TREATING INFLAMMATORY DISEASES BY ADMINISTRATION OF REV-ERB α LIGANDS

The invention relates to methods of modulating inflammatory diseases by administration of REV-ERB α nuclear receptor ligands, and to REV-ERB α ligands compounds.

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Cross-reference to Related Applications

The present application is claims benefit of priority from United States Provisional Patent Application No. 61/235,469 filed 20 August 2009.

Field of the Invention

The present invention relates to the use of the REV-ERB α nuclear receptor to influence inflammatory conditions, and is based upon the finding that this receptor plays a role in the regulation of the inflammatory response. The invention relates to the modulation of inflammatory responses using REV-ERB α ligands, including the treatment of inflammatory diseases, especially those associated with increased numbers of neutrophils, eosinophils, and macrophages. The invention also relates to compounds useful in binding to Rev-erb α.

Background

The Rev-ErbA proteins are members of the nuclear receptor family of intracellular transcription factors. There are two forms of the receptor, alpha (α) and beta (β), each encoded by a separate gene (NR1D1 and NR1D2 respectively).

Rev-ErbA α, also known as NR1D1 (nuclear receptor subfamily 1, group D, member 1) is a member of the Rev-ErbA family of nuclear receptors and is a transcriptional repressor. Rev-erbα is highly expressed in the liver, skeletal muscle, adipose tissue, and the brain, in mammals, participating in the development and circadian regulation of these tissues. Preitner N, et al.,
Rev-erbα regulates gene transcription by directly binding to target response elements (RevREs), comprises an A/T-hch flank followed by AGGTCA. Rev-erbα mediates repression by recruiting the corepressor N-CoR, which then activates the histone deacetylase (HDAC) 3. A number of target genes has been identified for Rev-erbα, including the lipoproteins ApoA1 and ApoCIII, hydratase dehydrogenase, the circadian factor BMAL, and the anti-fibrinolytic factor PAI-1. Wang J, et al., (2006). "The orphan nuclear receptor Rev-erb α regulates circadian expression of plasminogen activator inhibitor type 1". J. Biol. Chem. 281 (45): 33842-8. doi:10.1074/jbc.M607873200. PMID 16968709. Many of these genes are coordinately regulated by Rev-erbα and the RAR-related orphan receptor RORα, which share the same response elements but exert opposite effects on gene transcription. Crosstalk between Rev-erbα and RORα likely acts to fine-tune of their target physiologic networks, such as circadian rhythms, metabolic homeostasis, and inflammation. Forman BM, et al., (1994). "Cross-talk among ROR α 1 and the Rev-erb family of orphan nuclear receptors". Mol. Endocrinol. 8 (9): 1253-61. doi:10.1210/me.8.9.1253. PMID 7838158.

Rev-erbα mRNA is induced during adipogenesis and is highly expressed in adipose tissue. Fontaine C, et al., (2003). "The orphan nuclear receptor Rev-Erbα is a peroxisome proliferator-activated receptor (PPAR) gamma target gene and promotes PPARgamma-induced adipocyte differentiation". J. Biol.
One study reported that overexpression of Rev-erbα may enhance adipogenesis in cultured mouse adipocytes, but the mechanism of this effect remains to be elucidated. Chawla A, Lazar MA (1993). "Induction of Rev-ErbA α, an orphan receptor encoded on the opposite strand of the α-thyroid hormone receptor gene, during adipocyte differentiation". J. Biol. Chem. 268 (22): 16265-9. PMID 8344913.

Rev-erbα expression is also regulated at the post-translational level: it is phosphorylated on the amino terminus by glycogen synthase kinase (GSK3β), which contributes to its protein stability. It has been shown that lithium, which inhibits GSK3β, can destabilize Rev-erbα protein and affect its function in the circadian clock. Yin L, et al.,(2006). "Nuclear receptor Rev-erbα is a critical lithium-sensitive component of the circadian clock". Science 311 (5763): 1002-5. doi:10.1126/science.1121613. PMID 16484495.


There are many inflammatory disorders (diseases or conditions) which affect mammals, such as eczema, psoriasis, allergic dermatitis, neurodermatitis, pruhtis and hypersensitivity reactions; inflammatory conditions of the nose, throat or lungs such as asthma (including allergen-induced asthmatic reactions), rhinitis (including hayfever), nasal polyps, chronic obstructive pulmonary disease, interstitial lung disease, and fibrosis; inflammatory bowel conditions such as ulcerative colitis and Crohn's disease; and auto-immune diseases such as rheumatoid arthritis; as well as inflammatory conditions of the conjunctiva, such as conjunctivitis.

The chronic abnormal inflammation in COPD is marked by infiltration of T cells, and increased numbers of neutrophils and macrophages. This inflammation involves a number of specific inflammatory mediators and cytokines released by activated pulmonary epithelial cells and macrophages, Barnes, P.J. 2008. The cytokine network in asthma and chronic obstructive pulmonary disease. J Clin. Invest 118:3546-3556. These include growth factors, which stimulate fibroblast proliferation, pro-inflammatory cytokines (including Tumour Necrosis Factor α (TNF α) and IL-6), and chemokines (including JE and IL-8) which attract neutrophils and monocytes to the lung.

COPD is a complex condition that results from the interaction of genetic and environmental factors (particularly exposure to cigarette smoke and air pollution). Although the genetic factors that pre-dispose a patient to COPD are not fully understood, the familial clustering indicates their importance. Molfino, N.A. 2007. Current thinking on genetics of chronic obstructive pulmonary disease. Curr. Opin. Pulm. Med. 13:107-113. Several candidate genes have been associated with COPD, including TNF α and αI antitrypsin, Lomas, D.A., and Silverman, E.K. 2001. The genetics of chronic obstructive pulmonary disease. Respir. Res. 2:20-26. Some nuclear receptors have been shown to regulate pulmonary inflammation including: Retinoid-related Orphan Receptor α (RORα), Liver X receptor (LXR), Peroxisome Proliferator-activated receptors (PPARs) α and Y, steroid receptors and the vitamin D3 receptor, Stapleton, C.M. et ai, 2005. Enhanced susceptibility of staggerer...


Surprisingly, we have discovered that REV-ERB α plays an important role in the innate inflammatory response, and that REV-ERB α ligands are able to modulate inflammatory responses. Such ligands have utility for the treatment of inflammatory disorders, especially those associated with increased numbers of neutrophils, eosinophils, and macrophages. Such ligands also have utility as tools for studying the biology of the REV-ERB α receptor in modulation of inflammatory disorders, especially those associated with increased numbers of neutrophils, eosinophils, and macrophages.
SUMMARY OF THE INVENTION

The invention relates to a method of treating inflammatory diseases comprising the administration of REV-ERB α ligand to a patient in need thereof.

In one embodiment, the method comprises the treating a disease involving increased neutrophils comprising administering to a patient in need thereof REV-ERB α ligand.

In a further embodiment, the intention relates to a method of treating a disease involving increased macrophages comprising administering to a patient in need thereof REV-ERB α ligand.

In a still further embodiment, the invention involves a method of treating a disease involving inflammatory lung disease comprising administering to a patient in need thereof REV-ERB α ligand.

Further aspects of the present invention relates to a method of treating COPD, emphysema, cystic fibrosis, acute respiratory distress syndrome, or acute lung injury, comprising administration to a patient in need thereof REV-ERB α ligand.

Suitably, the REV-ERB α ligand is a REV-ERB α agonist.

Alternatively, the REV-ERB α ligand is a REV-ERB α modulator.

Still further, the invention involves a method for modulating IL-6 secretion in a patient comprising administering a REV-ERB α ligand. Suitably, in this method, the response modulated is IL-6 secretion. More suitably, the modulation is inhibition.
Suitably, the Rev-erb α ligand is one or more of the following compounds:

1,1-dimethylethyl N-[(4-chlorophenyl)methyl]-N-[(5-nitro-2-thienyl)methyl]glycinate; 

$\Lambda$-Benzyl-$\Lambda'$-(4-chlorobenzyl)-1-(5-nitrothiophen-2-yl)methanamine; 

$\Lambda$-Benzyl-$\Lambda'$-(3,4-dichlorobenzyl)-1-(5-nitrothiophen-2-yl)methanamine; or

2-((4-chlorobenzyl)((5-nitrothiophen-2-yl)methyl)amino)-N,N-dimethylacetamide.

In a further embodiment, REV-ERB α ligands, for example, 1,1-dimethylethyl N-[(4-chlorophenyl)methyl]-N-[(5-nitro-2-thienyl)methyl]glycinate; $\Lambda$-Benzyl-$\Lambda'$-(4-chlorobenzyl)-1-(5-nitrothiophen-2-yl)methanamine; $\Lambda$-Benzyl-$\Lambda'$-(3,4-dichlorobenzyl)-1-(5-nitrothiophen-2-yl)methanamine; and 2-((4-chlorobenzyl)((5-nitrothiophen-2-yl)methyl)amino)-N,N-dimethylacetamide, are also useful as tools for studying the biology of the REV-ERB α receptor in modulation of inflammatory disorders, especially those associated with increased numbers of neutrophils, eosinophils, and macrophages. Thus a further aspect of the invention relates to a method for modulating REV-ERB α induced inflammatory response comprising administration of a REV-ERB α ligand to a REV-ERB α nuclear receptor.
Fig. 1 depicts cytokines in BAL following in vivo LPS challenge (measured by bead array). Values are mean ± SEM. Cytokine levels were compared between genotypes at each dose using one Way ANOVA and post-hoc bonferroni tests. n=6-8, *P<0.05 and *** P<0.005.

Fig. 2 depicts quantification of total BAL cell numbers and types after in vivo LPS challenge. (A) Cell types were differentiated by the physical characteristics 'forward scatter' and 'side scatter' as illustrated. (B-F) Cell numbers in BAL from wild type (grey bars) and rev-erb α- mice (hatched bars), values are mean ± SEM. Differences between genotype at each dose were determined using one

Fig. 3 depicts Histology and immunohistochemistry on lung sections from control (saline) or LPS (2mg/ml) challenged mice. (A) H and E staining demonstrates the presence of inflammatory cells after LPS challenge (B) Neutrophils (detected using anti-NIMP/R14; deep purple) are present in large numbers around the airways after LPS, but not in control mice. (C) Macrophages (detected using anti-F4/80; brown) are present at similar levels before and after challenge. Lung sections were counterstained with haematoxylin (blue) after IHC, scale bar = 50µm.

Figure 4: Depicts Macrophage LPS stimulation. (A) RT-PCR confirms the presence of rev-erb α mRNA in wt but not rev-erb α- BMDM, both genotypes express cd11b mRNA. (B) Flow cytometry demonstrates that over 90% of BMDM stained positive for both CD11b and F4/80. (C) Release of cytokines after LPS challenge of BMDM, as measured by ELISA. Values are mean ± SEM.
Figure 5: Depicts the effects of a REV-ERB α ligand on the cytokine response of human alveolar macrophages to LPS. A. Application of the REV-ERB α ligand to LPS stimulated human alveolar macrophages reduced the release of IL-6 at all doses (One way ANOVA, Bonferroni *** P<0.005). B. IL-8. Values are mean ± SEM, n=4.

Figure 6: is a graphical depiction of the effect of GSK4112A on LPS stimulated human blood macrophages.
DETAILED DESCRIPTION

The present invention relates to a method of treating inflammatory diseases comprising the administration of a REV-ERB α ligand to a patient in need thereof.

In one embodiment, the method comprises the treating a disease involving increased neutrophils comprising administering to a patient in need thereof a REV-ERB α ligand.

In a further embodiment, the intention relates to a method of treating a disease involving increased macrophages comprising administering to a patient in need thereof a REV-ERB α ligand.

In a still further embodiment, the invention involves a method of treating a disease involving inflammatory lung disease comprising administering to a patient in need thereof a REV-ERB α ligand.

Further aspects of the present invention relates to a method of treating COPD, emphysema, cystic fibrosis, acute respiratory distress syndrome, or acute lung injury, comprising administration to a patient in need thereof a REV-ERB α ligand.

Suitably, the REV-ERB α ligand is a REV-ERB α agonist.

Alternatively, the REV-ERB α ligand is a REV-ERB α modulator.

Still further, the invention involves a method for modulating IL-6 secretion in a patient comprising administering a REV-ERB α ligand. Suitably, in this method, the response modulated is IL-6 secretion. More suitably, the modulation is inhibition.
Suitably, the REV-ERB α ligand is one or more of the following:

1,1-dimethylethyl N-[(4-chlorophenyl)methyl]-N-[(5-nitro-2-thienyl)methyl]glycinate;
\(\mathcal{N}\)-Benzyl-\(\mathcal{N}\)-(4-chlorobenzyl)-1-(5-nitrothiophen-2-yl)methanamine;
\(\mathcal{N}\)-Benzyl-\(\mathcal{N}\)-(3,4-dichlorobenzyl)-1-(5-nitrothiophen-2-yl)methanamine;
2-((4-chlorobenzyl)((5-nitrothiophen-2-yl)methyl)amino)-N,N-dimethylacetamide

COMPOUNDS

Compounds useful in the method of the present invention include, but are not limited to the following:

1,1-Dimethylethyl N-[(4-chlorophenyl)methyl]-N-[(5-nitro-2-thienyl)methyl]glycinate;
\(\mathcal{N}\)-Benzyl-\(\mathcal{N}\)-(4-chlorobenzyl)-1-(5-nitrothiophen-2-yl)methanamine;
\(\mathcal{N}\)-Benzyl-\(\mathcal{N}\)-(3,4-dichlorobenzyl)-1-(5-nitrothiophen-2-yl)methanamine; and
2-((4-chlorobenzyl)((5-nitrothiophen-2-yl)methyl)amino)-N,N-dimethylacetamide.

In a still further embodiment of the invention, REV-ERB α ligands, for example, 1,1-dimethylethyl N-[(4-chlorophenyl)methyl]-N-[(5-nitro-2-thienyl)methyl]glycinate; \(\mathcal{N}\)-Benzyl-\(\mathcal{N}\)-(4-chlorobenzyl)-1-(5-nitrothiophen-2-yl)methanamine; \(\mathcal{N}\)-Benzyl-\(\mathcal{N}\)-(3,4-dichlorobenzyl)-1-(5-nitrothiophen-2-yl)methanamine; and 2-((4-chlorobenzyl)((5-nitrothiophen-2-yl)methyl)amino)-N,N-dimethylacetamide, are also useful as tools for studying the biology of the REV-ERB α receptor in modulation of inflammatory disorders, especially...
those associated with increased numbers of neutrophils, eosinophils, and macrophages.

These above mentioned compounds may be produced as follows:

Solvents and reagents were reagent grade and used without purification unless otherwise noted. All \(^1\)H NMR spectra were recorded on a Varian 400MHz spectrometer. Chemical shifts (δ) are reported downfield from tetramethylsilane (Me_4Si) in parts per million (ppm) of the applied field. Peak multiplicities are abbreviated: singlet, s; broad singlet, bs; doublet, d; triplet, t; quartet, q; multiplet, m. Coupling constants (J) are reported in hertz. LCMS analyses were conducted using a Waters Acquity UPLC system with UV detection performed from 210 to 350 nm with the MS detection performed on a Waters Acquity SQD spectrometer. Purities of final compounds were >98% as determined by LCMS.

1. 1,1-Dimethylethyl \(N-(4\text{-chlorophenyl})\text{methyl}-N-(5\text{-nitro-2-thienyl})\text{methyl})\text{glycinate}

1,1-Dimethylethyl \(N\)-(4-chlorophenyl)methyl-\(N\)-(5-nitro-2-thienyl)methyl\)glycinate is commercially available (Sigma/Aldrich Product Number G0671). It may also be synthesised as follows:
To a solution of 4-chlorobenzylamine (1.34 ml, 11.0 mmol) in THF (25 mL) was added K$_2$CO$_3$ (3.46 g, 25.0 mmol). t-Butyl bromoacetate (1.48 mL, 10.0 mmol) was added drop wise over 3 min at room temperature, and the reaction mixture was stirred overnight at 40°C. The resultant slurry was then diluted with EtOAc (80 mL) and washed with 2 x 50 mL water and 50 mL brine. The organic solution was dried with MgSO$_4$ and concentrated under reduced pressure. The crude material was purified by silica gel chromatography (Biotage 4OM column with 0 to 50% EtOAc in CH$_2$Cl$_2$ elution) to yield the intermediate ferf-butyl N-[(4-chlorophenyl)methyl]glycinate as a clear, colorless oil: 2.08 g (81%) MS (ESI): m/z 256 (M+H)$^+$. To a stirring solution of t-butyl N-[(4-chlorophenyl)methyl]glycinate (1.05 g, 4.11 mmol) in CH$_2$Cl$_2$ (20 mL) was added successively HOAc (0.5 mL), 5-nitro-2-thiophenecarboxaldehyde (0.774 g, 4.93 mmol), and sodium thacetoxyborohydride (1.740 g, 8.21 mmol) The reaction mixture was allowed to stir overnight, after which time the it was diluted with 100 mL EtOAc and washed with 2 x 50 mL water and 50 mL brine. After drying over MgSO$_4$ and concentration under reduced pressure, the resulting yellow oil was purified by silica gel chromatography (Biotage 4OM column, 0 to 50% CH$_2$Cl$_2$ in hexanes elution) to yield the product as a pale yellow oil: $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 1.44 (s, 9H), 3.24 (s, 2H), 3.81 (s, 2H), 4.01 (s, 2H), 6.85 (d, $J = 4.0$ Hz, 1H), 7.29 (s, 4H), 7.75 (d, $J = 4.0$ Hz, 1H); $^{13}$C (100 MHz, CDCl$_3$) $\delta$ 28.5, 53.2, 54.8, 56.8, 81.4, 126.0, 129.1, 131.1, 132.6, 137.8, 150.2, 155.9, 170.2; MS (ESI): m/z 397 (M+H)$^+$. 

2. $^\Lambda$-Benzyl-$^\Lambda$-(4-chlorobenzyl)-1-(5-nitrothiophen-2-vDmethanamine
To a stirred solution of the appropriately substituted 4-chlorobenzylamine (432 µl, 3.5 mmol) in Λ,N-dimethylformamide (5 mL) was added successively benzaldehyde (356 µl, 3.5 mmol) and sodium thacetoxyborohydride (1.1 g, 5.5 mmol). The reaction mixture was allowed to stir for 5 hours at room temperature. To this reaction mixture was added successively 5-nitro-2-thiophenecarboxaldehyde (555 mg, 3.5 mmol) and sodium thacetoxyborohydride (1.1 g, 5.5 mmol) the reaction mixture was stirred overnight at room temperature. The reaction mixture was then partitioned between saturated aqueous sodium bicarbonate solution (40 mL) and EtOAc (3 x 50 mL), the organic fractions were combined, washed with brine, dried over Na₂SO₄, and evaporated to dryness under reduced pressure. The crude products were purified by silica gel column chromatography (EtOAc in Hexanes, 0-10%). 610 mg, 164 mmol, 46.3 % yield). ¹H NMR (400 MHz, CDCl₃) δ 3.59 (s, 2H), 3.62 (s, 2H), 3.72 (s, 2H), 6.84 (d, J = 3.9 Hz, 1 H), 7.24 - 7.41 (m, 9H), 7.76 (d, J = 3.9 Hz, 1H); ¹³C (100 MHz, CDCl₃) δ 52.9, 57.6, 58.3, 124.3, 127.8, 128.8, 128.9, 128.9, 130.2, 133.4, 137.0, 138.1, 151.0, 154.3. MS (ESI): m/z 373 (M+H)⁺.

3. ¹⁻BenzyL⁻(3,4-dichlorobenzyl)⁻1-(5-nitrothiophen-2-yl)methanamine
Prepared in a manner similar to Ν-Benzyl-Ν-(4-chlorobenzyl)-1-(5-nitrothiophen-2-yl)methanamine, but substituting 3,4-dichlorobenzylamine for 4-chlorobenzylamine. The crude products were purified by silica gel column chromatography (EtOAc in Hexanes, 0-10%). 578 mg, 1.405 mmol, 49.5 % yield. \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 3.58 (s, 2H), 3.63 (s, 2H), 3.73 (s, 2H), 6.86 (d, \(J = 4.3\) Hz, 1H), 7.26 - 7.31 (m, 2H), 7.33 - 7.41 (m, 4H), 7.43 (d, \(J = 8.2\) Hz, 1H), 7.48 (d, \(J = 1.95\) Hz, 1H), 7.78 (d, \(J = 4.1\) Hz, 1H); \(^{13}\)C (100 MHz, CDCl\(_3\)) \(\delta\) 53.0, 57.2, 58.4, 124.6, 127.9, 128.1, 128.9, 130.7, 130.8, 131.6, 132.8, 137.8, 138.9, 151.1, 153.8. MS (ESI): \(m/z\) 407 (M+H)\(^+\).
4. **2-((4-chlorobenzyl)((5-nitrothiophen-2-yl)methyl)amino)-N,N-dimethylacetamide**

To a stirred solution of (4-chlorobenzyl)amine (0.432 mL, 3.53 mmol) in tetrahydrofuran (THF) (10 mL) was added sodium hydride (60% suspension in mineral oil, 177 mg, 4.41 mmol) followed by 2-chloro-N,N-dimethylacetamide (0.363 mL, 3.53 mmol). The reaction was stirred for 14 hours at room temperature. The reaction mixture was then poured into a saturated sodium bicarbonate solution (50 mL) and extracted into EtOAc (70 mL). The organic phase was washed with brine (50 mL), dried over sodium sulfate, and concentrated in vacuo. The residue was purified by silica gel chromatography (0-15% Hex/EtOAc) to yield 2-((4-chlorobenzyl)amino)-N,N-dimethylacetamide as a yellow oil (524 mg, 1.041 mmol, 29.5% yield). The product was characterized by LCMS MS (ESI): m/z 227 (M+H)+ and taken on to the next step.

To a solution of 2-((4-chlorobenzyl)amino)-N,N-dimethylacetamide (524 mg, 2.3 mmol) in DMF (5 mL) was added successively acetic acid (0.5 mL), 5-nitro-2-thiophenecarboxaldehyde (363 mg, 2.3 mmol), and sodium thacetoxyborohydride (735 mg, 3.5 mmol). The reaction was allowed to stir overnight. The reaction mixture was then diluted with EtOAc (100 mL) and washed with saturated aqueous sodium bicarbonate solution (50 mL) and brine (50 mL). After drying over MgSO4 and concentration under reduced pressure, the resulting yellow oil was purified by silica gel chromatography
(EtOAc in Hexanes, 0-15%) to yield the title product as a pale yellow oil (182 mg, 0.466 mmol, 20.1% yield). 1H NMR (400 MHz, CDCl₃) δ 2.87 (s, 3H) 2.93 (s, 3H) 3.40 (s, 2H) 3.87 (s, 2H) 4.12 (s, 2H) 6.89 (d, J = 4.0 Hz, 1H) 7.34 (s, 4H) 7.80 (d, J = 4.0 Hz, 1H); 13C (100 MHz, CDCl₃) δ 35.4, 36.7, 53.2, 53.5, 57.4, 124.3, 128.8, 128.8, 130.3, 133.4, 136.6, 154.4, 169.7. MS (ESI): m/z 368 (M+H)+.

Pharmaceutical Formulations And Routes Of Administration

The compounds mentioned above can be administered to an animal host, including a human patient, by itself, or in pharmaceutical compositions where it is mixed with suitable carriers or excipient(s) at doses therapeutically effective to treat or ameliorate a variety of disorders, including the treatment of inflammatory diseases, especially those associated with increased numbers of neutrophils, eosinophils, and macrophages. A therapeutically effective dose further refers to that amount of the compound sufficient to result in amelioration of symptoms associated with such disorders. Techniques for formulation and administration of the compounds of the instant application may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, Pa., latest edition.

Effective Dosage

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve its intended purpose. More specifically, a therapeutically effective amount means an amount effective to prevent development of or to alleviate the existing symptoms of the subject being treated. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.
For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating concentration range that includes the IC50 (the dose where 50% of the cells show the desired effects) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans.

A therapeutically effective dose refers to that amount of the compound that results in amelioration of symptoms or a prolongation of survival in a patient. Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio between LD50 and ED50. Compounds which exhibit high therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g. Fingl et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 pi). Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety which are sufficient to maintain the desired effects.

In cases of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration.
The amount of composition administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician.

Composition And Formulation

The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, or lyophilizing processes. The compositions may also be manufactured by spray drying from solution or suspension.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmacetically. Proper formulation is dependent upon the route of administration chosen.

For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. Pharmaceutical preparations for oral use can be obtained solid excipient,
optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethylcellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.
For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, 1,1,1,2-tetrafluoroethane, 1,1,1,2,3,3,3-heptafluoropropane, carbon dioxide or other suitable gas, alone, or in combination. Pressurized aerosols may be formulated as suspensions or solutions, and include an appropriate propellant formulation, and various excipients, such as surfactants, co-solvents, etc. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may
also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

A pharmaceutical carrier for the hydrophobic compounds of the invention is a cosolvent system comprising benzyl alcohol, a nonpolar surfactant, a water-miscible organic polymer, and an aqueous phase. Naturally, the proportions of a co-solvent system may be varied considerably without destroying its solubility and toxicity characteristics. Furthermore, the identity of the co-solvent components may be varied.

Alternatively, other delivery systems for hydrophobic pharmaceutical compounds may be employed. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophobic drugs. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the compounds may be delivered using a sustained-release system, such as semipermeable matrices
of solid hydrophobic polymers containing the therapeutic agent. Various of sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for a few weeks up to over 100 days.

Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

Many of the compounds of the invention may be provided as salts with pharmaceutically compatible countehons. Pharmaceutically compatible salts may be formed with many acids, including but not limited to hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents that are the corresponding free base forms.
Routes Of Administration

Suitable routes of administration may, for example, include oral, rectal, transmucosal, transdermal, inhaled or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections.

Alternately, one may administer the compound in a local rather than systemic manner, for example, via injection of the compound directly into an affected area, often in a depot or sustained release formulation.

Routes of administration like suppository, intravenous, intramuscular, inhalational aerosol and sublingual avoid the first-pass effect because they allow drugs to be absorbed directly into the systemic circulation.

Furthermore, one may administer the drug in a targeted drug delivery system, for example, in a liposome coated with an antibody specific for affected cells. The liposomes will be targeted to and taken up selectively by the cells.

Packaging

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labelled for treatment of an indicated condition. Suitable conditions indicated on the label may include treatment of a disease such as one characterized by including the treatment
of inflammatory diseases, especially those associated with increased numbers of neutrophils, eosinophils, and macrophages.

**EXAMPLES:**

**Example 1**

A population sample set consisted of 925 COPD cases and 937 smoking controls, collected through the Department of Thoracic Medicine, Haukeland Hospital, Bergen, Norway. The ascertainment details and characteristics of the population are described in Zhu, G., Warren, L., Aponte, J., Gulsvik, A., Bakke, P., Anderson, W.H., Lomas, D.A., Silverman, E.K., and Pillai, S.G. 2007. The SERPINE2 gene is associated with chronic obstructive pulmonary disease in two large populations. Am. J Respir Crit Care Med 176:167-173, and in Pillai, S.G., Ge, D., Zhu, G., Kong, X., Shianna, K. K., Need, A.C, Feng, S., Hersh, C.P., Bakke, P., Gulsvik, A. et al 2009. A genome-wide association study in chronic obstructive pulmonary disease (COPD): identification of two major susceptibility loci. PLoS. Genet 5:el 000421. A total of 6,836 SNPs in 1855 genes were genotyped by a modification of the single base chain extension (SBCE) assay as described in, Roses, A.D., Burns, D.K., Chissoe, S., Middleton, L., and St Jean, P. 2005. Disease-specific target selection: a critical first step down the right road. Drug Discov. Today 10:177-189. Testing for genetic association was carried out using the Fisher's exact test procedure implemented in the SASv8.2 (SAS Institute, Cary, NC). A permutation test was carried out to determine the significance of this minimum p value obtained in the context of the number of SNPs analysed within each gene. Genes with a permutation p value < 0.01 were considered to be associated with COPD.

SNP rs939347 in the NR1 D1 (rev-erb a) gene showed a highly significant genotypic (P=0.0063) and allelic (P=0.0015) association with COPD with odds ratios of 1.47 (95% CI: 1.12 - 1.92) and 1.44 (95% CI: 1.15 - 1.80), respectively.
Example 2

Rev-erb $a^{-/-}$ mice in which exons 3 and 4 (encoding the DNA binding domain) and part of exons 2 and 5 of the gene were replaced by an in-frame LacZ allele (12) were bred as a heterozygous colony, and offspring genotyped to identify rev-erb $a^{-/-}$ and wild-type (WT) animals. Genomic DNA was extracted using Illustra tissue and cell genomic prep mini spin kit (GE Healthcare, Buckinghamshire, UK), and utilised in two PCR reactions to determine whether the samples had copies of WT rev-erb $a^{-/-}$ and/or a LacZ insert. The following primers were utilised: REV WT: 5' CAC CTT ACA CAG TAG CAC CAT GCC ATT CA 3'; LacZ: 5' AAA CCA GGC AAA GCG CCA TTC GCC ATT CA 3'; and Common: 5' CCA GGA AGT CTA CAA GTG GCC ATG GAA GA 3'. Animals were maintained under a 12h light:12h dark lighting schedule.

In vivo LPS challenge was undertaken using rev-erb $a^{-/-}$ (n=38) and WT (n=40) mice (13-28 wks). Animals were divided into five groups (n=6-8; equally matched for sex) to receive either aerosolised saline or one of four LPS doses: 0.05mg/ml, 0.2mg/ml, 0.5mg/ml or 2mg/ml. Four hours after lights on, mice were placed into Perspex chambers and exposed to aerosolised saline (vehicle control) or LPS (from E.coli 0127:B8; Sigma) for 20 min. Five hours post-exposure, mice were sacrificed. Bronchoalveolar lavage (BAL) was performed by instilling and removing BAL fluid (10mM EDTA in PBS with 0.1% BSA and protease inhibitors) via a tracheal cannula. The lungs were inflated with 1ml 4% paraformaldehyde and removed for histological analysis.

BAL samples were centrifuged, the supernatant was utilised for cytokine assays and cell pellets re-suspended in 0.5ml BAL fluid for subsequent analysis by flow cytometry. Cytokine analysis was performed using custom made bead array assays as per kit instructions (Luminex corporation, Netherlands). The assays were performed using the Luminex 100 IS and data acquired using the Star Station acquisition programme (Luminex...
For flow cytometry, 50 µl re-suspended pellet was processed using a Coulter TQprep (Beckman Coulter) to fix cells and lyse red blood cells. Fluorosphere beads (50 µl Beckman Coulter Flow-count) were added and samples assayed using a Coulter EPICS XL-MCL flow cytometer. Eosinophils, lymphocytes, neutrophils and macrophages were differentiated by the physical characteristics of 'forward scatter' and 'side scatter' (determining size and granularity of individual cells respectively, Fig. 2a). Each sample was assayed for a minimum 7000 cells or 180 seconds, and cell quantification determined by reference to the Flow-count beads.

BAL levels of IL-6, TNF α, MIP-2, KC, RANTES, MIP1 α, IL-10 and G-CSF increased in a dose-dependent manner following LPS challenge (Fig. 1). JE levels did not increase above baseline at any dose. IL-1, IFN Y and M-CSF were undetectable in all samples. TNFα and G-CSF concentrations were significantly elevated (2 fold) in BAL from KO mice administered 2mg/ml LPS. At 0.5mg/ml LPS WT samples had significantly elevated levels of MIP1 α, but at 2mg/ml the reverse was observed. KC and MIP-2 levels were dramatically augmented in KO mice following LPS treatment, with concentrations 4 to 5-fold higher than WT mice.

In response to LPS challenge, total cell numbers in BAL fluid increased in a dose-dependent manner in both KO and WT mice. Rev-erb a−/−-mice exhibited significantly higher total cell counts than WT mice at all LPS dose concentrations (Fig. 2). Most strikingly, there was a highly significant LPS dose-dependent elevation in neutrophil numbers in the BAL of KO mice. A significant elevation in eosinophil numbers in KO mice was observed at the highest dose of LPS in KO mice but not in WT mice.

Fixed lung tissue was processed, embedded, and 5µm sections mounted onto slides. Sections were utilised for routine H and E staining. Additionally, lung sections from OVA sensitised mice were taken for Periodic Acid Schiff (PAS;
Sigma) and Masson Trichrome staining using standard procedures (Sigma reagents). Immunohistochemical staining was carried out using antibodies raised against F4/80 (to detect macrophages; Abeam Ab6640) and NIMP-R14 (to detect fibroblasts; Abeam Ab2557) using established methods (17), sections were counterstained with haematoxylin. Immunohistochemistry revealed substantial neutrophilic recruitment in LPS challenged lung tissue (Fig. 3a and b) which was localised to the bronchioles. In confirmation of flow cytometry, no changes in lung macrophage numbers were observed in either genotype (Fig. 3c).
Example 3

Bone marrow was flushed from the femurs and tibias of rev-erb a+ and WT mice to harvest macrophages, as described previously (18), and incubated in the presence or absence of LPS (1-200ng/ml from E Coli 017:B8; Sigma). Supernatant was collected at +24h and cells harvested for either RT-PCR (to detect rev-erb a and cd11b mRNA) or flow cytometry to confirm the purity of the population. For flow cytometry, cells were scraped from culture plates and re-suspended in buffer (phosphate buffered saline and 5% fetal bovine serum). Cells were pre-incubated with mouse Fc Block (BD Biosciences, Oxford UK) before double staining with phycoerythrin-labelled anti-CD11b (1:200, ebioscience, Hatfield UK) and allophycocyanin-labelled anti-F4/80 (1:200, ebioscience) for 30 min. Results were acquired on a FACSCaliber flow cytometer, and analysed using CellQuest Pro software (BD Biosciences). The percentage of cells which stained positive for either one or both antibodies was determined.

RNA was extracted from BMDM using Trizol reagent (Invitrogen, Paisley UK), quantified, and 1µg transcribed to cDNA using oligo dT oligonucleotides and Superscript II RNase (Invitrogen). The resultant cDNA was used in PCR reactions with primers designed against cd11b (forward: 5'-CAG ATC AAC AAT GTG ACC GTA TGG-3' and reverse: 5'-CAT CAT GTC CTT GTA CTG CCG C-3') or rev-erb α (forward: 5'-AAT GGC ATG GTG GTA CTG CTG TG-3' and reverse: 5'-GCA TCC GTT GCT TCT CTC TC-3'). RT-PCR confirmed the absence of rev-erb a RNA in cells derived from KO mice, and as expected, both populations were positive for cd11b (Fig. 4a). The purity of the population was confirmed by flow cytometry, with over 90% of cells stained positive for both CD11b and F4/80 (Fig. 4b). KC and MIP-2 production were significantly elevated in KO macrophages (Fig 4c). TNF-α production increased with LPS dose, with a trend for KO macrophages to
exhibit reduced levels compared to WT. IL-6 production increased with LPS and did not differ between genotypes.

Example 4
Surgical specimens were collected, with consent, from patients undergoing lung resection (Wythenshawe Hospital, Manchester, UK). Ethical approval was obtained from the local research and ethics committee. Aveolar macrophages were collected from lung tissue as described previously (19), re-suspended in media and stimulated with LPS (026:B6, 1mg/ml) in the presence or absence of the REV-ERB α ligand GSK4112, 1,1-dimethylethyl N-[(4-chlorophenyl)methyl]-N-[(5-nitro-2-thienyl)methyl]glycinate, (0.1-10 μg/ml in DMSO) 1h prior to LPS challenge (100ng/ml) (20) and supernatants were collected after 24h for ELISAs.

The REV-ERB α ligand GSK4112 significantly inhibited IL-6 secretion in a dose-dependent manner and at 10μM caused a 69 ± 3% inhibition of release. (Fig 5).

Example 5
Anti-inflammatory activity of a Rev-erbα agonist in synchronized human macrophages

Human peripheral blood mononuclear cells were isolated from heparinised blood using a density gradient. The monocyte population was purified by positive selection using anti CD14 magnetic Macs beads. Monocytes were resuspended in RPMI + 10% FCS +L-glutamine + penicillin/streptomycin + 5ng/ml GM-CSF at 1x10^6 cells/ml. Cells were plated in 96 well TC plates at 100μl/well. Plates were incubated at 37°C, 5% CO₂ for 4 days to allow macrophage differentiation.
Media on the cells was replaced with 100 µl/well fresh RPMI + 50% FCS + L-glutamine + penicillin/streptomycin. Plates were incubated at 37°C, 5% CO₂ for 2hrs before the media was replaced with 100 µl/well fresh serum free RPMI + L-glutamine + penicillin/streptomycin.

Rev-erb α agonist GSK4112 (1,1-dimethylethyl N-[(4-chlorophenyl)methyl]-N-[(5-nitro-2-thienyl)methyl]glycinate) was added to the cells at a final concentration of 10 µM. Plates were incubated for 1hr at 37°C before the addition of LPS (Sigma L2654) at a final concentration of 10 ng/ml. After a further 24hr incubation at 37°C supernatants were harvested and stored at -20°C until analysed.

IL-6 was measured using a Luminex assay. Briefly, samples were diluted in PBS+1%BSA and were incubated with anti IL-6 (Endogen M620) coupled fluorescent beads for 2hrs at room temperature. After a wash with PBS+0.05% tween 20, biotinylated anti IL-6 secondary antibody (Endogen M621 B) was added and incubated for 1hr at room temperature. Plates were washed again and streptavidin-PE (Prozyme PJ31 S) added and incubated for a further 30mins. After a final wash the beads were resuspended in Luminex sheath fluid and analysed on the Luminex using StarStation 2.3 software to determine IL-6 levels. Results are shown in Fig. 6.

REV-ERB α ligands, for example, 1,1-dimethylethyl N-[(4-chlorophenyl)methyl]-N-[(5-nitro-2-thienyl)methyl]glycinate; N-Benzyl-L-N-(4-chlorobenzyl)-1-(5-nitrothiophen-2-yl) methanamine; N-Benzyl-L-N-(3,4-dichlorobenzyl)-1-(5-nitrothiophen-2-yl) methanamine; and 2-[(4-chlorobenzyl)-(5-nitrothiophen-2-yl)methyl]amino)-N,N-dimethylacetamide, are also useful as tools for studying the biology of the REV-ERB α receptor in modulation of inflammatory disorders, especially those associated with increased numbers of neutrophils, eosinophils, and macrophages. Thus in a
further aspect of the invention, administration of a REV-ERB α ligand, including the above mentioned ligands, to a REV-ERB α nuclear receptor may be used in the study of the manner in which REV-ERB α induced inflammatory response. Thus, these compounds are also useful tools in the study of how REV-ERB α impacts inflammation, especially those inflammation associated with increased numbers of neutrophils, eosinophils, and macrophages.

All publications, patents and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.
Claims

1. A method of treating inflammatory diseases comprising the administration of a REV-ERB α ligand to a patient in need thereof.

2. A method of treating a disease involving increased neutrophils comprising administering to a patient in need thereof a REV-ERB α ligand.

3. A method of treating a disease involving increased macrophages comprising administering to a patient in need thereof a REV-ERB α ligand.


5. A method of treating COPD, emphysema, cystic fibrosis, acute respiratory distress syndrome, or acute lung injury, comprising administration to a patient in need thereof a REV-ERB α ligand.

6. A method of any one of claims 1 to 5 wherein the REV-ERB α ligand is a REV-ERB α agonist.

7. A method of any one of claims 1 to 5 wherein the REV-ERB α ligand is a REV-ERB α modulator.

8. A method for modulating IL-6 secretion in a patient comprising administering a REV-ERB α ligand.
9. The method of claim 8, wherein the response modulated is IL-6 secretion.

10. The method of claim 8 wherein the modulation is inhibition.

11. The method of claim 8, wherein the preceding claims, wherein the ligand is 1,1-dimethylethyl N-[(4-chlorophenyl)methyl]-N-[(5-nitro-2-thienyl)methyl]glycinate.

12. The method of claim 8, wherein the preceding claims, wherein the ligand is \( \Lambda -\text{Benzyl-}\Lambda -\text{-(4-chlorobenzyl)-1-(5-nitrothiophen-2-yl)methanamine.} \)

13. The method of claim 8, wherein the preceding claims, wherein the ligand is \( \Lambda -\text{Benzyl-}\Lambda -\text{-(3,4-dichlorobenzyl)-1-(5-nitrothiophen-2-yl)methanamine.} \)

14. The method of claim 8, wherein the preceding claims, wherein the ligand is 2-\(((4\text{-chlorobenzyl})\text{((5-nitrothiophen-2-yl)methyl)amino})\text{-N,N-dimethylacetamide.} \)

15. A method for modulating REV-ERB \( \alpha \) induced inflammatory response comprising administration of a REV-ERB \( \alpha \) ligand to a REV-ERB \( \alpha \) nuclear receptor.

16. The method of claim 16, wherein the REV-ERB \( \alpha \) ligand is selected from the group consisting essentially of 1,1-dimethylethyl N-[(4-chlorophenyl)methyl]-N-[(5-nitro-2-thiienyl)methyl]glycinate; \( \Lambda -\text{Benzyl-}\Lambda -\text{-(4-chlorobenzyl)-1-(5-nitrothiophen-2-yl)methanamine;} \) \( \Lambda -\text{Benzyl-}\Lambda -\text{-(3,4-dichlorobenzyl)-1-(5-nitrothiophen-2-yl)methanamine;} \) and 2-\(((4\text{-chlorobenzyl})\text{((5-nitrothiophen-2-yl)methyl)amino})\text{-N,N-dimethylacetamide,} \)

are also useful as tools for studying the biology of the REV-ERB \( \alpha \) receptor in modulation of inflammatory disorders, especially those associated with increased numbers of neutrophils, eosinophils, and macrophages.
Figure 1: Cytokines in BAL following in vivo LPS challenge (measured by bead array).

Values are mean ± SEM. Cytokine levels were compared between genotypes at each dose using one Way ANOVA and post-hoc bonferroni tests. n=6-8, *P<0.05 and ***P<0.005.
Figure 2: Quantification of total BAL cell numbers and types after in vivo LPS challenge. (A) Cell types were differentiated by the physical characteristics 'forward scatter' and 'side scatter' as illustrated. (B-F) Cell numbers in BAL from wild type (grey bars) and rev-erb α−/− mice (hatched bars), values are mean ± SEM. Differences between genotype at each dose were determined using one way ANOVA and post-hoc bonferonni tests, ***P<0.005; n=6-8).
**Figure 3:** Histology and immunohistochemistry on lung sections from control (saline) or LPS (2mg/ml) challenged mice. (A) H and E staining demonstrates the presence of inflammatory cells after LPS challenge (B) Neutrophils (detected using anti-NIMP/R14; deep purple) are present in large numbers around the airways after LPS, but not in control mice. (C) Macrophages (detected using anti-F4/80; brown) are present at similar levels before and after challenge. Lung sections were counterstained with haematoxylin (blue) after IHC, scale bar = 50μm.
Figure 4: Macrophage LPS stimulation. (A) RT-PCR confirms the presence of rev-erb α mRNA in wt but not rev-erb α- BMDM, both genotypes express cd11b mRNA. (B) Flow cytometry demonstrates that over 90% of BMDM stained positive for both CD11b and F4/80. (C) Release of cytokines after LPS challenge of BMDM, as measured by ELISA. Values are mean ± SEM, ANOVA and bonferroni,

*p<0.05  ***p<0.005; n=3.
Figure 5: The effects of a REV-ERB α ligand on the cytokine response of human alveolar macrophages to LPS. (A) Application of the REV-ERB α ligand to LPS stimulated human alveolar macrophages reduced the release of IL-6 at all doses (One way ANOVA, Bonferroni ***P<0.005), but not IL-8 (B).

Values are mean ± SEM, n=4.
Figure 6

Effect of GSK414112A on LPS stimulated human blood macrophages at 10uM - mean of 3 donors
**INTERNATIONAL SEARCH REPORT**

**International application No**
PCT/US 10/46120

**A** CLASSIFICATION OF SUBJECT MATTER

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According to International Patent Classification (IPC) or to both national classification and IPC

**B** FIELDS SEARCHED

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**C** DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<td>the Nuclear Hormone Receptor Rev-erb alpha ACS Chem Biol 02 August 2010, 5(10)</td>
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**D** Further documents are listed in the continuation of Box C

| "A" document defining the general state of the art which is not considered to be of particular relevance |
| "E" earlier application or patent but 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 prior 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 |
| "Z" document member of the same patent family |

**Date of the actual completion of the international search**

20 November 2010 (20.11.2010)

**Date of mailing of the international search report**

03 DEC 2010

**Date**

03 DEC 2010

**Page and number of pages included in the international preliminary report**

Form PCT/ISA/210 (second sheet) (July 2009)

**Name and mailing address of the ISA/US**

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Lee W Young

**PCT Helpdesk** 571-272-3000

**PCT OSP** 571-272-7774
# INTERNATIONAL SEARCH REPORT

**International application No**

PCT/US 10/46120

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**Box No. H Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

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

1. **L** Claims NOS because they relate to subject matter not required to be searched by this Authority, namely:

2. **D** 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. **X** Claims Nos 11-14 because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 64(a)

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**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

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

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

2. **I** As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. **I** 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.

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**Remark on Protest**

- **□** The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

- **□** The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

- **□** No protest accompanied the payment of additional search fees.

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Form PCT/ISA/210 (continuation of first sheet (2)) (July 2Q09)