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(54) Title: PPAR-ALPHA-GAMMA LIGANDS OR AGONISTS FOR THE TREATMENT OF INFLAMMATION

(57) Abstract: The invention encompasses a method for treating or preventing an inflammatory disease or condition in a mammalian patient in need of such treatment or prevention comprising administering to said patient a compound that is capable of simultaneously binding PPARα and PPARγ or concomitantly administering a compound that selectively binds PPARα with a compound that selectively binds PPARγ in an amount that is effective to treat or prevent the inflammatory disease or condition. The invention also encompasses a method for treating or preventing an inflammatory disease or condition in a mammalian patient in need of such treatment or prevention comprising administering to said patient a compound that is capable of simultaneously binding and activating PPARα and PPARγ or concomitantly administering a compound that selectively binds and activates PPARα with a compound that selectively binds and activates PPARγ in an amount that is effective to treat or prevent the inflammatory disease or condition.
TITLE OF THE INVENTION
PPAR-ALPHA-GAMMA LIGANDS OR AGONISTS FOR THE TREATMENT OF INFLAMMATION

BACKGROUND OF THE INVENTION
A key step in the process of inflammation is the ingress of leukocytes into inflamed tissue. During this process, the affected tissue responds to the inflammatory insult by the elaboration of cytokines, chemotactic factors, and adhesion molecules. These molecules serve in the attraction of leukocytes. Simultaneously, leukocytes respond to these factors by adhesion, migration, and elaboration of further cytokines and chemotactic factors. It is thus clear that inflammation requires the response of both the target tissue and the leukocytes. This dual contribution is illustrated by the failure of inflammation to occur in animals deficient in leukocyte adhesion molecules (beta-2 integrins) or in animals deficient in the tissue expression of the counterreceptor for integrins, ICAM-1. This invention involves administration of an agent that binds to transcription factors both in tissues and in leukocytes.

Peroxisome proliferator-activated receptors (PPARs) are transcription factors belonging to the nuclear receptor supergene family. Three distinct PPARs, termed α, δ and γ, have been described. Each one is encoded by a separate gene.

PPARs are characterized by distinct tissue distribution patterns and metabolic functions.

PPARγ is a transcription factor expressed in adipose tissues, cells of the colon, and in tissue macrophages. Several studies indicate that ligands for PPARγ suppress the expression of proinflammatory molecules (Nature 391:82, Nature 391;79: Cell 93, 241; Cell 93; 229 (1998)) and may have anti-inflammatory action in vivo (JCI 104: 383, 1999). It is noteworthy that strong anti-inflammatory activity was observed in a model of colon inflammation and PPARγ is strongly expressed in the colon. PPARα is a homologous transcription factor with a distinct expression pattern being present in liver, monocytes, smooth muscle cells and other tissues.

Recent studies indicate that agonists of PPARα blunt production of pro-inflammatory cytokines (Nature 393: 790 (1998), Circulation 99: 3125 (1999)).

SUMMARY OF THE INVENTION
The invention encompasses a method for treating or preventing an inflammatory disease or condition in a mammalian patient in need of such treatment.
or prevention comprising administering to said patient a compound that is capable of simultaneously binding PPARα and PPARγ or concomitantly administering a compound that selectively binds PPARα with a compound that selectively binds PPARγ in an amount that is effective to treat or prevent the inflammatory disease or condition. The invention also encompasses a method for treating or preventing an inflammatory disease or condition in a mammalian patient in need of such treatment or prevention comprising administering to said patient a compound that is capable of simultaneously binding and activating PPARα and PPARγ or concomitantly administering a compound that selectively binds PPARα with a compound that selectively binds and activates PPARγ in an amount that is effective to treat or prevent the inflammatory disease or condition.

DETAILED DESCRIPTION OF THE INVENTION

The present invention encompasses a method for treating or preventing an inflammatory disease or condition in a mammalian patient in need of such treatment or prevention comprising administering to said patient a compound that is capable of simultaneously binding PPARα and PPARγ or concomitantly administering a compound that selectively binds PPARα with a compound that selectively binds PPARγ in an amount that is effective to treat or prevent the inflammatory disease or condition.

Compounds that are capable of simultaneously binding PPARα and PPARγ or “dual ligands” are defined as those compounds with half-maximal concentration potencies (IC50’s or KI’s) for displacement of radioligand binding to hPPARγ vs. hPPARα that differ by less than 30-fold as measured by the human PPARα and PPARγ binding assays described below. All other compounds binding to PPARα and/or PPARγ that fall outside this definition are considered compounds that selectively bind either PPARα or PPARγ for purposes of this specification.

An embodiment of the invention encompasses administering the compound that is capable of simultaneously binding PPARα and PPARγ.

An embodiment of the invention encompasses the instant method wherein the compound that is capable of simultaneously binding PPARα and PPARγ has a half-maximal concentration potency (IC50 or KI) for displacement of radioligand binding to hPPARγ vs. hPPARα that differs by less than 20-fold as measured by the human PPARα and PPARγ binding assays. Another embodiment is wherein the compound that is capable of simultaneously binding PPARα and PPARγ
has a half-maximal concentration potency (IC$_{50}$ or $K_l$) for displacement of radioligand binding to hPPAR$_\gamma$ vs. hPPAR$_\alpha$ that differs by less than 10-fold as measured by the human PPAR$_\alpha$ and PPAR$_\gamma$ binding assays. Another embodiment is wherein the compound that is capable of simultaneously binding PPAR$_\alpha$ and PPAR$_\gamma$ has a half-maximal concentration potency (IC$_{50}$ or $K_l$) for displacement of radioligand binding to hPPAR$_\gamma$ vs. hPPAR$_\alpha$ that differs by less than 5-fold as measured by the human PPAR$_\alpha$ and PPAR$_\gamma$ binding assays. Another embodiment is wherein the compound that is capable of simultaneously binding PPAR$_\alpha$ and PPAR$_\gamma$ has a half-maximal concentration potency (IC$_{50}$ or $K_l$) for displacement of radioligand binding to hPPAR$_\gamma$ vs. hPPAR$_\alpha$ differ by less than 2-fold as measured by the human PPAR$_\alpha$ and PPAR$_\gamma$ binding assays.

Another embodiment of the invention encompasses the instant method wherein the compound that is capable of simultaneously binding PPAR$_\alpha$ and PPAR$_\gamma$ is orally active. For purposes of this specification, compounds that are orally active means compounds that produce a therapeutic response following oral ingestion of the compound.

Another embodiment of the invention encompasses the instant method wherein the compound that is capable of simultaneously binding PPAR$_\alpha$ and PPAR$_\gamma$ possesses a long duration of action. For purposes of this specification, compounds that possess a long duration of action means compounds that have a half-life equal to or exceeding about 1 hour.

Another embodiment of the invention encompasses the instant method wherein the inflammatory disease or condition is inflammatory bowel syndrome.

Another embodiment of the invention encompasses the instant method wherein the inflammatory disease or condition is arthritis. Within this embodiment is encompassed the instant method wherein the inflammatory disease or condition is selected from the group consisting of: rheumatoid arthritis, ankylosing spondylitis, gout, psoriasis, osteoarthritis, and juvenile arthritis.

The compounds of the present invention that can be used in the treatment or prevention of an inflammatory disease or condition are compounds that have both PPAR$_\alpha$ and PPAR$_\gamma$ activity, which are defined in terms of PPAR agonism or in terms of binding to the PPAR receptors, partially displacing other compounds that are excellent PPAR ligands.
Another embodiment of the invention encompasses a method for treating or preventing an inflammatory disease or condition in a mammalian patient in need of such treatment or prevention comprising administering to said patient a compound that is capable of simultaneously binding and activating PPARα and PPARγ or concomitantly administering a compound that selectively binds and activates PPARα with a compound that selectively binds and activates PPARγ in an amount that is effective to treat or prevent the inflammatory disease or condition.

Compounds that are capable of simultaneously binding and activating PPARα and PPARγ or “dual PPARα/PPARγ agonists” are defined as those compounds that exhibit both significant PPARα and PPARγ agonism as well as half-maximal concentration potencies (EC50’s) for activation of hPPARγ vs. hPPARα that differ by less than 30-fold as measured by the cell-based transactivation assay or cell-free co-activator association assay, which are described below. Compounds that exhibit both significant PPARα and PPARγ agonism are those compounds exhibiting ≥50% of the maximal effects of rosiglitazone (on human PPARγ) and ≥50% of the maximal effects of fenofibrate (on human PPARα) on both receptors as measured by the cell-based transactivation assay or cell-free co-activator association assay. It is these compounds that also exhibit half-maximal concentration potencies (EC50’s) for activation of hPPARγ vs. hPPARα that differ by less than 30-fold that are considered “dual PPARα/PPARγ agonists” for purposes of this specification.

A compound that exhibits ≥50% of the maximal effects of rosiglitazone on human PPARγ but is outside the 30 fold activation difference described above is considered a compound that selectively binds and activates PPARγ. Rosiglitazone is an example of such a compound. Likewise, a compound that exhibits ≥50% of the maximal effects of fenofibrate on human PPARα but is outside the 30 fold activation difference described above is considered a compound that selectively binds and activates PPARα. Fenofibrate is an example of such a compound.

An embodiment of the invention encompasses administering the compound that is capable of simultaneously binding and activating PPARα and PPARγ.

Within this embodiment of the invention is encompassed the above method wherein the compound that is capable of simultaneously binding and activating PPARα and PPARγ has a half-maximal concentration potency (EC50) for activation of hPPARγ vs. hPPARα that differs by less than 20-fold as measured by the cell-based transactivation assay or cell-free co-activator association assay. Also
within this embodiment is encompassed the above method wherein the compound that is capable of simultaneously binding and activating PPARα and PPARγ has a half-maximal concentration potency (EC50) for activation of hPPARγ vs. hPPARα that differs by less than 10-fold as measured by the cell-based transactivation assay or cell-free co-activator association assay. Also within this embodiment is encompassed the above method wherein the compound that is capable of simultaneously binding and activating PPARα and PPARγ has a half-maximal concentration potency (EC50’s) for activation of hPPARγ vs. hPPARα that differs by less than 5-fold as measured by the cell-based transactivation assay or cell-free co-activator association assay. Also within this embodiment is encompassed the above method wherein the compound that is capable of simultaneously binding and activating PPARα and PPARγ has a half-maximal concentration potency (EC50’s) for activation of hPPARγ vs. hPPARα differ by less than 2-fold as measured by the cell-based transactivation assay or cell-free co-activator association assay.

Within this embodiment of the invention is also encompassed the above method wherein the compound that is capable of simultaneously binding and activating PPARα and PPARγ is orally active. Also within this embodiment of the invention is encompassed the above method wherein the compound that is capable of simultaneously binding and activating PPARα and PPARγ possesses a long duration of action.

Also within this embodiment of the invention is encompassed the above method wherein the inflammatory disease or condition is inflammatory bowel syndrome.

Also within this embodiment of the invention is encompassed the above method wherein the inflammatory disease or condition is arthritis. Within this group is encompassed the above method wherein the inflammatory disease or condition is selected from the group consisting of: rheumatoid arthritis, ankylosing spondylitis, gout, psoriasis, osteoarthritis, and juvenile arthritis.

For purposes of this specification “concomitantly administering” means administering one compound by a route and in an amount commonly used therefor, contemporaneously or sequentially with another compound. When compounds are referred to as administered concomitantly, a pharmaceutical composition in unit dosage form containing the two drugs is preferred.

For purposes of this specification, a “PPARα agent” means a compound that selectively binds or binds and activates PPARα as defined above.
“PPARγ agent” means a compound that selectively binds or binds and activates PPARγ.

Examples of compounds that are capable of simultaneously binding or simultaneously binding and activating PPARα and PPARγ, as well as examples of selective PPARα agents and selective PPARγ agents, are found in the following patents and published applications: WO 97/28115 published on August 7, 1997; WO 00/78312 published on December 28, 2000; WO 00/78313 published on December 28, 2000; U.S. No. 5,847,008 granted on December 8, 1998; U.S. No. 5,859,051 granted on January 12, 1999; U.S. No. 6,008,237 granted on December 28, 1999; U.S. No. 6,090,836 granted on July 18, 2000; U.S. No. 6,090,839 granted on July 18, 2000; U.S. No. 6,160,000 granted on December 12, 2000; and U.S. No. 6,200,998 granted on March 13, 2001, all of which are hereby incorporated by reference in their entirety.

Utilities

Compounds that are capable of simultaneously binding or simultaneously binding and activating PPARα and PPARγ, or the concomitant use of selective PPARα and PPARγ ligands/agonists, are useful for the treatment of inflammatory diseases or conditions. For example, the present invention encompasses the treatment of arthritis, including but not limited to rheumatoid arthritis, ankylosing spondylitis, gout, psoriasis, osteoarthritis, systemic lupus erythematosus and juvenile arthritis. The invention also includes the treatment of: asthma, bronchitis, menstrual cramps, tendinitis, bursitis, and skin related conditions such as psoriasis, eczema, burns and dermatitis; gastrointestinal conditions such as inflammatory bowel disease, Crohn’s disease, gastritis, irritable bowel syndrome and ulcerative colitis; inflammation in such diseases as vascular diseases, migraine headaches, periarthritis nodosa, thyroiditis, aplastic anemia, Hodgkin’s disease, sclerodema, rheumatic fever, vasculitis, systemic lupus erythematosus (SLE), Alzheimer’s disease, atherosclerosis, acute respiratory distress syndrome (ARDS), myasthenia gravis, multiple sclerosis, sarcoidosis, nephrotic syndrome, Behcet’s syndrome, polymyositis, gingivitis, hypersensitivity, swelling occurring after injury, and myocardial ischemia; ophthalmic diseases, such as retinitis, retinopathies, conjunctivitis, uveitis, ocular photophobia, and of acute injury to the eye tissue; and the treatment of pulmonary inflammation, such as that associated with viral infections and cystic fibrosis.
Pharmaceutical Compositions

The pharmaceutical compositions of the present invention comprise a compound that is capable of simultaneously binding or simultaneously binding and activating PPARα and PPARγ, or a combination of a selective PPARα agent with a selective PPARγ agent, as an active ingredient or a pharmaceutically acceptable salt thereof, and may also contain a pharmaceutically acceptable carrier and optionally other therapeutic ingredients. The term "pharmaceutically acceptable salts" refers to salts prepared from pharmaceutically acceptable non-toxic bases or acids including inorganic bases or acids and organic bases or acids.

The term "composition", as in pharmaceutical composition, is intended to encompass a product comprising the active ingredient(s), and the inert ingredient(s) that make up the carrier, as well as any product which results, directly or indirectly, from combination, complexation or aggregation of any two or more of the ingredients, or from dissociation of one or more of the ingredients, or from other types of reactions or interactions of one or more of the ingredients. Accordingly, the pharmaceutical compositions of the present invention encompass any composition made by admixing a compound of the present invention and a pharmaceutically acceptable carrier.

The compositions include compositions suitable for oral, rectal, topical, parenteral (including subcutaneous, intramuscular, and intravenous), ocular (ophthalmic), pulmonary (nasal or buccal inhalation), or nasal administration, although the most suitable route in any given case will depend on the nature and severity of the conditions being treated and on the nature of the active ingredient. They may be conveniently presented in unit dosage form and prepared by any of the methods well-known in the art of pharmacy.

In practical use, the present compounds can be combined as the active ingredient in intimate admixture with a pharmaceutical carrier according to conventional pharmaceutical compounding techniques. The carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g., oral or parenteral (including intravenous). In preparing the compositions for oral dosage form, any of the usual pharmaceutical media may be employed, such as, for example, water, glycols, oils, alcohols, flavoring agents, preservatives, coloring agents and the like in the case of oral liquid preparations, such as, for example, suspensions, elixirs and solutions; or carriers such as starches, sugars, microcrystalline cellulose, diluents, granulating agents, lubricants, binders, disintegrating agents and the like in the case of oral solid preparations such as, for example, powders, hard and soft
capsules and tablets, with the solid oral preparations being preferred over the liquid preparations.

Because of their ease of administration, tablets and capsules represent the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are obviously employed. If desired, tablets may be coated by standard aqueous or nonaqueous techniques. Such compositions and preparations should contain at least 0.1 percent of active compound. The percentage of active compound in these compositions may, of course, be varied and may conveniently be between about 2 percent to about 60 percent of the weight of the unit. The amount of active compound in such therapeutically useful compositions is such that an effective dosage will be obtained. The active compounds can also be administered intranasally as, for example, liquid drops or spray.

The tablets, pills, capsules, and the like may also contain a binder such as gum tragacanth, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin. When a dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier such as a fatty oil.

Various other materials may be present as coatings or to modify the physical form of the dosage unit. For instance, tablets may be coated with shellac, sugar or both. A syrup or elixir may contain, in addition to the active ingredient, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and a flavoring such as cherry or orange flavor.

The present compounds may also be administered parenterally.

Solutions or suspensions of these active compounds can be prepared in water suitably mixed with a surfactant such as hydroxy-propylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols and mixtures thereof in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier
can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (e.g. glycerol, propylene glycol and liquid polyethylene glycol), suitable mixtures thereof, and vegetable oils.

5 **Salts**

The term "pharmaceutically acceptable salts" refers to salts prepared from pharmaceutically acceptable non-toxic bases or acids including inorganic or organic bases and inorganic or organic acids. Salts derived from inorganic bases include aluminum, ammonium, calcium, copper, ferric, ferrous, lithium, magnesium, manganic salts, manganous, potassium, sodium, zinc, and the like. Particularly preferred are the ammonium, calcium, magnesium, potassium, and sodium salts. Salts in the solid form may exist in more than one crystal structure, and may also be in the form of hydrates. Salts derived from pharmaceutically acceptable organic non-toxic bases include salts of primary, secondary, and tertiary amines, substituted amines including naturally occurring substituted amines, cyclic amines, and basic ion exchange resins, such as arginine, betaine, caffeine, choline, N,N'-dibenzylethylendiamine, diethylamine, 2-diethylaminoethanol, 2-dimethylaminoethanol, ethanolamine, ethylenediamine, N-ethyl-morpholine, N-ethylpiperidine, glucamine, glucosamine, histidine, hydramine, isopropylamine, lysine, methylglucamine, morpholine, piperazine, piperidine, polyamine resins, procaine, purines, theobromine, triethylamine, trimethylamine, tripropylamine, tromethamine, and the like.

When the compound of the present invention is basic, salts may be prepared from pharmaceutically acceptable non-toxic acids, including inorganic and organic acids. Such acids include acetic, benzenesulfonic, benzoic, camphorsulfonic, citric, ethanesulfonic, fumaric, gluconic, glutamic, hydrobromic, hydrochloric, isethionic, lactic, maleic, malic, mandelic, methanesulfonic, mucic, nitric, pamoic, pantothenic, phosphoric, succinic, sulfuric, tartaric, p-toluene sulfonic acid, and the like. Particularly preferred are citric, hydrobromic, hydrochloric, maleic, phosphoric, sulfuric, and tartaric acids.

It will be understood that, as used herein, references to compounds capable of simultaneously binding or simultaneously binding and activating PPARα and PPARγ are meant to also include the pharmaceutically acceptable salts thereof. Likewise, references to PPARα agents or PPARγ agents are meant to include pharmaceutically acceptable salts thereof.
Optical Isomers - Diastereomers - Geometric Isomers - Tautomers

The compounds of the present invention may contain one or more asymmetric centers and can thus occur as racemates and racemic mixtures, single enantiomers, diastereomeric mixtures and individual diastereomers. The present invention is meant to comprehend all such isomeric forms.

The compounds encompassed by the present invention may contain olefinic double bonds, and unless specified otherwise, are meant to include both E and Z geometric isomers.

The compounds encompassed by the present invention may exist with different points of attachment of hydrogen, referred to as tautomers. Such an example may be a ketone and its enol form, known as keto-enol tautomers. The individual tautomers as well as mixtures thereof are encompassed with compounds of Formula II and IIa.

The compounds encompassed by the present invention may be separated into diastereoisomeric pairs of enantiomers by, for example, fractional crystallization from a suitable solvent, for example methanol or ethyl acetate or a mixture thereof. The pair of enantiomers thus obtained may be separated into individual stereoisomers by conventional means, for example by the use of an optically active acid as a resolving agent.

Alternatively, any enantiomer of the compounds of the present invention may be obtained by stereospecific synthesis using optically pure starting materials or reagents of known configuration.

Administration and Dose Ranges

Any suitable route of administration may be employed for providing a mammal, and especially a human, with an effective dosage of the present compounds for the treatment of inflammatory diseases or conditions. For example, oral, rectal, topical, parenteral, ocular, pulmonary, nasal, and the like may be employed. Dosage forms include tablets, troches, dispersions, suspensions, solutions, capsules, creams, ointments, aerosols, and the like. Preferably the compounds are administered orally.

The effective dosage of the active ingredient employed may vary depending on the particular compound employed, the mode of administration, the
condition being treated and the severity of the condition being treated. Such dosage may be ascertained readily by a person skilled in the art.

When treating or preventing inflammatory diseases or conditions generally satisfactory results are obtained when the compound is administered at a daily dosage of from about 0.1 milligram to about 100 milligram per kilogram of animal body weight, preferably given as a single daily dose or in divided doses two to six times a day, or in sustained release form. For most large mammals, the total daily dosage is from about 1.0 milligrams to about 1000 milligrams, preferably from about 1 milligrams to about 50 milligrams. In the case of a 70 kg adult human, the total daily dose will generally be from about 7 milligrams to about 350 milligrams. This dosage regimen may be adjusted to provide the optimal therapeutic response.

**Combination Therapy**

The compounds of the present invention for use in treating or preventing inflammatory diseases or conditions may be used in combination with other drugs for the treatment or prevention of inflammatory diseases or conditions. Such other drugs may be administered, by a route and in an amount commonly used therefor, contemporaneously or sequentially with a compound of the present invention. When a compound of the present invention is used contemporaneously with one or more other drugs, a pharmaceutical composition in unit dosage form containing such other drugs and the compound capable of simultaneously binding or simultaneously binding and activating PPARα and PPARγ, or the combination of a selective PPARα agents with a selective PPARγ agents with such other drug, is preferred. It is also contemplated that when used in combination with one or more other active ingredients, the compound of the present invention and the other active ingredients may be used in lower doses than when each is used singly. Accordingly, the pharmaceutical compositions of the present invention include those that contain one or more other active ingredients, in addition to a compound of the present invention.

Examples of other active ingredients that may be combined with the present compounds for treating an inflammatory disease or condition, either administered separately or in the same pharmaceutical compositions, include, but are not limited to:

1. a salicylate, including acetylsalicylic acid,
2. a non-steroidal anti-inflammatory drug, including indomethacin, sulindac, mefenamic, meclofenamic, tolfenamic, tolmetin,
ketorolac, dicofenac, ibuprofen, naproxen, fenoprofen, ketoprofen, flurbiprofen and oxaprozin,
(3) a cyclooxygenase-2 selective inhibitor such as rofecoxib, etoricoxib, celecoxib, parecoxib or valdecoxib,
5 a corticosteroid, including dexamethasone and prednisolone
(4) a TNF inhibitor, including etanercept and infliximab,
(5) an IL-1 receptor antagonist,
(6) a cytotoxic or immunosuppressive drug, including methotrexate, leflunomide, azathioprine and cyclosporine,
10 a gold compound,
(8) hydroxychloroquine or sulfasalazine,
(9) penicillamine,
(10) darbufelone,
(11) a p38 kinase inhibitor,
(12) an integrin αv antagonist, including the compounds disclosed in U.S. Patent No. 6,017,926 and U.S. Patent No. 6,048,861,
15 and
(13) a prostaglandin receptor ligand, such as the compounds disclosed in WO 00/20398, WO 99/47497 and WO 00/20371.

Biological Assays

Standardized Cell-Based GAL4 Chimeric Receptor Transactivation Assay (Cell-Based Transactivation Assay)


Expression constructs are prepared by inserting cDNA sequences encoding the ligand binding domains of human PPARγ or PPARα adjacent to the yeast GAL4 transcription factor DNA binding domain in the mammalian expression vector pcDNA3 to create pcDNA3-hPPARγ/GAL4 and pcDNA3-hPPARα/GAL4, respectively. The GAL4-responsive reporter construct, pUAS(5X)-tk-luc, contains 5
copies of the GAL4 response element placed adjacent to the thymidine kinase minimal promoter and the luciferase reporter gene. The transfection control vector, pCMV-lacZ, contains the galactosidase Z gene under the regulation of the cytomegalovirus promoter. COS-1 cells are seeded at 1.2 \times 10^4 \text{ cells/well} in 96 well plates in Dulbecco’s modified Eagle medium (high glucose) containing 10% charcoal stripped fetal calf serum, nonessential amino acids, 100 units/ml Penicillin G and 100 \mu g/ml Streptomycin sulfate at 37°C in a humidified atmosphere of 10% CO2. After 24 h, transfections are performed with Lipofectamine (Gibco-BRL, Gaithersburg, MD) according to the instructions of the manufacturer. Transfection mixes contain 0.00075 \mu g of PPARγ/GAL4 or PPARα/GAL4 expression vector, 0.045 \mu g of reporter vector pUAS(5X)-tk-luc and 0.0002 \mu g of pCMV-lacZ vector as an internal control of transfection efficiency. Compounds are characterized by incubation with transfected cells for 48h across a range of 8-12 concentrations from 0.1 nM to 50 \mu M. Cell lysates are prepared from washed cells using Reporter Lysis Buffer (Promega) according to the manufacturer’s directions. Luciferase activity in cell extracts is determined using Luciferase Assay Buffer (Promega) in a ML3000 luminometer (Dynatech Laboratories). β-galactosidase activity is determined using β-D-galactopyranoside (Calbiochem-Novabiochem, LaJolla, CA) as described by Hollons and Yoshimura (Anal. Biochem, 182,411-418, 1989). Rosiglitazone can be used as a standard for human PPARγ activity. EC50 values for Rosiglitazone in the hPPARγ/GAL4 assay usually range from 20-40 nM. Fenofibrate can be used as a standard for hPPARα activity. EC50 values for Fenofibrate in the hPPARα/GAL4 assay usually range from 5-20 \mu M. Similarly, methods involving the co-transfection of full-length PPARγ or PPARγ along with a relevant reporter gene into one of several mammalian (or yeast) cell types could be employed as an alternative method to identify compounds with both PPARα and PPARγ agonist activity.

Cell-Free Co-Activator Association Assay
This assay measures the ability of compounds to promote the association of PPARγ (or its isolated ligand binding domain) or PPARα (or its isolated ligand binding domain) with a protein (or portion of a protein) that is (or is derived from) a co-activator molecule such as Creb Binding Protein (CBP) or Steroid Receptor Coactivator 1 (SRC-1) and can be used to identify compounds with both PPARα and PPARγ agonist activity. This assay is described in: Zhou G, Cummings R, Li Y, Mitra S, Wilkinson H, Elbrecht A, Hermes JD, Schaeffer JM, Smith RG,
Moller DE. Nuclear receptors have distinct affinities for co-activators: characterization by fluorescence resonance energy transfer. Mol Endocrinol 1998 12:1594-1604, herein incorporated by reference in its entirety.

5 Human PPARα and PPARγ binding assays

An alternative to measuring agonist activity of compounds in cell-based transactivation assays or cell-free co-activator association assays is to determine that compounds can function as ligands by binding to both PPARγ and PPARα. Compounds with half-maximal concentration potencies (IC50’s or Ki’s) for displacement of radioligand binding to hPPARγ vs. hPPARα that differ by less than 30-fold and preferably less than 10-fold can be considered as dual ligands. For these assays, the methods described below can be employed (as also described in: Berger J, Leibowitz MD, Doebber TW, Elbrecht A, Zhang B, Zhou G, Biswas C, Cullinan CA, Hayes NS, Li Y, Tanen M, Venture J, Wu MS, Berger GD, Mosley R, Marquis R, Santini C, Sahoo SP, Tolman RL, Smith RG, Moller DE. Novel peroxisome proliferator-activated receptorγ (PPARγ) and PPARδ ligands produce distinct biological effects, 1999 J Biol Chem 274: 6718-6725, herein incorporated by reference in its entirety):

Human PPARγ2 and human PPARα were expressed as a GST-fusion protein in E. coli. The full length human cDNA for PPARγ2 was subcloned into the pGEX-2T expression vector (Pharmacia). The full length human cDNA for PPARα was subcloned into the pGEX-KT expression vector (Pharmacia). E. coli containing the respective plasmids were propagated, induced, and harvested by centrifugation. The resuspended pellet was broken in a French press and debris was removed by centrifugation at 12,000Xg. Recombinant human PPAR receptors were purified by affinity chromatography on glutathione sepharose. After application to the column, and one wash, receptor was eluted with glutathione. Glycerol (10%) was added to stabilize the receptor and aliquots were stored at -80 °C.

For each assay, an aliquot of receptor was incubated in TEGM (10 mM Tris, pH 7.2, 1 mM EDTA, 10% glycerol, 7 μL/100 ml β-mercaptoethanol, 10 mM Na molybdic acid, 1 mM dithiothreitol, 5 μg/mL aprotinin, 2 μg/mL leupeptin, 2 μg/mL benzamidine and 0.5 mM PMSF) containing 0.1% non-fat dry milk and 10 nM [3H2] L-746,962, (21 Ci/mmmole), ± test compound. Assays were incubated for ~16 hr at 4 °C in a final volume of 150 μL. Unbound ligand was removed by incubation with 100
μL dextran/gelatin-coated charcoal, on ice, for 10 min. After centrifugation at 3000 rpm for 10 min at 4 °C, 50 μL of the supernatant fraction was counted in a Topcount. In this assay the KD for L-746,962 is ≈ 1 nM.

For a human PPARα binding assay, an aliquot of receptor was incubated in TEGM (10 mM Tris, pH 7.2, 1 mM EDTA, 10% glycerol, 7 μL/100 ml β-mercaptoethanol, 10 mM Na molybdate, 1 mM dithiothreitol, 5 μg/mL aprotinin, 2 μg/mL leupeptin, 2 μg/mL benzamidine and 0.5 mM PMSF) containing 0.1% non-fat dry milk and 5.0 nM [3H]L-783483, ± test compound. Assays were incubated for ~16 hr at 4 °C in a final volume of 150 μL. Unbound ligand was removed by incubation with 100 μL dextran/gelatin-coated charcoal, on ice, for ~10 min. After centrifugation at 3000 rpm for 10 min at 4 °C, 50 μL of the supernatant fraction was counted in a Topcount.

Cell Proliferation Assay

This assay measures the ability of cells to convert MTS tetrazolium into formazan, using the AQeuous cell proliferation assay kit (Promega, Madison, WI). This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. The assay is described in Shu, et al., Biochemical and Biophysical Research Communications, vol. 267, pp. 345-349 (2000).

MMP-9 ELISA

This assay is used for measuring the amount of MMP-9 secreted from cultured human monocyctic THP-1 in response to lipopolysaccharide (LPS) stimulation. The assay is described in Shu, et al., Biochemical and Biophysical Research Communications, vol. 267, pp. 345-349 (2000). Cultured THP-1 cells were treated with PPARα and/or γ agonists for 2 hr at 37°C. The cells were then stimulated with bacteria LPS (1 ng/ml). After 48 hr incubation at 37°C, culture supernatants were collected. MMP-9 secreted in the media were measured by ELISA using antibodies specific to MMP-9.

To demonstrate additive anti-inflammatory action of PPARα and PPARγ agents in combination or the action of a compound capable of simultaneously binding and activating PPARα and PPARγ, the release of a pro-inflammatory protein, MMP-9, in response to lipopolysaccharide (LPS) was measured. Dosing with PPARα
and PPARγ agents together or with a dual PPARα/γ agent produces an unexpectedly superior decrease in the secretion of MMP-9 as compared to the either a PPARα or PPARγ agent alone.

While the invention has been described and illustrated with reference to certain particular embodiments thereof, those skilled in the art will appreciate that various adaptations, changes, modifications, substitutions, deletions, or additions of procedures and protocols may be made without departing from the spirit and scope of the invention. For example, effective dosages other than the particular dosages as set forth herein above may be applicable as a consequence of variations in the responsiveness of the mammal being treated for any of the indications with the compounds of the invention indicated above. Likewise, the specific pharmacological responses observed may vary according to and depending upon the particular active compounds selected or whether there are present pharmaceutical carriers, as well as the type of formulation and mode of administration employed, and such expected variations or differences in the results are contemplated in accordance with the objects and practices of the present invention. It is intended, therefore, that the invention be defined by the scope of the claims which follow and that such claims be interpreted as broadly as is reasonable.
WHAT IS CLAIMED IS:

1. A method for treating or preventing an inflammatory disease or condition in a mammalian patient in need of such treatment or prevention comprising administering to said patient a compound that is capable of simultaneously binding PPARα and PPARγ or concomitantly administering a compound that selectively binds PPARα with a compound that selectively binds PPARγ in an amount that is effective to treat or prevent the inflammatory disease or condition.

2. The method according to Claim 1 comprising administering the compound that is capable of simultaneously binding PPARα and PPARγ.

3. The method according to Claim 2 wherein the compound that is capable of simultaneously binding PPARα and PPARγ has a half-maximal concentration potency (IC₅₀ or Kᵢ) for the displacement of radioligand binding to hPPARγ vs. hPPARα that differs by less than 20-fold as measured by the human PPARα and PPARγ binding assays.

4. The method according to Claim 2 wherein the compound that is capable of simultaneously binding PPARα and PPARγ has a half-maximal concentration potency (IC₅₀ or Kᵢ) for the displacement of radioligand binding to hPPARγ vs. hPPARα that differs by less than 10-fold as measured by the human PPARα and PPARγ binding assays.

5. The method according to Claim 2 wherein the compound that is capable of simultaneously binding PPARα and PPARγ has a half-maximal concentration potency (IC₅₀ or Kᵢ) for the displacement of radioligand binding to hPPARγ vs. hPPARα that differs by less than 5-fold as measured by the human PPARα and PPARγ binding assays.

6. The method according to Claim 2 wherein the compound that is capable of simultaneously binding PPARα and PPARγ has a half-maximal concentration potency (IC₅₀ or Kᵢ) for the displacement of radioligand binding to
hPPARγ vs. hPPARα that differs by less than 2-fold as measured by the human PPARα and PPARγ binding assays.

7. The method according to Claim 2 wherein the compound that is capable of simultaneously binding PPARα and PPARγ that is capable of simultaneously binding PPARα and PPARγ is orally active.

8. The method according to Claim 2 wherein the compound that is capable of simultaneously binding PPARα and PPARγ possess a long duration of action.

9. The method according to Claim 1 wherein the inflammatory disease or condition is inflammatory bowel syndrome.

10. The method according to Claim 1 wherein the inflammatory disease or condition is arthritis.

11. The method according to Claim 10 wherein the inflammatory disease or condition is selected from the group consisting of: rheumatoid arthritis, ankylosing spondylitis, gout, psoriasis, osteoarthritis, and juvenile arthritis.

12. A method for treating or preventing an inflammatory disease or condition in a mammalian patient in need of such treatment or prevention comprising administering to said patient a compound that is capable of simultaneously binding and activating PPARα and PPARγ or concomitantly administering a compound that selectively binds and activates PPARα with a compound that selectively binds and activates PPARγ in an amount that is effective to treat or prevent the inflammatory disease or condition in accordance with Claim 1.

13. The method according to Claim 12 comprising administering the compound that is capable of simultaneously binding and activating PPARα and PPARγ.
14. The method according to Claim 13 wherein the compound that is capable of simultaneously binding and activating PPARα and PPARγ has a half-maximal concentration potency (EC_{50}) for activation of hPPARγ vs. hPPARα that differs by less than 20-fold as measured by the cell-based transactivation assay or cell-free co-activator association assay.

15. The method according to Claim 13 wherein the compound that is capable of simultaneously binding and activating PPARα and PPARγ has a half-maximal concentration potency (EC_{50}) for activation of hPPARγ vs. hPPARα that differs by less than 10-fold as measured by the cell-based transactivation assay or cell-free co-activator association assay.

16. The method according to Claim 13 wherein the compound that is capable of simultaneously binding and activating PPARα and PPARγ has a half-maximal concentration potency (EC_{50}) for activation of hPPARγ vs. hPPARα that differs by less than 5-fold as measured by the cell-based transactivation assay or cell-free co-activator association assay.

17. The method according to Claim 13 wherein the compound that is capable of simultaneously binding and activating PPARα and PPARγ has a half-maximal concentration potency (EC_{50}) for activation of hPPARγ vs. hPPARα that differs by less than 2-fold as measured by the cell-based transactivation assay or cell-free co-activator association assay.

18. The method according to Claim 13 wherein the compound that is capable of simultaneously binding and activating PPARα and PPARγ is orally active.

19. The method according to Claim 13 wherein the compound that is capable of simultaneously binding and activating PPARα and PPARγ possesses a long duration of action.

20. The method according to Claim 12 wherein the inflammatory disease or condition is inflammatory bowel syndrome.
21. The method according to Claim 12 wherein the inflammatory disease or condition is arthritis.

22. The method according to Claim 21 wherein the inflammatory disease or condition is selected from the group consisting of: rheumatoid arthritis, ankylosing spondylitis, gout, psoriasis, osteoarthritis, and juvenile arthritis.
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
   IPC(7) : A01N 61/00, 37/18; A61K 31/00, 38/00
   US CL : 514/1, 2

   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. : 514/1, 2

   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)
   WEST, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
</table>

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

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 *P* document published prior to the international filing date but later than the priority date claimed

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