pages 355-381, see especially pages 358-360. | 14-18 |

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/06446

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/06446

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, STN, BIOSCIENCE, MEDLINE, CAPLUS, WPIDS, BIOSIS, EMBASE, SCISEARCH

search terms: ppar?, peroxisom?(5a)receptor?, fatty acid, diet?, food

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-13, drawn to a method to determine if a test compound is a ligand.

Group II, claim(s) 14-18, drawn to a method to monitor a fatty acid containing foodstuff.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features because the method of claim 1 is anticipated by KLIEWER et al. and thus does not constitute a special technical feature as defined by PCT Rule 13.2.

The methods of group I and II do not share a special technical feature because the methods have materially different process steps and are practiced for materially different purposes, and each defines a separate invention over the art.