

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
13 January 2011 (13.01.2011)

(10) International Publication Number
WO 2011/006087 A1

(51) International Patent Classification:
A01N 45/00 (2006.01)

(21) International Application Number:
PCT/US2010/041560

(22) International Filing Date:
9 July 2010 (09.07.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/224,656 10 July 2009 (10.07.2009) US

(71) Applicant (for all designated States except US): **THE REGENTS OF THE UNIVERSITY OF CALIFORNIA** [US/US]; 1111 Franklin Street, Oakland, California 94607-5200 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **PARHAMI, Farhad** [US/US]; UCLA Med-Cardio, Box 951679, 47-123 CHS, Los Angeles, California 90095-1679 (US). **KIM, Woo-Kyun** [KR/CA]; Department of Animal Science, University of Manitoba, Winnipeg, Manitoba R3T 2N2 (CA). **JUNG, Michael E.** [US/US]; UCLA Chem & Biochem, Box 951569, 3505A Mol. Sci. Bldg., Los Angeles, California 90095-1569 (US). **NGUYEN, Khanhlinh** [US/US]; UCLA Chem & Biochem, Box 951569, 3505A Mol. Sci. Bldg., Los Angeles, California 90095-1569 (US).

(74) Agents: **AXELROD, Nancy J.** et al.; **VENABLE LLP**, P.O. Box 34385, Washington, District of Columbia 20043-9998 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

- as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))
- as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))
- of inventorship (Rule 4.17(iv))

Published:

- with international search report (Art. 21(3))

(54) Title: INHIBITION OF PPAR GAMMA EXPRESSION IN PREADIPOCYTE CELLS BY OXYSTEROLS

(57) Abstract: This invention relates, e.g., to methods and agents to inhibit peroxisome proliferator activated receptor expression (PPAR) in preadipocytes.



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INHIBITION OF PPAR GAMMA EXPRESSION IN PREADIPOCYTE CELLS BY OXYSTEROLS

This application claims the benefit of the filing date of U.S. provisional patent application 61/224,656, filed July 10, 2009, which is incorporated by reference in its entirety herein.

Aspects of the invention were made with U.S. government support provided by NIAMS/NIH grant number RO1AR050426. The government has certain rights in the invention.

BACKGROUND INFORMATION

Oxysterols form a large family of oxygenated derivatives of cholesterol that are present in the circulation, and in human and animal tissues. Oxysterols that have been identified in human plasma to date include 7 α -hydroxycholesterol, 24S-hydroxycholesterol, and 4 α - and 4 β -hydroxycholesterol, which are present at concentrations ranging from 5-500 ng/ml. These oxysterols have a variety of half-lives in circulation ranging from 0.5-60 hours, and their levels can be altered by aging, drug interventions, and disease processes. Oxysterols may be formed either by autooxidation, as a secondary byproduct of lipid peroxidation, or by the action of specific monooxygenases, most of which are members of the cytochrome P450 family of enzymes. Examples of these enzymes are cholesterol 7 α -hydroxylase (CYP7A1) that forms 7 α -hydroxycholesterol, cholesterol 25-hydroxylase that forms 25-hydroxycholesterol, cholesterol 24S-hydroxylase (CYP46) that forms 24S-hydroxycholesterol, and others. In addition, oxysterols may be derived from the diet. Cytochrome P450 enzymes are also involved in the further oxidation of oxysterols and their metabolism into active or inactive metabolites that leads to their eventual removal from the system. Certain oxysterols have potent effects on cholesterol metabolism. In addition, the presence of oxysterols in atherosclerotic lesions has prompted studies of their potential role in the pathogenesis of this disorder. A role for specific oxysterols has been implicated in various physiologic processes including cellular differentiation, inflammation, apoptosis, and steroid production.

Recently, several reports have noted the possible role of oxysterols in cellular differentiation. Specific oxysterols induce the differentiation of suitable progenitor cells to human keratinocytes *in vitro*, while the differentiation of suitable progenitors to monocytes can be induced by the oxysterol 7-ketocholesterol. Previous reports by the

present inventors have shown that specific oxysterols induce the differentiation of multipotent mesenchymal cells into osteoblastic cells, while inhibiting their differentiation into adipocytes. Differentiation of keratinocytes by oxysterols is mediated by the nuclear hormone receptor, liver X receptor β (LXR β). LXR α and LXR β , initially identified as
5 orphan nuclear receptors, act as receptors for oxysterols. However, many of the effects of oxysterols are mediated by LXR-independent mechanisms. These include their effects on mesenchymal cells, since activation of LXR by specific LXR ligands inhibited, rather than stimulated, the osteogenic differentiation of mesenchymal cells. Furthermore, marrow stromal cells derived from LXR null mice were able to respond to osteogenic oxysterols
10 as well as their wild type counterparts. Additional oxysterol binding proteins have been reported that can regulate the activity of signaling molecules such as mitogen-activated protein kinase (MAPK).

Hedgehog molecules have been shown to play key roles in a variety of processes including tissue patterning, mitogenesis, morphogenesis, cellular differentiation and
15 embryonic developments. In addition to its role in embryonic development, hedgehog signaling plays a crucial role in postnatal development and maintenance of tissue/organ integrity and function. Studies using genetically engineered mice have demonstrated that hedgehog signaling is important during skeletogenesis as well as in the development of osteoblasts *in vitro* and *in vivo*. In addition to playing a pro-osteogenic role, hedgehog
20 signaling also inhibits adipogenesis when applied to multipotent mesenchymal cells, C3H-10T 1/2.

Hedgehog signaling involves a very complex network of signaling molecules that includes plasma membrane proteins, kinases, phosphatases, and factors that facilitate the shuffling and distribution of hedgehog molecules. Hedgehog molecules are produced from
25 a subset of producing/signaling cells that are involved in its synthesis, autoprocessing and lipid modification. Lipid modification of hedgehog, which appears to be essential for its functionality, involves the addition of a cholesterol molecule to the C-terminal domain of the auto-cleaved hedgehog molecule and palmitoylation at its N-terminal domain. Additional accessory factors help shuttle hedgehog molecules to the plasma membrane of
30 the signaling cells, release them into the extracellular environment, and transport them to the responding cells.

In the absence of hedgehog molecules, Patched (Ptch), present on the plasma membrane of the responding cells, keeps hedgehog signaling in a silent mode by

inhibiting the activity of another plasma membrane associated signal transducer molecule, Smoothed (Smo). In the presence of hedgehog, the inhibition of Smo by Ptch is alleviated and Smo transduces the signal for the regulation of transcription of hedgehog-regulated genes. This transcriptional regulation involves, in part, the Ci/Gli transcription factors that enter the nucleus from the cytoplasm after a very intricate interaction between the members of a complex of accessory molecules that regulate Gli and its conversion from a 75 kd transcriptional repressor to a 155 kd transcriptional activator. The details of this highly complex signaling network have been extensively reviewed. (Cohen (2003) *Am J Med Gen* 123A, 5-28; Mullor *et al.* (2002) *Trends Cell Bio* 12, 562-569).

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BRIEF DESCRIPTION OF THE FIGURES

Figure 1A presents images of M2 cells after various treatments.

Figure 1B presents a bar graph illustrating the number of adipocytes after various treatments.

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Figure 2 presents bar graphs illustrating PPAR γ mRNA expression by M2 cells after various treatments. Figure 2A: after 24 hours. Figure 2B: after 48 hours. Figure 2C: after 96 hours.

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Figure 3 presents bar graphs illustrating C/EBP α mRNA expression by M2 cells after various treatments. Figure 3A: after 24 hours. Figure 3B: after 48 hours. Figure 3C: after 96 hours.

Figure 4 presents bar graphs illustrating aP2 mRNA expression by M2 cells after various treatments. Figure 4A: after 24 hours. Figure 4B: after 48 hours. Figure 4C: after 96 hours.

Figure 5 presents bar graphs illustrating reporter activity by M2 cells after various treatments. Figure 5A: M2 cells transfected with PPRE-TK-LUC or pTK-LUC and pTK-Renilla-Luciferase plasmid. Figure 5B: M2 cells transfected with PPRE-TK-LUC or pTK-LUC and CMX PPAR γ expression plasmid and pTK-Renilla-Luciferase plasmid. Figure 5C: M2 cells transfected with PPRE-TK-LUC or pTK-LUC and CMX PPAR γ expression plasmid and pTK-Renilla-Luciferase plasmid.

Figures 6A and 6B present bar graphs illustrating number of adipocytes generated from M2 cells after various treatments.

5 Figures 6C and 6D present bar graphs illustrating relative expression of PPAR γ by M2 cells after various treatments.

Figure 6E presents bar graphs illustrating relative luciferase activity in M2 cells after various treatments.

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Figure 7A presents bar graphs illustrating the number of adipocytes generated from M2 cells after various treatments.

15 Figure 7B presents bar graphs illustrating PPAR γ expression by M2 cells after various treatments.

Figure 8 shows that 20(S)-hydroxycholesterol (20S) inhibits adipogenic differentiation and PPAR γ expression in 3T3-L1 preadipocytes. 3T3-L1 cells were treated with control vehicle, DMI, (1 μ M dexamethasone, 0.5mM 3-isobutyl-1-methylxanthine and 15 μ g/ml insulin), DMI + 20S, or 5 μ M 20S. Fig. 8a shows adipocyte formation, as visualized by Oil-red O staining on Day 10. Fig. 8b shows PPAR γ mRNA expression on Day 4, measured by quantitative real-time PCR. The values are reported as the mean of triplicate determinations \pm SD ($p < 0.0001$ for control vs. DMI and DMI vs. DMI+20S).

25 Figure 9 shows the effects of 20(S)-hydroxycholesterol (20S) on Hh target genes Gli1 and Patched1 in 3T3-L1 preadipocytes. 3T3-L1 cells were treated with control vehicle, 5 μ M 20S for 96hr. Gli1 and Patched1 mRNA expression was measured by quantitative real-time PCR. Fold changes in gene expression relative to control are reported as the mean of triplicate determinations \pm SD (a, b: $p < 0.0001$ control or 20S).

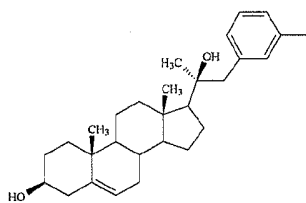
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DESCRIPTION

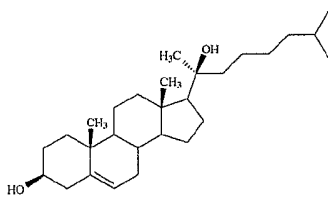
This application relates, *e.g.*, to a method for inhibiting expression of a peroxisome proliferator activated receptor (PPAR) in a cell, in particular in a preadipocyte cell,

comprising contacting at least one oxysterol compound with the cell in an amount effective to inhibit expression of the PPAR and, optionally, measuring the amount of inhibition of the PPAR. The PPAR can be PPAR gamma. In aspects of the invention, the at least one oxysterol compound inhibits adipogenesis of the cell; is an activator of the hedgehog signaling pathway; inhibits the dysregulated differentiation of the cell into an adipocyte; and/or inhibits the expression of a PPAR target gene, such as adipocyte protein 2 (aP2) or lipoprotein lipases (LPL). In a method of the invention, the preadipocyte cell may *in vitro*, *ex vivo*, or in a subject, including a human.

The oxysterol compound can be, for example, 20(S)-hydroxycholesterol (*e.g.*, at a concentration of about 5 μ M), 22(R)-hydroxycholesterol, 22(S)-hydroxycholesterol, 25-hydroxycholesterol, 25(S)-hydroxy cholesterol, 5-cholesten-3-beta-20-alpha-diol-3-acetate, 24-hydroxycholesterol, 24(S)-hydroxy- cholesterol, 24(S),25-epoxycholesterol, 26-hydroxycholesterol, 4-beta-hydroxy- cholesterol, pregnenolone,

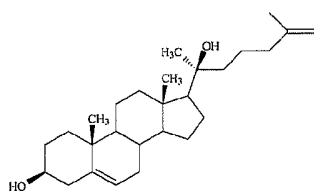


Oxy 11

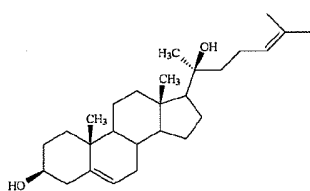


Oxy 12

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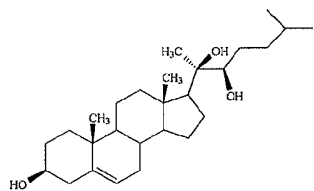
Oxy 13



Oxy 14

, or

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Oxy 16

or analogs thereof, derivatives thereof, or combinations thereof.

The expression of PPAR in the cell can be inhibited relative to a baseline value. For example, the baseline value can be the level of PPAR expression (*e.g.*, the amount or activity of the protein, or the amount of mRNA that encodes PPAR) in cells in culture that are stimulated to express PPAR by any of a variety of well-known PPAR stimulatory agents. Such stimulatory agents can include, *e.g.*, a PPAR agonist; the well-characterized adipogenic inducer, DMI (a cocktail of dexamethasone, 3-isobutyl-1-methylxanthine and insulin); a member of the drug class of thiazolidinediones (*e.g.*, troglitazone, pioglitazone, or rosiglitazone); or the like. Some of the discussion herein refers to "PPAR agonists"; it is to be understood that the PPAR stimulatory agents listed above, and others, are also included in such discussions. Such PPAR stimulatory agents can induce adipogenesis by activating the adipogenic differentiation program at any of a variety of steps at different levels in the complex differentiation program.

In one embodiment of the invention, "inhibiting" means reducing or decreasing expression or activity by 50%, 60%, 70%, 80%, 90%, 99%, or 100%, etc. compared to control conditions (*e.g.*, contacting a cell or subject with an oxysterol versus no contact).

The baseline value can be indicative of the level of PPAR expression in a subject exhibiting a pathology or condition associated with excessive levels (increased amounts, aberrant levels) of adipogenesis and/or with excessive accumulation of intracellular and/or extracellular fats and/or lipids (*e.g.*, obesity or other well-recognized conditions, including osteoporosis, diabetes, aging, xanthomas, etc.), prior to treatment with an oxysterol compound to reduce expression to a lower, *e.g.* healthier, level. The baseline value can be an average or mean of the measurements of a pool or population of such subjects. Suitable baseline values can be determined by those of skill in the art without undue experimentation. Suitable baseline values may be available in a database compiled from the values and/or may be determined based on published data or retrospective studies of samples from subjects, and other information that would be apparent to a person of ordinary skill implementing a method of the invention.

Another aspect of the invention is a method for treating or preventing a condition in a subject that is associated with excessive amounts (increased amounts, aberrant levels) of adipogenesis and/or with excessive accumulation of intracellular and/or extracellular fats and/or lipids, comprising administering to the subject an amount of an oxysterol compound that is effective to treat or prevent the condition. In one embodiment of the invention, the oxysterol compound has been shown to be in an amount that is effective to

inhibit PPAR expression in a preadipocyte (*e.g.*, *in vitro*, *ex vivo*, or *in vivo*). In embodiments of the invention, the oxysterol compound is administered systemically or locally to a target tissue of interest. The condition can be, for example, obesity, osteoporosis, diabetes, muscular atrophy, xanthoma formation, or aging.

5 A method of the invention can be applied to treating a subject having any of a variety of PPAR expression related conditions, which will be evident to a skilled worker.

 Another aspect of the invention is a kit that includes a dosage form of a pharmaceutical composition comprising an oxysterol compound effective to inhibit expression of PPAR in a preadipocyte; the compound may be in a container. The
10 oxysterol compound may be one of more of any of the oxysterols described herein, or others. The kit can include a label indicating use in treating or preventing one or more of the conditions described herein that are associated with excessive levels of adipogenesis and/or with excessive accumulation of intracellular and/or extracellular fats and/or lipids, *e.g.*, obesity, osteoporosis, diabetes, muscular atrophy, aging, or xanthoma formation in an
15 animal or a human.

 Another aspect of the invention is a method for identifying an oxysterol compound that inhibits expression of PPAR, comprising screening a candidate oxysterol compound for the ability to inhibit expression of PPAR in a preadipocyte cell in an *in vitro* assay. The method can comprise, *e.g.*, measuring the ability of the candidate oxysterol to reduce
20 the stimulation of PPAR expression by a PPAR-stimulatory agent; and selecting a candidate oxysterol compound that measurably inhibits the PPAR expression. For example, a PPAR stimulatory agent can be contacted with the cell in the presence and absence of a candidate oxysterol; and a candidate can be selected for which the amount of expression of PPAR is at least about 3-fold less (*e.g.*, at least about 10-fold, or 100-fold
25 less) when measured in the presence of the candidate oxysterol than when measured in the absence of the candidate oxysterol.

 The term "about," as used herein, means plus or minus 10%.

 Much of the discussion herein is directed to the effect of oxysterols on multipotent
30 bone marrow stromal cells, sometimes referred to herein as mesenchymal stem cells (MSCs). These cells are present in a variety of tissues and are prevalent in bone marrow stroma. However, much of this discussion applies as well to the effects of oxysterols on preadipocytes. Of course, the discussion of the ability of oxysterols to stimulate

osteogenesis in MSCs does not apply to preadipocytes, because preadipocytes, as defined herein, are already at least partially committed to become adipocytes and thus cannot differentiate into osteoblasts.

Age-related bone loss is associated with a progressive decrease in bone formation and an increase in adipogenesis in the bone marrow, increasing the risk of bone fractures. MSCs are common progenitors of osteoblasts and adipocytes, and a potential reciprocal relationship between osteogenic and adipogenic differentiation of MSCs has been suggested. Furthermore, an increase in adipose tissue volume and a decrease in trabecular bone volume in bone marrow have been observed with aging and in patients with osteoporosis. However, the molecular mechanisms underlying the reciprocal relationship between osteogenic and adipogenic differentiation during aging and pathological states are not well understood.

Preadipocytes are derived from mesenchymal stem cells and can be either completely determined or partially determined to become adipocytes. Without wishing to be bound by any particular mechanism, it is suggested that there are multiple populations of preadipocyte cells, some with the ability to differentiate into many cell types that come from the mesenchymal origin, some that may be able to differentiate into several but not all lineages of cells, and some that can differentiate only into adipocytes. For example, a predetermined adipocyte such as the 3T3-L1 cell line is unipotent, meaning that it can only spontaneously differentiate into an adipocyte and not other cell types. Therefore the term preadipocyte, as used herein, refers to a cell that can become an adipocyte, but does not limit the level of multipotency of that cell, except that multipotent cells, such as MSCs, and pluripotent cells, such as embryonic stem cells or induced pluripotent stem cells (iPSCs), are excluded from the term, preadipocytes, as it is used herein.

As used herein, the term "pluripotent" refers to the ability of a cell to form all the lineages of the body or soma (the embryo proper). For example, embryonic stem cells are a type of pluripotent stem cells that are able to form cells from each of the three germ layers: the ectoderm, the mesoderm and the endoderm. As used herein, the term "multipotent" refers to the ability of an adult stem cell to form multiple cell types of one lineage. For example, hematopoietic stem cells are capable of forming all cells of the blood lineage, *e.g.*, lymphoid and myeloid cells. Another non-limiting example of multipotent stem cells are mesenchymal stem cells, which are capable of forming

osteoblasts, chondrocytes, fibroblasts, myocytes, adipocytes, and other mesenchymal cell types.

Peroxisome proliferator-activated receptor γ (PPAR γ) is a member of the nuclear hormone receptor superfamily and a key regulator of adipogenic differentiation. In early adipogenic differentiation, CCAAT/enhancer-binding protein a (C/EBP β) and C/EBP δ induce the expression of PPAR γ and C/EBP α . PPAR γ and C/EBP α regulate each other's expression through a positive feedback mechanism and induce other adipogenic genes that establish terminal adipogenic differentiation. PPAR γ consists of two protein isoforms produced by alternative promoter use and splicing. PPAR γ 1 is expressed at low levels in many tissues, whereas PPAR γ 2 is expressed at high levels in adipose tissue. The introduction of PPAR γ 2 into fibroblastic cells using retroviral infection stimulates adipocyte differentiation, whereas PPAR γ null embryonic stem (ES) cells fail to differentiate into adipocytes.

PPAR γ inhibition can induce a shift in MSC differentiation from the adipogenic to the osteogenic pathway. Oxysterols can be used to shift MSC differentiation pathways. Oxysterols, a large family of 27-carbon oxygenated products of cholesterol, are present in the circulation and in human and animal tissues, and can be formed from cholesterol by either enzymatic or nonenzymatic oxidation. Oxysterols have been identified as bioactive compounds involved in various biological and pathological processes, such as cholesterol efflux, lipoprotein metabolism, calcium uptake, cell differentiation, atherosclerosis, and apoptosis. We previously reported that specific oxysterols including 20(S)-hydroxycholesterol (20S) induce osteoblastic differentiation markers, such as alkaline phosphatase activity, osteocalcin expression, and matrix mineralization in murine M2-10B4 (M2) MSCs. See, e.g., Kha *et al.* (2004) *J Bone Miner Res* 19, 830-840; Richardson *et al.* (2007) *J Cell Biochem* 100, 1131-1145. Furthermore, the osteogenic oxysterols inhibit adipocyte formation and the expression of adipogenic differentiation marker genes, such as lipoprotein lipase (LPL) and adipocyte-specific fatty acid binding protein 2 (ap2). See, e.g., Kha *et al.* (2004) (*supra*). Recently, we reported that oxysterols are novel activators of the hedgehog signaling pathway (Dwyer *et al.* (2007) *J Biol Chem* 282, 8959-8968. Inhibitory effects of oxysterols on adipogenic differentiation of MSCs may be mediated by hedgehog signaling.

Because 20S is a naturally occurring osteogenic oxysterol that we have identified, in this study we further investigated the molecular mechanisms by which it inhibits

adipogenic differentiation of MSCs. We found that similar to sonic hedgehog (Shh), 20S inhibited PPAR γ mRNA expression induced by the thiazolidinedione, troglitazone (Tro), which stimulates adipogenesis by activating PPAR γ . The inhibitory effects of 20S and Shh on PPAR γ expression were completely blocked by the hedgehog signaling inhibitor, cyclopamine. Furthermore, 20S and Shh significantly inhibited PPAR γ promoter activity
5 induced by C/EBP α overexpression. However, 20S did not inhibit the transcriptional activity of PPAR γ . This suggests that the inhibition of adipogenesis by 20S may be mediated predominantly through a hedgehog pathway-dependent mechanism(s).

Increased adipogenesis is associated with a variety of conditions including obesity,
10 osteoporosis, and xanthoma formation. The transcription factor peroxisome proliferator activated receptor gamma (PPAR γ) is understood to control the expression of target genes that allow for the formation of adipocytes. Therefore PPAR γ antagonists have the potential to have clinical potential for the treatment of conditions associated with increased adipogenesis. For example, PPAR γ antagonists have potential as combined anti-
15 obesity and anti-diabetic drugs. Few molecules have been heretofore been shown to inhibit the expression of PPAR γ . Compounds reported as having PPAR γ antagonistic activity include the following: SR202, Oxazole, Tesaglitazar (by AstraZeneca), compounds 501516 and 590735 (by GlaxoSmithKline), T0070907 (Cayman Chemical), bisphenol A diglycidylether (BADGE). To our knowledge, none of these compounds
20 belongs to the oxysterol class of molecules.

This application presents certain oxysterols that can inhibit the expression of PPAR γ . For example, oxysterols that activate the hedgehog signaling pathway and that have osteogenic and anti-adipogenic properties can inhibit the expression of PPAR γ . Osteogenic oxysterols can inhibit the mRNA expression of PPAR γ . Such inhibition can be
25 useful for controlling the dysregulated differentiation of cells into adipocytes. Oxysterol molecules effective in inhibiting PPAR γ expression, analogs of these molecules, and/or active regions of these molecules, alone or as a part of a large molecule, e.g. a carrier molecule, can be systemically or locally administered to a subject to inhibit PPAR γ expression in a target tissue of interest, for example, to decrease differentiation of cells
30 into adipocytes.

Thus, adipogenesis is associated with increased expression of PPAR γ and its target genes, adipocyte protein 2 (aP2) and lipoprotein lipase (LPL). The osteogenic oxysterol 20(S)-hydroxycholesterol (20S) inhibits the mRNA expression of PPAR γ in MSCs. The

inhibition is at the level of mRNA expression: 20S does not inhibit the transcriptional activity of exogenous PPAR γ when expressed in cells using an artificial expression vector. Therefore, inhibition appears to be at a transcriptional level. Because of the association of expression of PPAR γ with adipogenesis, oxysterols exhibiting anti-adipogenic and osteogenic characteristics, in addition to 20(S)-hydroxycholesterol (20S), can similarly inhibit the expression and/or activity of PPAR γ in MSCs and other cell types. Furthermore, natural and synthetic analogs and molecules incorporating active portions of such anti-adipogenic and osteogenic oxysterols are expected to similarly inhibit the expression and/or activity of PPAR γ in marrow stromal cells and other cell types.

10 Oxysterols exhibiting anti-adipogenic and osteogenic characteristics, their natural and synthetic analogs, and molecules incorporating active portions of anti-adipogenic and osteogenic oxysterols can also modulate the expression and/or activity of other members of the PPAR family of proteins, including but not limited to PPAR α and PPAR δ (also known as PPAR γ).

15 Because of the association of expression of PPAR γ with adipogenesis, the discovery that certain oxysterol compounds exhibit anti-adipogenic and osteogenic characteristics leads to the possibility of using oxysterol compounds in addition to 20(S)-hydroxycholesterol (20S) that can similarly inhibit the expression and/or activity of PPAR γ in MSCs and other cell types. Furthermore, natural and synthetic analogs and molecules incorporating active portions of such anti-adipogenic and osteogenic oxysterols can similarly inhibit the expression and/or activity of PPAR γ in marrow stromal cells and other cell types. Oxysterols exhibiting anti-adipogenic and osteogenic characteristics, their natural and synthetic analogs, and molecules incorporating active portions of anti-adipogenic and osteogenic oxysterols can further modulate the expression and/or activity of other members of the PPAR family of proteins, including but not limited to PPAR α and PPAR δ (also known as PPAR β). Such oxysterol compounds can be identified by measuring their effect *in vitro* or in other assays of PPAR expression or activity, as taught in this application.

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For example, anti-adipogenic oxysterols, oxysterol analogs, or active portions of oxysterols can be administered to cells, such as cells *in vitro* or in a human or animal subject to be treated, in an amount effective to inhibit a PPAR-mediated response in the cell (*e.g.*, in a preadipocyte cell). In one embodiment, anti-adipogenic oxysterols are administered to control the dysregulated differentiation of a cell into an adipocyte. In

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another embodiment, anti-adipogenic oxysterols are administered to a human or animal subject to be treated to treat or prevent diseases and disorders associated with PPAR over-expression, including dysregulated and excessive accumulation of intracellular and/or extracellular fats and/or lipids and/or excessive adipogenesis. In another embodiment,
5 anti-adipogenic oxysterols are administered to treat or prevent diseases and disorders such as obesity, osteoporosis, diabetes, muscular atrophy, aging, and/or xanthoma formation in an animal or a human.

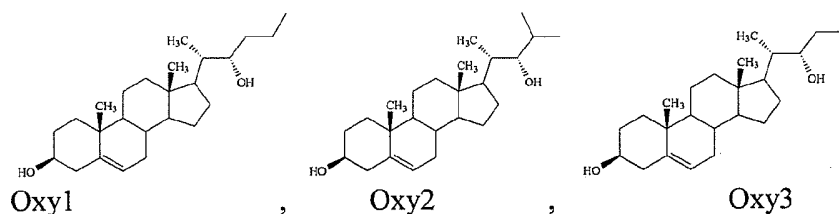
Thus, in a method according to the invention, PPAR expression inhibiting oxysterol compounds can be administered to treat a physiological and/or pathological
10 condition in which PPAR is a key regulator and target for intervention, for the treatment of a human or animal disease.

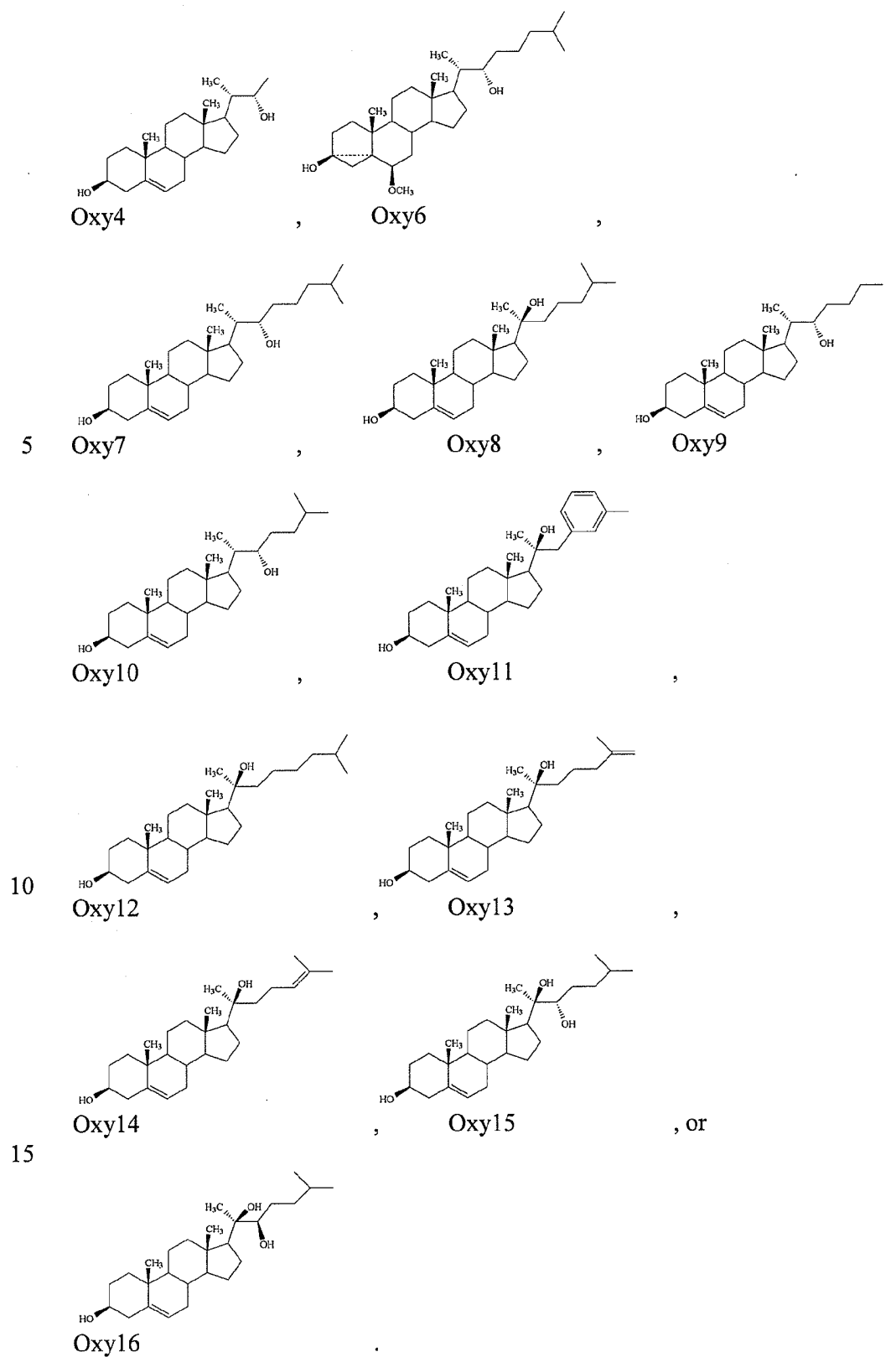
A subject can be selected for treatment by administration of PPAR expression inhibiting oxysterol compounds (oxysterols, oxysterol analogs, or active portions of oxysterols), for example, on the basis of the subject having a condition, disease, or
15 disorder related to PPAR expression, (otherwise referred to as a PPAR expression related condition), or on the basis of the subject having a measured abnormal level of PPAR expression, for example, systemically, in a region of tissue, or in cells, or on the basis of other diagnostic tests. During a course of treatment by administration of anti-adipogenic oxysterol compounds (*e.g.*, oxysterols, oxysterol analogs, or active portions of oxysterols),
20 the treatment can be modified. For example, a dosage can be increased or decreased or terminated if the subject's measured level of PPAR protein expression moves into a normal range, as determined systemically, in a region of tissue, or in cells, or on the basis of other diagnostic tests, such as inhibition of adipogenesis or serum markers indicative of adipogenesis including, but not limited to, adiponectin, leptin, and triglycerides. Thus, a
25 PPAR expression related condition can be identified in a subject by measuring an abnormal level of PPAR expression and/or fat cell formation in the adipose tissue, as well as non-adipose tissues including, but not limited to, bone, bone marrow, skeletal muscle, and organs, such as the liver, heart, and kidney, and the oxysterol compounds of the invention can be administered to the subject to bring the level into a normal range. PPAR
30 expression can be measured directly, for example, in an *in vitro* study or in a cell culture obtained by biopsy of a tissue of interest in an animal or a human subject. PPAR expression can be measured indirectly, for example, in a non-invasive procedure, such as through an X-ray of an animal or a human subject and/or analysis of symptoms and/or

indications by a medical practitioner. For example, an X-ray may indicate excessive fat in a tissue, which may indicate overexpression of PPAR. The PPAR expression related conditions may be, for example, obesity, osteoporosis, diabetes, muscular atrophy, aging, and other conditions associated with increased adipogenesis, as well as xanthoma formation and other conditions associated with dysregulated and excessive accumulation of intracellular and/or extracellular fats and/or lipids.

A kit of the invention can include an oxysterol, such as an anti-adipogenic oxysterol, effective to inhibit expression of a PPAR protein in a preadipocyte. The oxysterol can be formulated as a pharmaceutical composition. The kit can include a label indicating use in treating and/or preventing a condition, disease, or disorder, such as obesity, osteoporosis, diabetes, muscular atrophy, aging, or xanthoma formation in a subject, such as an animal or a human.

In one embodiment of the present invention, a candidate oxysterol, oxysterol analog, or active portion of an oxysterol is screened for the ability to inhibit expression of a PPAR protein in a preadipocyte cell an *in vitro* assay. A candidate oxysterol, oxysterol analog, or active portion of an oxysterol can be selected that inhibits PPAR expression. The candidate oxysterol, oxysterol analog, or active portion of an oxysterol screened and/or selected can be a compound that has not previously been isolated, purified, or synthesized, or can be a compound that has not previously been recognized as having hedgehog activating, anti-adipogenic, and/or osteogenic characteristics. For example, the candidate oxysterol, oxysterol analog, or active portion of an oxysterol screened and/or selected can be other than 5-cholesten-3-beta-20-alpha-diol-3-acetate, 4- β -hydroxycholesterol, 7-ketocholesterol, 7-ketohydroxycholesterol, 7 α -hydroxycholesterol, 20(S)-hydroxycholesterol, 22(R)-hydroxycholesterol, 22(S)-hydroxycholesterol, 24-hydroxycholesterol, 24(S)-hydroxycholesterol, 24(S),25-epoxycholesterol, 25-hydroxycholesterol, 25(S)-hydroxycholesterol, 26-hydroxycholesterol, pregnenolone,





The oxysterol compound can be present in various forms and, if appropriate, as a pharmaceutically acceptable acid, base, or salt form.

A screening method in the invention can comprise contacting a PPAR agonist and the candidate oxysterol, oxysterol analog, or active portion of an oxysterol with a preadipocyte cell and measuring the level of PPAR protein expression. The level of PPAR protein expression by the cell contacted with the PPAR agonist and the candidate can then
5 be compared to the level of PPAR protein expression in a cell of the same type contacted with PPAR agonist, but not the candidate oxysterol, oxysterol analog, or active portion of an oxysterol. The ratio of the level of PPAR protein expression by the cell contacted with PPAR agonist, but not the candidate, to the level of PPAR protein expression by the cell contacted with the PPAR agonist and the candidate oxysterol, oxysterol analog, or active
10 portion of an oxysterol can be determined. The ratio can be used as a metric of PPAR expression inhibition. A candidate oxysterol, oxysterol analog, or active portion of an oxysterol can be selected for exhibiting PPAR expression inhibition if the ratio of the level of PPAR protein expression by the cell contacted with PPAR agonist, but not the candidate, to the level of PPAR protein expression by the cell contacted with the PPAR
15 agonist and the candidate oxysterol, oxysterol analog, or active portion of an oxysterol is greater than a predetermined ratio. For example, the predetermined ratio can be chosen to be 3, so that the level of PPAR protein expression by the cell contacted with PPAR agonist, but not the candidate, must be three-fold greater than the level of PPAR protein expression by the cell contacted with the PPAR agonist and the candidate oxysterol,
20 oxysterol analog, or active portion of an oxysterol for the candidate to be selected. Alternatively, the predetermined ratio can be chosen to be 10, 100, or another value that one of skill in the art deems appropriate. Stated otherwise, the oxysterol compound may reduce PPAR expression to, *e.g.*, about 1%, 5%, 10%, 30%, or a higher or lower level compared to the baseline level of expression. The cells used in the method can be
25 preadipocytes. The level of PPAR protein expression measured can be the level of PPAR γ protein. The PPAR agonist used can be troglitazone.

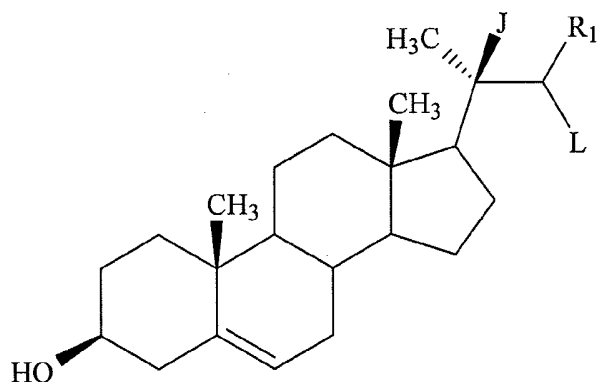
The oxysterols used can be natural or synthetic. The oxysterols can exhibit any of a variety of activities, including the stimulation of osteomorphogenesis or osteoproliferation, and/or the inhibition of adipocyte morphogenesis or adipocyte
30 proliferation, and thus can be used to treat conditions mediated by, or exhibiting aberrant expression of, those physiological phenomena. Certain oxysterols act by stimulating the hedgehog (Hh) signaling pathway. Thus oxysterols, including naturally occurring molecules as well as synthetic ones, can enhance this pathway, either *in vitro*, *ex vivo*, or

in vivo (in a subject) and can be used to treat conditions mediated by elements of the Hh pathway.

Advantages of oxysterols of the invention and methods for using them, *e.g.* for the treatment of suitable subjects, include that the compounds are inexpensive to manufacture, can be easily administered (*e.g.* locally or systemically), and exhibit great efficacy and potency. Bone morphogenic proteins (BMPs) can be used to enhance bone healing, but very large amounts of those proteins are required. Because oxysterols of the invention act synergistically with certain BMPs, lower doses of the proteins are required when they are co-administered with an oxysterol of the invention. This is another advantage of oxysterols of the invention. In some embodiments, administration of the compounds of the invention allows one to circumvent surgery, which can lead to scarring, *e.g.* in cosmetically sensitive areas.

One aspect of the invention is an oxysterol (*e.g.*, an isolated oxysterol) represented by Formula 1.

15



Formula 1

In Formula 1, J can be hydrogen (H) or hydroxyl (OH), L can be hydrogen (H) or hydroxyl (OH), and R₁ can be a linear or branched alkane of from 1 to 6 carbons, a linear or branched alkene of from 2 to 6 carbons, or phenyl optionally substituted with methyl. For example, at least one of J and L can be hydroxyl (OH) and/or at least one of J and L can be hydrogen (H). For example, R₁ can be other than 3-methylbutyl. For example, when J is OH, R₁ can be other than 3-methyl-2-butenyl, and when L is OH, R₁ can be other than n-propyl.

25

In one embodiment of the invention, J is hydroxyl (OH) and L is hydrogen (H). R₁ can be an alkane of from 5 to 6 carbons, for example, an alkane of from 5 to 6 carbons other than 3-methylbutyl. For example, R₁ can be 4-methylpentyl (Oxy 12). R₁ can be an alkene of from 5 to 6 carbons, for example, an alkene of from 5 to 6 carbons other than 3-methyl-2-butenyl. For example R₁ can be 3-methyl-3-butenyl (Oxy 13). R₁ can be phenyl optionally substituted with methyl. For example, R₁ can be 3-methylphenyl (Oxy 11).

In another embodiment, J is hydrogen (H) and L is hydroxyl (OH). R₁ can be an alkane of from 1 to 6 carbons. For example, R₁ can be methyl (Oxy 4), ethyl (Oxy 3), n-butyl (Oxy 9), or 4-methylpentyl (Oxy 7).

10 In another embodiment, J is hydroxyl (OH) and K is hydroxyl (OH). R₁ can be an alkane of from 1 to 6 carbons. For example, R₁ can be 3-methylbutyl (Oxy 15 and Oxy 16).

In another embodiment, a compound has Formula I and J is H or OH and L is H or OH. At least one of J and L is H and at least one of J and L is OH. R₁ is selected from the group consisting of alkane of from 1 to 6 carbons, alkene of from 2 to 6 carbons, and phenyl optionally substituted with methyl. R₁ is not 3-methylbutyl. When J is OH, R₁ is not 3-methyl-2-butenyl. When L is OH, R₁ is not n-propyl.

20 One embodiment is a pharmaceutical composition that comprises a compound having Formula I and a pharmaceutically acceptable carrier. J is H or OH, and L is H or OH. At least one of J and L is OH. R₁ is selected from the group consisting of alkane of from 1 to 6 carbons, alkene of from 2 to 6 carbons, and phenyl optionally substituted with methyl. When one of J and L is H, R₁ is not 3-methylbutyl. In another embodiment, the pharmaceutical composition further includes at least one additional oxysterol.

25 In one embodiment, the pharmaceutical composition includes at least two of Oxy 3, Oxy 4, Oxy 7, Oxy 9, Oxy 11, Oxy 12, Oxy 13, Oxy 14, and Oxy 15. The pharmaceutical composition may further comprise at least one of 20(S)-hydroxycholesterol, 22(S)-hydroxycholesterol, or 22(R)-hydroxycholesterol, or any other oxysterol. In one embodiment, the pharmaceutical composition includes Oxy 16.

30 Another aspect of the invention is a complex (*in vitro*, *ex vivo*, or *in vivo*) comprising an oxysterol of the invention and any of variety of intracellular oxysterol binding molecules (*e.g.*, proteins, receptors, etc.), examples of which will be evident to the skilled worker.

As used herein, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. For example, "an" oxysterol" includes multiple oxysterols, *e.g.* 2, 3, 4, 5 or more oxysterols, which can be the same or different.

Another aspect of the invention is a combination or pharmaceutical composition
5 comprising an oxysterol of the invention (optionally in combination of other agents as discussed above) and at least one additional agent, selected, *e.g.*, from the group consisting of parathyroid hormone, sodium fluoride, insulin-like growth factor I (ILGF-I), insulin-like growth factor II (ILGF-II), transforming growth factor beta (TGF- β), a cytochrome P450 inhibitor, a phospholipase activator, arachadonic acid, a COX enzyme
10 activator, an osteogenic prostanoid, an ERK activator, BMP 2, 4, 7 and 14.

Another aspect of the invention is a kit for performing any of the methods discussed herein, comprising one or more oxysterols of the invention, individually or in combination with one another, or in combination with naturally occurring oxysterols and/or with BMPs or other agents noted herein, optionally packaged in one or more
15 containers. When the kit is for treating a subject, the oxysterol(s) may be in the form of a pharmaceutically acceptable composition.

Another aspect of the invention is a method for modulating a hedgehog (Hh) pathway mediated response in a cell (*e.g.*, a preadipocyte cell) or tissue comprising the cells (*e.g.*, preadipocyte cells), comprising contacting the cell or tissue with an effective
20 amount of an oxysterol or a pharmaceutical composition of the invention. The cell or tissue may be *in vitro* or in a subject (*in vivo*). In the latter case, the subject can be one who would benefit, *e.g.*, from the stimulation of osteomorphogenesis, osteoproliferation or hair growth; or the inhibition of adipocyte morphogenesis or adipocyte proliferation.

A "subject," as used herein, includes any animal that exhibits a symptom of a
25 condition that can be treated with an oxysterol of the invention. Suitable subjects (patients) include laboratory animals (such as mouse, rat, rabbit, or guinea pig), farm animals, and domestic animals or pets (such as a cat or dog). Non-human primates and, preferably, human patients, are included. Typical subjects include animals that exhibit aberrant amounts (lower or higher amounts than a "normal" or "healthy" subject) of one or
30 more physiological activities that can be modulated by an oxysterol of the invention (*e.g.* stimulation of osteomorphogenesis or osteoproliferation, and/or the inhibition of adipocyte morphogenesis or adipocyte proliferation). Subjects exhibiting non-pathogenic conditions, such as alopecia, are also included. The ability of an oxysterol to "modulate" a

response, as used herein, includes the ability to increase or to decrease the level of the response compared to the response elicited in the absence of the oxysterol. The aberrant activities may be regulated by any of a variety of mechanisms, including activation of a hedgehog activity, etc. The aberrant activities can result in a pathological condition.

5 An "effective amount," as used herein, includes an amount that can bring about a detectable effect. A "therapeutically effective amount," as used herein, includes an amount that can bring about a detectable therapeutic effect (*e.g.* the amelioration of a symptom).

Another aspect of the invention is a method for treating a subject suffering from a condition known to be mediated by oxysterols or by the hedgehog pathway, comprising
10 administering to the subject an effective amount of an oxysterol or a pharmaceutical composition of the invention. Some such conditions are discussed elsewhere herein.

Another aspect of the invention is a method for inducing osteoblastic differentiation of a mammalian mesenchymal stem cell, comprising contacting the cell with an effective amount of an oxysterol or a pharmaceutical composition of the
15 invention. This method can further comprise treating the mammalian mesenchymal cell with at least one secondary agent, selected from the group consisting of parathyroid hormone, sodium fluoride, insulin-like growth factor I (ILGF-I), insulin-like growth factor II (ILGF-II), transforming growth factor beta (TGF- β), a cytochrome P450 inhibitor, a phospholipase activator, arachadonic acid, a COX enzyme activator, an osteogenic
20 prostanoid and an ERK activator.

Other aspects of the invention using an oxysterol or a pharmaceutical composition of the invention include methods for (1) stimulating a mammalian cell (*e.g.* a mesenchymal stem cell, an osteoprogenitor cell or a cell in a calvarial organ culture) to express a level of a biological marker of osteoblastic differentiation (*e.g.* an increase in at
25 least one of alkaline phosphatase activity, calcium incorporation, mineralization or expression of osteocalcin mRNA) which is greater than the level of the biological marker in an untreated cell; (2) treating a subject (patient) to increase the differentiation of MSCs into osteoblasts; (3) treating a subject to induce bone formation (to increase bone mass); or (4) treating a patient exhibiting clinical symptoms of osteoporosis. Methods for treating
30 a subject may comprise administering an oxysterol or a pharmaceutical composition of the invention at a therapeutically effective dose, in an effective dosage form, and at a selected interval to effectively carry out the elicit the desired response (*e.g.* to increase bone mass,

to increase the number of osteoblasts present in bone tissue, to ameliorate the symptoms of the osteoporosis, respectively).

Another aspect of the invention is a method for treating a subject to induce bone formation comprising: harvesting mammalian mesenchymal stem cells; treating the mammalian mesenchymal cells with an oxysterol or a pharmaceutical composition of the invention, wherein the oxysterol induces the mesenchymal stem cells to express at least one cellular marker of osteoblastic differentiation; and administering the differentiated cells to the subject.

Another aspect of the invention is an implant for use in an animal (*e.g.* human) body, comprising a substrate having a surface, wherein at least the surface of the implant includes an oxysterol or a pharmaceutical composition of the invention, in an amount sufficient to induce bone formation in the surrounding bone tissue. The substrate may be formed into the shape of, *e.g.*, a pin, screw, plate, or prosthetic joint.

Another aspect of the invention is a method for inhibiting adipocyte differentiation of a mammalian mesenchymal stem cell, comprising contacting the mesenchymal stem cell with an effective amount of an oxysterol or a pharmaceutical composition of the invention. The cell may be *in vitro*, *ex vivo*, or in a subject (*in vivo*).

Another aspect of the invention is a method for identifying a modulator of a hedgehog pathway-mediated activity, comprising screening candidate oxysterols for the ability to modulate an activity in one of the hedgehog-related *in vitro* assays discussed herein (*e.g.*, induction of expression of the *Gli-1* gene, for example by stimulation of a *Gli1* promoter; activation of a reporter construct driven by a multimerized *Gli-1* responsive element; induction of expression of *Patched*; inhibition of a putative oxysterol-induced effect by cyclopamine; etc).

Another aspect of the invention is in a method for modulating a hedgehog (Hh) pathway mediated response in a cell or tissue (*in vitro*, *ex vivo*, or in a subject), the improvement comprising contacting the cell or tissue with an oxysterol of the invention. Another aspect of the invention is in a method for treating a subject for one of the indications as described herein (*e.g.*, to increase the differentiation of MSCs into osteoblasts, or to induce bone formation, the improvement comprising contacting the cell or tissue with an oxysterol of the invention).

One aspect of the invention is an oxysterol (*e.g.* an isolated oxysterol) of the invention as represented by Formula I, above. Examples of oxysterols, designated as Oxy

1 through Oxy 4 and Oxy 6 through Oxy 16 are presented in Figure 9. For example, the compounds designated as Oxy 7, Oxy 9, Oxy11, Oxy12, Oxy13, Oxy 14, and Oxy 15 can stimulate at least a measurable amount of a hedgehog-mediated pathway and/or a PPAR- γ -mediated pathway, and/or osteomorphogenesis or osteoproliferation (or a marker thereof), and/or can inhibit at least a measurable amount of adipocyte morphogenesis or adipocyte proliferation (or a marker thereof). Oxy 3 and Oxy 4 can act as enhancers of activity in combination with other oxysterols. For example, the combination of Oxy 3 and 20(S)-hydroxycholesterol, as well as the combination of Oxy4 and 20(S)-hydroxycholesterol were found to enhance the incorporation of ^{45}Ca in an assay used to measure mineralization in M2 cells over the incorporation when only 20(S)-hydroxycholesterol was applied. Oxy 7 was found to be minimally enhancing of activity.

Other oxysterols have not been demonstrated to modulate one of the activities mentioned above. However, these molecules, which share structural features with the oxysterols discussed above, would be expected to act as competitive inhibitors of those compounds and, in some cases, to act as antagonists of one of the mentioned activities (*e.g.*, of osteomorphogenesis or osteoproliferation, etc.).

In some aspects of the invention (*e.g.*, methods in which oxysterols are used to stimulate members of the Hh pathway, naturally occurring oxysterols (*e.g.*, 22(S)-hydroxycholesterol (sometimes referred to herein as "22S"); 22(R)-hydroxycholesterol (sometimes referred to herein as "22R"); 20(S)-hydroxycholesterol (also known as 20-alpha hydroxycholesterol, and sometimes referred to herein as "20S"); 5-cholesten-3beta, 20alpha-diol 3-acetate; 24-hydroxycholesterol; 24(S), 25-epoxycholesterol; pregnenolone, 26-hydroxycholesterol; 4beta-hydroxycholesterol; can also be used.

By "isolated" is meant removed from its original environment (*e.g.*, the natural environment if it is naturally occurring), and/or separated from at least one other component with which it is naturally associated. For example, a naturally-occurring oxysterol present in its natural living host is not isolated, but the same oxysterol, separated from some or all of the coexisting materials in the natural system, is isolated. Such an oxysterol can be part of a composition (*e.g.* a pharmaceutical composition), and still be isolated in that such composition is not part of its natural environment. Also, an intermediate product in the synthesis of another oxysterol, wherein the intermediate product is not purified or separated from other components in the reaction pathway, is not isolated.

It was observed that the hydroxyl groups in 20(S)-hydroxycholesterol and 22(S)-hydroxycholesterol are about 12-14 Å apart. Therefore, the putative receptor that mediates the effects of osteoinductive oxysterols may have a requirement for a diol in which the two hydroxyl groups are approximately 12-14 Å apart. In this light, we have synthesized and envision reaction schemes for the synthesis of synthetic oxysterols and derivatives thereof in which the functional group at the steroid 17 position is modified. With respect to modification of the functional group at the steroid 17 position, variants include, for example, the following: placement of a hydroxyl group at the steroid 20 position, the steroid 22 position, or both; inclusion of only single carbon-carbon bonds (alkane), double bonds (alkene), triple bonds (alkyne), or aromatic groups (e.g., phenyl, methylphenyl) in the functional group; and variation of stereochemistry. It is desirable to produce synthetic oxysterols that are derivatives of 20S-hydroxycholesterol and that are active even in the absence of 22S-hydroxycholesterol or 22R-hydroxycholesterol. For example, such synthetic oxysterols can be active in that they induce a measurable amount of a hedgehog-mediated pathway and/or a PPAR- γ -mediated pathway, and/or osteomorphogenesis or osteoproliferation (or a marker thereof), and/or inhibit at least a measurable amount of adipocyte morphogenesis or adipocyte proliferation (or a marker thereof).

Combinations of oxysterols of the invention, with one another and/or with other oxysterols, including naturally occurring oxysterols, can also be used in methods of the invention. Among the naturally occurring oxysterols that can be used are: 22(S)-hydroxycholesterol; 22(R)-hydroxycholesterol; 20(S)-hydroxycholesterol (also known as 20- α hydroxycholesterol); 5-cholesten-3 β , 20 α -diol 3-acetate; 24-hydroxycholesterol; 24(S), 25-epoxycholesterol; 26-hydroxycholesterol; and/or 4 β -hydroxycholesterol.

Example III, below, provides illustrative synthetic procedures, as well as bibliographic citations.

The oxysterols discussed herein can be used to modulate a variety of responses or activities in a cell or tissue, *in vitro* or *in vivo* (in a subject). By "modulate" is meant is to increase or decrease the degree of the response.

The Examples herein illustrate some of the many activities that are exhibited by oxysterols of the invention. The present inventors and colleagues previously demonstrated that naturally occurring oxysterols (e.g. 22(S)-hydroxycholesterol (sometimes referred to herein as "22S"); 22(R)-hydroxycholesterol (sometimes referred to herein as "22R");

20(S)-hydroxycholesterol (also known as 20-alpha hydroxycholesterol, and sometimes referred to herein as "20S"); 5-cholesten-3beta, 20alpha-diol 3-acetate; 24-hydroxycholesterol; 24(S), 25-epoxycholesterol; pregnenolone, 26-hydroxycholesterol; and 4beta-hydroxycholesterol; individually or in combination, exhibit osteogenic and anti-adipogenic properties. See, *e.g.*, the commonly owned and published PCT international applications WO2004/019884, WO2005/020928, WO2005/020928; and WO2006/12902, all of which are incorporated herein by reference in their entirety. See also Dwyer *et al.* (2007), *J. Biol. Chem.*, Epub ahead of print; Parhami *et al.* (2002) *J. Bone Miner. Res.* 17, 1997-2003; Kha *et al.* (2004) *J Bone Miner Res.* 19, 830-840; Shouhed *et al.* (2005) *J Cell Biochem* 95, 1276-1283; Richardson *et al.* (2006) (*J Cell Biochem*, in press); and Aghaloo *et al.* (2006) *J Orthop Res*, in press). In the present application, the inventors report that the novel oxysterols of the invention exhibit similar activities, as well as further activities. Such activities were demonstrated by a variety of markers of such activities.

In still further embodiments, the subject method can be employed for the generation of bone (osteogenesis) at a site in the animal where such skeletal tissue is deficient. Indian hedgehog is particularly associated with the hypertrophic chondrocytes that are ultimately replaced by osteoblasts. For instance, administration of a hedgehog agent of the present invention can be employed as part of a method for treating bone loss in a subject, *e.g.* to prevent and/or reverse osteoporosis and other osteopenic disorders, as well as to regulate bone growth and maturation. Periodontal implants are also contemplated. For example, preparations comprising oxysterol compounds can be employed, for example, to induce endochondral ossification, at least so far as to facilitate the formation of cartilaginous tissue precursors to form the "model" for ossification. Therapeutic compositions of hedgehog agonists can be supplemented, if required, with other osteoinductive factors, such as bone growth factors (*e.g.* TGF- β factors, such as the bone morphogenetic factors BMP-2, BMP-4, BMP-7 or BMP 14 as well as activin), and may also include, or be administered in combination with, an inhibitor of bone resorption such as estrogen, bisphosphonate, sodium fluoride, calcitonin, or tamoxifen, or related compounds. However, it will be appreciated that hedgehog proteins are likely to be upstream of BMPs, so that treatment with a hedgehog polypeptide and/or a hedgehog agonist will have the advantage of initiating endogenous expression of BMPs along with other factors.

The oxysterols discussed herein can be formulated into various compositions, *e.g.*, pharmaceutical compositions, for use in therapeutic treatment methods. The pharmaceutical compositions can be assembled as a kit. Generally, a pharmaceutical composition of the invention comprises an effective amount of an oxysterol or combination of the invention. An "effective amount," as used herein, is an amount that is sufficient to effect at least a detectable therapeutic response in the individual over a reasonable time frame. For example, it can ameliorate, at least to a detectable degree, the symptoms of a hedgehog-mediated condition and/or a PPAR- γ -mediated condition, etc. The composition can comprise a carrier, such as a pharmaceutically acceptable carrier. By "pharmaceutically acceptable" is meant a material that is not biologically or otherwise undesirable, *i.e.*, the material may be administered to a subject without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained. The carrier would naturally be selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject, as would be well known to one of skill in the art. For a discussion of pharmaceutically acceptable carriers and other components of pharmaceutical compositions, see, *e.g.*, Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Company, 1990.

A pharmaceutical composition or kit of the invention can contain other pharmaceuticals, as noted elsewhere herein, in addition to the oxysterols of the invention. The other agent(s) can be administered at any suitable time during the treatment of the patient, either concurrently or sequentially.

One skilled in the art will appreciate that the particular formulation will depend, in part, upon the particular agent that is employed, and the chosen route of administration. Accordingly, there is a wide variety of suitable formulations of compositions of the present invention.

Formulations suitable for oral administration can consist of liquid solutions, such as an effective amount of the agent dissolved in diluents, such as water, saline, or fruit juice; capsules, sachets or tablets, each containing a predetermined amount of the active ingredient, as solid, granules or freeze-dried cells; solutions or suspensions in an aqueous liquid; and oil-in-water emulsions or water-in-oil emulsions. Tablet forms can include one or more of lactose, mannitol, corn starch, potato starch, microcrystalline cellulose, acacia, gelatin, colloidal silicon dioxide, croscarmellose sodium, talc, magnesium stearate, stearic

acid, and other excipients, colorants, diluents, buffering agents, moistening agents, preservatives, flavoring agents, and pharmacologically compatible carriers. Suitable formulations for oral delivery can also be incorporated into synthetic and natural polymeric microspheres, or other means to protect the agents of the present invention
5 from degradation within the gastrointestinal tract.

Formulations suitable for parenteral administration (*e.g.* intravenous) include aqueous and non- aqueous, isotonic sterile injection solutions, which can contain anti-oxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can
10 include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water, for injections, immediately prior to use. Extemporaneous injection solutions and suspensions can be
15 prepared from sterile powders, granules, and tablets of the kind previously described.

The oxysterols of the invention, alone or in combination with other therapeutic agents, can be made into aerosol formulations to be administered via inhalation. These aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen and the like.

20 The oxysterols of the invention, alone or in combinations with other therapeutic agents, can be made into suitable formulations for transdermal application and absorption (Wallace *et al.*, 1993, *supra*). Transdermal electroporation or iontophoresis also can be used to promote and/or control the systemic delivery of the agents and/or pharmaceutical compositions of the present invention through the skin (*e.g.*, see Theiss *et al.* (1991),
25 *Meth. Find. Exp. Clin. Pharmacol.* 13, 353-359).

Formulations which are suitable for topical administration include lozenges comprising the active ingredient in a flavor, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin, or sucrose and acacia; mouthwashes comprising the active ingredient in a suitable liquid
30 carrier; or creams, emulsions, suspensions, solutions, gels, creams, pastes, foams, lubricants, sprays, suppositories, or the like.

One skilled in the art will appreciate that a suitable or appropriate formulation can be selected, adapted or developed based upon the particular application at hand.

Dosages for an oxysterols of the invention can be in unit dosage form, such as a tablet or capsule. The term "unit dosage form" as used herein refers to physically discrete units suitable as unitary dosages for animal (*e.g.* human) subjects, each unit containing a predetermined quantity of an agent of the invention, alone or in combination with other therapeutic agents, calculated in an amount sufficient to produce the desired effect in association with a pharmaceutically acceptable diluent, carrier, or vehicle.

One skilled in the art can easily determine the appropriate dose, schedule, and method of administration for the exact formulation of the composition being used, in order to achieve the desired effective amount or effective concentration of the agent in the individual patient. One skilled in the art also can readily determine and use an appropriate indicator of the "effective concentration" of the compounds of the present invention by a direct or indirect analysis of appropriate patient samples (*e.g.*, blood and/or tissues).

The dose of an oxysterol of the invention, or composition thereof, administered to an animal, particularly a human, in the context of the present invention should be sufficient to effect at least a therapeutic response in the individual over a reasonable time frame. The exact amount of the dose will vary from subject to subject, depending on the species, age, weight and general condition of the subject, the severity or mechanism of any disorder being treated, the particular agent or vehicle used, its mode of administration and the like. The dose used to achieve a desired concentration *in vivo* will be determined by the potency of the particular oxysterol employed, the pharmacodynamics associated with the agent in the host, the severity of the disease state of infected individuals, as well as, in the case of systemic administration, the body weight and age of the individual. The size of the dose also will be determined by the existence of any adverse side effects that may accompany the particular agent, or composition thereof, employed. It is generally desirable, whenever possible, to keep adverse side effects to a minimum.

For example, a dose can be administered in the range of from about 5 ng (nanograms) to about 1000 mg (milligrams), or from about 100 ng to about 600 mg, or from about 1 mg to about 500 mg, or from about 20 mg to about 400 mg. For example, the dose can be selected to achieve a dose to body weight ratio of from about 0.0001 mg/kg to about 1500 mg/kg, or from about 1 mg/kg to about 1000 mg/kg, or from about 5 mg/kg to about 150 mg/kg, or from about 20 mg/kg to about 100 mg/kg. For example, a dosage unit can be in the range of from about 1 ng to about 5000 mg, or from about 5 ng to about 1000 mg, or from about or from about 100 ng to about 600 mg, or from about 1

mg to about 500 mg, or from about 20 mg to about 400 mg, or from about 40 mg to about 200 mg of a compound of according to the present invention. A dose can be administered once per day, twice per day, four times per day, or more than four times per day as required to elicit a desired therapeutic effect. For example, a dose administration regimen
5 can be selected to achieve a blood serum concentration of a compound of the present invention in the range of from about 0.01 to about 1000 nM, or from about 0.1 to about 750 nM, or from about 1 to about 500 nM, or from about 20 to about 500 nM, or from about 100 to about 500 nM, or from about 200 to about 400 nM. For example, a dose administration regime can be selected to achieve an average blood serum concentration
10 with a half maximum dose of a compound of the present invention in the range of from about 1 µg/L (microgram per liter) to about 2000 µg/L, or from about 2 µg/L to about 1000 µg/L, or from about 5 µg/L to about 500 µg/L, or from about 10 µg/L to about 400 µg/L, or from about 20 µg/L to about 200 µg/L, or from about 40 µg/L to about 100 µg/L.

15 A therapeutically effective dose of an oxysterol compound or other agent useful in this invention is one which has a positive clinical effect on a patient as measured by the ability of the agent to improve adipogenesis or PPAR expression related conditions. The therapeutically effective dose of each agent can be modulated to achieve the desired clinical effect, while minimizing negative side effects. The dosage of the agent may be
20 selected for an individual patient depending upon the route of administration, severity of the disease, age and weight of the patient, other medications the patient is taking and other factors normally considered by an attending physician, when determining an individual regimen and dose level appropriate for a particular patient.

By way of example, the invention may include elevating endogenous, circulating
25 oxysterol levels over the patient's basal level. In a normal adult levels are about 10-400 ng/ml depending on age and type of oxysterol, as measured by mass spectrometry. Those skilled in the art of pharmacology would be able to select a dose and monitor the same to determine if an increase in circulating levels over basal levels has occurred.

When given in combined therapy, the other agent can be given at the same time as
30 the oxysterol, or the dosing can be staggered as desired. The two (or more) drugs also can be combined in a composition. Doses of each can be less when used in combination than when either is used alone.

The invention may include treatment with an additional agent which acts independently or synergistically with at least a first oxysterol compound to reduce adipogenesis, etc. Additional agents may be agents which, *e.g.*, stimulate the mechanistic pathway by which oxysterols reduce adipogenesis and enhance osteoblastic differentiation. Among such suitable agents are bone morphogenic proteins (*e.g.*, BMP 2, 4, 7, and/or 14), which have been shown by the inventors to act synergistically with oxysterols.

Therefore, the invention may include the use of a combination of at least one oxysterol of the invention and at least one BMP to induce osteoblastic differentiation or bone formation. This combination of agents to maintain bone homeostasis, enhance bone formation and/or enhance bone repair may be desirable at least in that the dosage of each agent may be reduced as a result of the synergistic effects. In one example, BMP2 may be used for localized use in fracture healing studies. The dosages used vary depending on mode of delivery. For example, beads coated with 10-100 micrograms of BMP2 have been used in mouse bone fracture studies. In studies with monkeys, BMP7 has been used in dosages ranging from 500-2000 micrograms. In studies with dogs, BMP2 has been used between 200-2000 micrograms. In studies where BMP2 was delivered in a sponge implanted in the fracture site, the dosage used was 1.5mg/ml. In a spinal fusion trial where fusion was achieved, a large dose of 10 mg of BMP2 was used. In a human study of tibial non-union fractures in humans, BMP7 was used at several mg dosages.

Additional classes of agents which may be useful in this invention alone or in combination with oxysterols include, but are not limited to cytochrome P450 inhibitors, such as SKF525A. Other classes of agents useful in the invention include phospholipase activators, or arachadonic acid. Other classes of agents useful in the invention include COX enzyme activators, or prostaglandins or osteogenic prostanoids. Other classes of agents useful in the invention include ERK activators.

The invention may include combination treatments with oxysterols and other therapeutics. For example, oxysterols in combination with bisphosphonates, hormone therapy treatments, such as estrogen receptor modulators, calcitonin, and vitamin D1 calcium supplementation, PTH (such as Forteo or teriparatide, Eli Lilly), sodium fluoride and growth factors that have a positive effect on bone, such as insulin-like growth factors I and II and transforming growth factor beta. Those skilled in the art would be able to

determine the accepted dosages for each of the therapies using standard therapeutic dosage parameters.

In this aspect of the invention, MSCs or preadipocytes may be treated with an agent(s) to reduce adipogenesis and/or optionally to stimulate osteoblastic differentiation, as measured by any one of the increase in alkaline phosphatase activity, calcium
5 incorporation, mineralization or osteocalcin mRNA expression, or other indicators of osteoblastic differentiation. In one embodiment of the invention MSCs are harvested from a patient, treated with at least one oxysterol of the invention, and osteoblastic cells are administered to the patient.

10 The invention may include administering osteoblastically differentiated MSCs systemically to the patient.

The invention may include placing osteoblastically differentiated MSCs at selected locations in the body of a patient. In one embodiment of the invention, cells may be injected at a location at which bone homeostasis, formation and/or repair is desired.

15 In one application of the invention, the agents and methods may be applied to, but are not limited to the treatment or to slow the progression of bone related disorders, such as osteoporosis.

In applications of the invention, the agents and methods may be applied to, but are not limited to application of cells or agents to a surgical or fracture site, in periodontitis,
20 periodontal regeneration, alveolar ridge augmentation for tooth implant reconstruction, treatment of non-union fractures, sites of knee/hip/joint repair or replacement surgery.

In one embodiment, the invention may include implants for use in the human body, comprising a substrate having a surface, wherein at least the surface of the implant includes at least one oxysterol of the invention in an amount sufficient to induce bone
25 formation in the surrounding bone tissue, or the implant may include mammalian cells capable of osteoblastic differentiation, or osteoblastic mammalian cells (*e.g.*, mammalian MSCs), or a combination thereof for inducing bone formation or enhancing bone repair. For example, implants may include, but are not limited to pins, screws, plates or prosthetic joints which may be placed in the proximity of or in contact with a bone that are used to
30 immobilize a fracture, enhance bone formation, or stabilize a prosthetic implant by stimulating formation or repair of a site of bone removal, fracture or other bone injury. The invention may also include the application of at least one agent or differentiated cells

in the proximity of or in contact with a bone at a site of bone removal, fracture or other bone injury where bone formation or bone repair is desired.

Another embodiment of the invention is a kit useful for any of the methods disclosed herein, either *in vitro*, *ex vivo*, or *in vivo*. Such a kit can comprise one or more of the oxysterols or pharmaceutical compositions discussed herein. Optionally, the kits
5 comprise instructions for performing the method. Optional elements of a kit of the invention include suitable buffers, pharmaceutically acceptable carriers, or the like, containers, or packaging materials. The reagents of the kit may be in containers in which the reagents are stable, *e.g.*, in lyophilized form or stabilized liquids. The reagents may
10 also be in single use form, *e.g.*, in single dosage form. A skilled worker will recognize components of kits suitable for carrying out any of the methods of the invention.

In the foregoing and in the following examples, all temperatures are set forth in uncorrected degrees Celsius; and, unless otherwise indicated, all parts and percentages are by weight.

15 What a "statistically significant amount" is depends on a number of factors, such as the technique of the experimenter and the quality of the equipment used. For example, in certain cases, a statistically significant amount may be a change of 1%. In other cases, a statistically significant amount can be represented by a change of at least about 5%, 10%, 20%, 50%, 75%, double, or more. In relation to inhibition, the significant reduction
20 may be to a level of less than about 90%, 75%, 50%, 25%, 10%, 5%, 1%, or less.

EXAMPLES

Example I - Materials and methods

Guidance for the performance of the assays described below can be found, *e.g.*, in
25 the commonly owned and published PCT international applications WO2004/019884, WO2005/020928, WO2005/020928; and WO2006/12902. See also Dwyer *et al.* (Jan. 2, 2007), *J. Biol. Chem*, Epub ahead of print; Parhami *et al.* (2002) *J. Bone Miner. Res.* 17, 1997-2003; Kha *et al.* (2004) *J Bone Miner Res.* 19, 830-840; Shouhed *et al.* (2005) *J Cell Biochem* 95, 1276-1283; Richardson *et al.* (2006) (*J Cell Biochem*, in press); and Aghaloo
30 *et al.* (2006) *J Orthop Res*, in press).

Example II - *In vivo* anti-adipogenic effects of oxysterols

We previously reported that both the inducer oxysterol, 20S, and the stimulatory oxysterols 22S and 22R, inhibit the adipogenic differentiation of M2 cells. Without wishing to be bound by any specific mechanism, this appears to suggest that the mechanism by which these oxysterols inhibit adipogenic differentiation might be distinct from that which induces osteogenic differentiation, and that therefore even some of the analogues that may be inactive in our osteoinductive tests may still inhibit adipogenesis. M2 cells are treated with PPAR γ agonist, troglitazone (Tro) at 10 μ M which induces adipogenesis in a variety of multipotent cells including the M2 marrow stromal cells. The synthetic analogues are tested by treating M2 cells with Tro in the absence or presence of the individual oxysterols. After 8 days of treatment, at which time fully formed adipocytes are produced in M2 cultures treated with Tro, oil red O staining is performed to detect adipocytes that stain red due to the accumulation of neutral lipids. Adipocyte numbers are quantified by counting fields under a phase contrast microscope by conventional procedures. Those oxysterols that exhibit anti-adipogenic effects *in vitro* are also expected to inhibit adipogenesis *in vivo*.

Example III - Syntheses of Oxysterols

Some sources pertaining to the synthesis of oxysterols are as follows: Drew, J. et al., *J. Org. Chem.*, 52 (1987) 4047-4052; Honda, T. et al., *J. Chem. Soc., Perkin Trans. 1*, (1996) 2291-2296; Gen, A. V. D. et al. *J. Am. Chem. Soc.*, 95 (1973) 2656-2663; Mazzocchi, P. H. et al. S. *J. Org. Chem.*, 48 (1983) 2981-2989; Byon C. et al., *J Org Chem*, 41 (1976) 3716-3722; Rao, A.S., *Comprehensive Organic Synthesis*, Pergamon Press, Eds. Trost BM, Fleming I., 7 (chapter 3.1) (1991) 376-380.

25

A. Method of Synthesis of Oxy11 and Oxy12**1. Route to Synthesis of Oxy11**

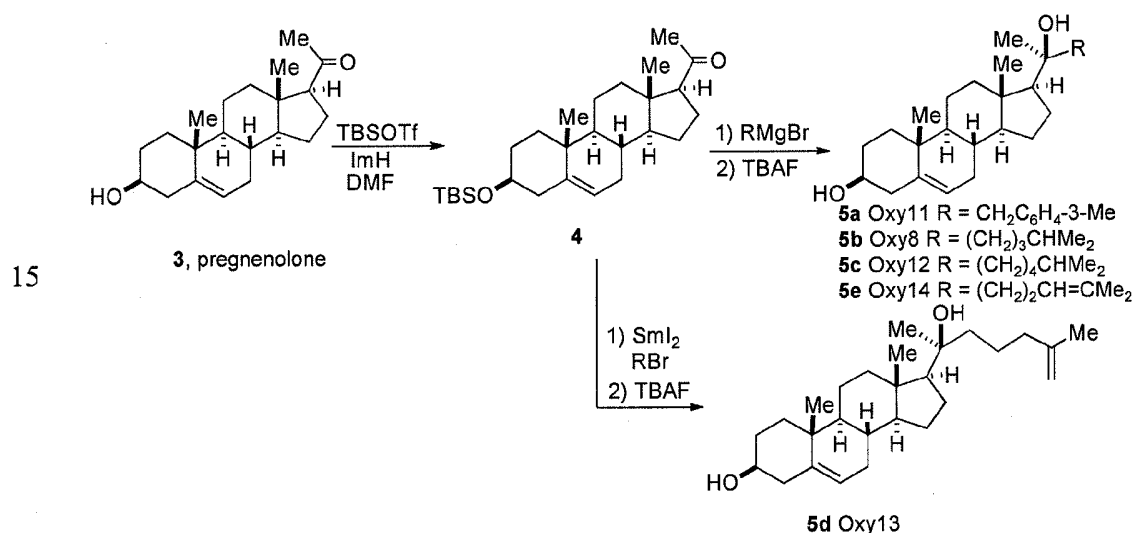
Imidazole (ImH) can be added to a solution of pregnenolone (compound 3, see Scheme 1) in anhydrous dimethylformamide (DMF). Tert-butyltrimethylsilyltrifluoromethanesulfonate can then be added to the solution. The reaction product can be purified to obtain compound 4, 1-((3S,8S,9S,10R,13S,14S,17S)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3-[(1,1-

dimethylethyl)dimethylsilyloxy]-10,13-dimethyl-1*H*-cyclopenta[*a*]phenanthren-17-yl) ethanone, as shown in Scheme 2.

The Grignard reagent 3-methylbenzylmagnesium bromide can then be reacted with 4 in a mixture of diethyl ether and tetrahydrofuran (THF). The silyl ether can be removed by the addition of tetrabutylammonium fluoride to yield compound 5a (Oxy 11) as shown in Scheme 1.

2. Route to Synthesis of Oxy12

The Grignard reagent isoheptylmagnesium bromide can then be reacted with 4 in a mixture of diethyl ether and THF. The silyl ether can be removed by the addition of tetrabutylammonium fluoride to yield compound 5c (Oxy 12) as shown in Scheme 1.



Scheme 1

20

B. Method of Synthesis of Oxy12 and Oxy13

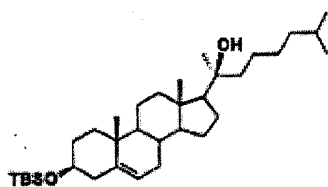
1. Alternative Route to Synthesis of Oxy12

25 1-((3*S*,8*S*,9*S*,10*R*,13*S*,14*S*,17*S*)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3-[(1,1-dimethylethyl)dimethylsilyloxy]-10,13-dimethyl-1*H*-cyclopenta[*a*]phenanthren-17-yl) ethanone, 1

To a stirred solution of pregnenolone (5.0 g, 15.8 mmol) in anhydrous dimethylformamide (DMF, 180 mL) was added imidazole (2.7 g, 39.7 mmol). The reaction was allowed to stir for 20 min followed by slow addition of *tert*-butyldimethylsilyl chloride (3.6 g, 23.9 mmol). After stirring for 12 h at ambient
5 temperature, the reaction mixture was poured over ice. The precipitates were collected and dissolved in diethyl ether. The organic phases were washed with brine, dried over Na₂SO₄ and evaporated *in vacuo* to yield compound **1** (6.7 g, 15.6 mmol, 98%) as a white powder which was used without further purification. The spectroscopic data was identical to those reported in the literature (Drew *et al.* (1987) *J. Org. Chem.* 52, 4047-4052).

10 **(3*S*,8*S*,9*S*,10*R*,13*S*,14*S*,17*S*)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3--[(1,1-dimethylethyl)dimethylsilyloxy]-17-((*S*)-2-hydroxy-7-methyloctan-2-yl)-10,13-dimethyl-1*H*-cyclopenta[*a*]phenanthrene, **2****

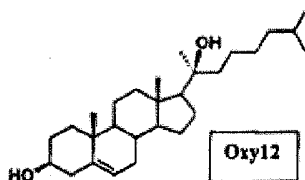
To a stirred suspension of samarium metal (758 mg, 5.0 mmol) and 3 Å molecular sieves (0.5 g) in anhydrous tetrahydrofuran (THF, 9.5 mL) was slowly added a solution of
15 1,2-diiodoethane (1.3 g, 4.6 mmol) in THF (9.5 mL) at ambient temperature. After the reaction stirred for 30 min, hexamethylphosphoramide (HPMA, 3.0 mL, 17.2 mmol) was added to the reaction mixture and continued stirring for an additional 20 min. Then, a solution of ketone **1** (500.0 mg, 1.16 mmol) in THF (6.0 mL) was added followed by a solution of 1-bromo-5-methylhexane (208.0 mg, 1.16 mmol) in THF (2.0 mL). The
20 reaction was allowed to stir for an additional hour until the starting material was completely consumed. After this, the reaction mixture was slowly treated with saturated NaHCO₃, filtered through Celite and rinsed three times with an excess amount of diethyl ether. The filtrate was treated with water and extracted with diethyl ether. The ether
25 extracts were washed with brine, dried over Na₂SO₄ and evaporated *in vacuo* to give a residue which was purified via silica gel chromatography. Elution with hexane-diethyl ether (4:1, v/v) afforded compound **2** (350.0 mg, 0.6 mmol, 57%) as a white powder (Honda *et al.* (1996) *J. Chem. Soc., Perkin Trans.* 1, 2291-2296).



¹H NMR (500 MHz, CDCl₃) δ 0.05 (s, 6H), 0.86 (s, 3H), 0.86 (d, *J* = 6.6 Hz, 6H), 0.89 (s, 9H), 1.00 (s, 3H), 1.02-1.17 (m, 8H), 1.26 (s, 3H), 1.29-1.81 (m, 18H), 1.95-1.99 (m, 1H), 2.07-2.10 (m, 1H), 2.14-2.18 (m, 1H), 2.24-2.26 (m, 1H), 3.46-3.50 (m, 1H), 5.31 (app t, *J* = 5.2 Hz, 1H). ¹³C NMR (125 MHz, CDCl₃) δ -4.7, 13.5, 18.1, 19.3, 20.8, 22.2, 22.4, 22.5, 23.7, 24.4, 25.8, 26.3, 27.8, 27.9, 31.2, 31.7, 32.0, 36.5, 37.3, 38.9, 40.0, 42.5, 42.7, 43.9, 50.0, 56.8, 57.4, 72.4, 75.0, 120.9, 141.4.

(3*S*,8*S*,9*S*,10*R*,13*S*,14*S*,17*S*)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-17-((*S*)-2-hydroxy-7-methyloctan-2-yl)-10,13-dimethyl-1*H*-cyclopenta[*a*]phenanthren-3-ol, Oxy12

To a solution of compound **2** (300.0 mg, 0.57 mmol) in anhydrous THF was added a 1.0 M solution of tetrabutylammonium fluoride in THF (2.5 mL, 2.5 mmol) and the solution was allowed to stir at ambient temperature. After 12 h, the reaction was treated with water and extracted three times with diethyl ether. The organic phases were collected, dried over Na₂SO₄ and concentrated *in vacuo* to give an oil. Flash column chromatography of this oil (silica gel, 1:3 hexane/diethyl ether) yielded the compound **Oxy12** (210.0 mg, 0.50 mmol, 88%) as a white powder.



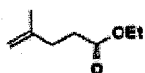
¹H NMR (500 MHz, CDCl₃) δ 0.86 (s, 3H), 0.86 (d, *J* = 6.6 Hz, 6H), 1.01 (s, 3H), 1.02-1.25 (m, 11H), 1.26 (s, 3H), 1.42-1.76 (m, 14H), 1.82-1.85 (m, 2H), 1.95-1.99 (m, 1H), 2.07-2.11 (m, 1H), 2.23-2.30 (m, 2H), 3.49-3.55 (m, 1H), 5.35 (app t, *J* = 5.2 Hz, 1H). ¹³C NMR (125 MHz, CDCl₃) δ 13.5, 19.3, 20.8, 22.2, 22.5, 23.7, 24.4, 26.3, 27.8, 27.9, 31.2, 31.5, 31.7, 36.4, 37.1, 38.9, 39.0, 40.0, 42.2, 42.5, 44.0, 56.8, 57.5, 71.7, 75.1, 121.5, 140.7.

25

2. Route to Synthesis of Oxy13

Ethyl 4-methylpent-4-enoate, 7

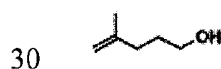
A solution of 2-methyl-2-propen-1-ol (12.9 g, 0.18 mol), triethyl orthoacetate (230.0 mL, 1.3 mol) and propionic acid (0.9 mL, 0.12 mol) was heated to 170 °C (external). The reaction apparatus was equipped with a Vigreux Claisen adapter with a collection flask to remove the ethanol produced. The reaction mixture was left under
5 reflux overnight. The excess amount of triethyl orthoacetate was gently distilled off at 130 mm Hg until the temperature in the reaction flask began to increase. After the reaction was cool, the remaining liquid was treated with 300 mL of 10% monobasic potassium phosphate and the left reaction was stirred for 90 min at ambient temperature. The reaction mixture was extracted with diethyl ether (3 x 100 mL). The combined organic
10 phase was dried over Na₂SO₄ and concentrated *in vacuo* to give a yellow oil. Flash column chromatography of this oil (silica gel, 4:1 hexane/diethyl ether) afforded compound 7 as a colorless oil (17.0 g, 0.12 mmol, 67%) (Gen *et al.* (1973) *J. Am. Chem. Soc.* 95, 2656-2663).



15 ¹H NMR (500 MHz, CDCl₃) δ 1.25 (t, *J* = 7.2 Hz, 3H), 1.74 (s, 3H), 2.33 (t, *J* = 7.9 Hz, 2H), 2.45 (t, *J* = 8.0 Hz, 2H), 4.13 (q, *J* = 7.1 Hz, 2H), 4.68 (s, 1H), 4.74 (s, 1H).

4-Methylpent-4-en-1-ol, 8

To a flame-dried flask that was purged under argon for 20 min was added LiAlH₄ followed by 150 mL of anhydrous THF. The reaction mixture was cooled to 0 °C and a
20 solution of compound 7 in THF (20 mL) was added slowly. The resulting solution was allowed to warm to room temperature and was stirred for 3 h until the starting material was completely consumed as indicated by TLC. The reaction was quenched by slow addition of the mixture to 300 mL of ice cold 1M NaOH. The mixture was then allowed to stir for another hour and was filtered through Celite. A large amount of diethyl ether was
25 used for rinsing. The filtrate was treated with water and extracted twice with diethyl ether. The combined organic phase was dried over Na₂SO₄ and evaporated *in vacuo* to give a residue which was purified via distillation at 20 mm Hg (bp 65-68 °C) to afford compound 8 as a yellow oil (9.5 g, 0.095 mol, 79%) (Mazzocchi *et al.* (1983) *J. Org. Chem.* 48, 2981-2989).



30

¹H NMR (500 MHz, CDCl₃) δ 1.47 (br, 1H), 1.69-1.74 (m, 5H), 2.1 (t, *J* = 7.5 Hz, 2H), 3.66 (t, *J* = 6.5 Hz, 2H), 4.71 (d, *J* = 0.8 Hz, 1H), 4.73 (d, *J* = 0.8 Hz, 1H), 4.73 (d, *J* = 0.4 Hz, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 22.22, 30.41, 33.98, 62.64, 110.08, 145.40.

5-Bromo-2-methyl-1-pentene, 9

5 To a solution of compound **8** (8.8 g, 0.088 mol) in pyridine (150 mL) cooled to 0 °C was added *p*-toluenesulfonyl chloride (35.0 g, 0.18 mol) in small portions. After the reaction mixture stirred for 20 minutes, it was allowed the reaction mixture to warm to room temperature over 3 h. The solution was acidified with 1 M HCl and extracted three times with diethyl ether. The ether extracts were washed with 1 M HCL, saturated
10 NaHCO₃ and brine. The combined organic layers were dried over Na₂SO₄ and evaporated *in vacuo* to yield the crude tosylate which was used without further purification.

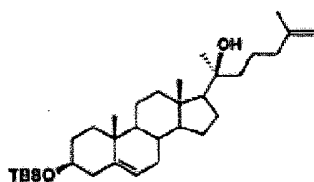
The tosylate (23.8 g, 0.094 mol) was dissolved in acetone (150 mL) and LiBr (17.0 g, 0.20 mol) was added slowly at ambient temperature. The reaction was left under reflux at 75 °C for 3 h. The solution was poured into ice water and extracted with diethyl ether (3
15 x 200 mL). The combined the organic layers were dried over Na₂SO₄ and concentrated *in vacuo* to afford a yellow oil. Flash column chromatography of this oil (silica gel, 9:1 hexane/diethyl ether) gave compound **9** (7.0 g, 0.043 mol, 49%) as a colorless oil.



¹H NMR (500 MHz, CDCl₃) δ 1.73 (s, 3H), 1.97-2.02 (m, 2H), 2.16 (t, *J* = 7.2 Hz, 2H),
20 3.41 (t, *J* = 6.7 Hz, 2H), 4.72 (d, *J* = 1.0 Hz, 1H), 4.76 (d, *J* = 0.5 Hz, 1H); ¹³C NMR (125 MHz, CDCl₃) δ 22.18, 30.47, 33.17, 35.92, 110.88, 143.82.

(3*S*,8*S*,9*S*,10*R*,13*S*,14*S*,17*S*)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-3-[(1,1-dimethylethyl)dimethylsilyloxy]-17-((*S*)-2-hydroxy-6-methylhept-6-en-2-yl)-10,13-dimethyl-1*H*-cyclopenta[*a*]phenanthrene, 10

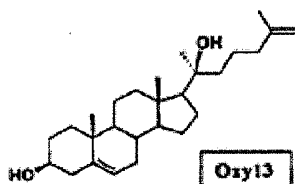
25 The coupling reaction of the protected pregnenolone **1** (500.0 mg, 1.16 mmol) with 5-bromo-2-methyl-1-pentene **9** (199.0 mg, 1.22 mmol) in the presence of samarium diiodide was performed under similar condition as described for the preparation of **2** to afford the 20*S*-hydroxy steroid **10** (419.0 mg, 0.82 mmol, 71%) as a white powder.



¹H NMR (500 MHz, CDCl₃) δ 0.05 (s, 6H), 0.86 (s, 3H), 0.89 (s, 9H), 1.00 (s, 3H), 1.13-1.22 (m, 5H), 1.28 (s, 3H), 1.32-1.55 (m, 11H), 1.71 (s, 3H), 1.72-1.79 (m, 5H), 1.97-2.0 (m, 6H), 3.47-3.48 (m, 1H), 4.67 (s, 1H), 4.70 (s, 1H), 5.31 (app t, *J* = 5.3 Hz, 1H). ¹³C NMR (125 MHz, CDCl₃) δ -4.7, 13.5, 18.1, 19.3, 20.8, 22.1, 22.2, 22.3, 23.7, 25.8, 26.3, 31.2, 31.7, 32.0, 36.5, 37.3, 38.2, 40.0, 42.6, 42.7, 43.4, 50.0, 56.8, 57.7, 72.5, 75.0, 109.8, 120.9, 141.5, 145.7.

(3*S*,8*S*,9*S*,10*R*,13*S*,14*S*,17*S*)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-17-((*S*)-2-hydroxy-6-methylhept-6-en-2-yl)-10,13-dimethyl-1*H*-cyclopenta[*a*]phenanthren-3-ol, Oxy13

The deprotection of the silyl ether **10** was carried out under similar conditions as those used for the preparation of the compound **Oxy12** to afford compound **Oxy13** (300.0 mg, 0.75 mmol, 91%) as a white powder.

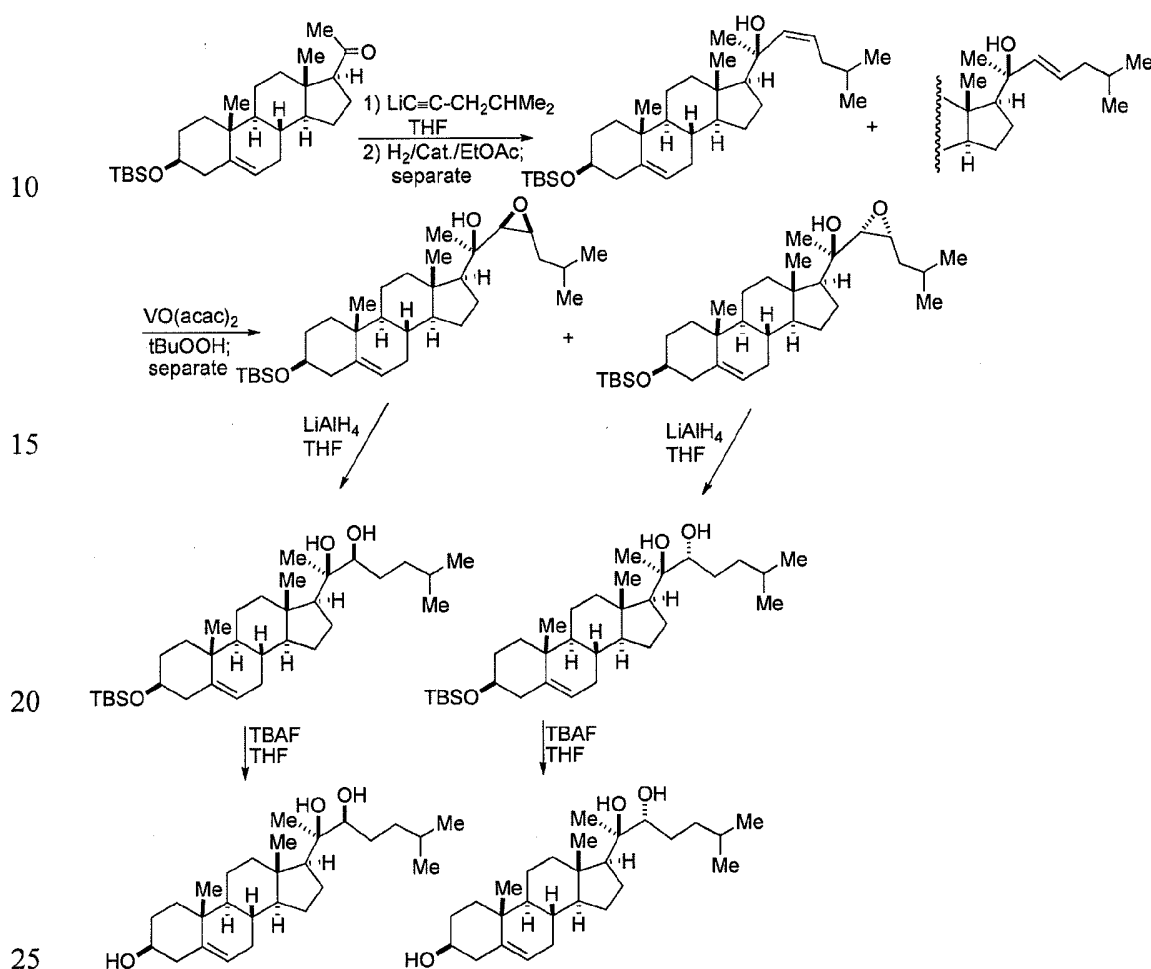


¹H NMR (500 MHz, CDCl₃) δ 0.86 (s, 3H), 1.00 (s, 3H), 1.12-1.20 (m, 5H), 1.28 (s, 3H), 1.32-1.65 (m, 14H), 1.73 (s, 3H), 1.83-2.0 (m, 5H), 2.07-2.09 (m, 1H), 2.23-2.28 (m, 2H), 2.48 (br, 1H), 3.52-3.54 (m, 1H), 4.67 (s, 1H), 4.70 (s, 1H), 5.35 (app t, *J* = 2.0 Hz, 1H). ¹³C NMR (125 MHz, CDCl₃) δ 13.5, 19.3, 20.8, 22.1, 22.2, 22.3, 23.7, 26.3, 31.2, 31.5, 31.7, 36.4, 37.1, 38.2, 40.0, 42.2, 42.6, 43.4, 49.9, 56.8, 57.7, 71.6, 75.0, 109.8, 121.5, 140.7, 145.7.

C. Method of Synthesis of Oxy15 and Oxy16

The pregnenolone silyl ether (compound **4**, see Schemes 1 and 2) can be reacted with 4-methylpentynyllithium in tetrahydrofuran (THF) and the resulting alcohol was then

reduced using Lindlar's catalyst to give a mixture of cis and trans alkenes which were separated. The cis isomer was epoxidized using t-butyl hydroperoxide and vanadyl acetoacetate to give a mixture of the two epoxides (the first shown in Scheme 2 being major). Hydride reduction of the hydroxy epoxides individually gave the diols. Final
 5 removal of the silyl ether of the two diols gave the triols, Oxy15 and Oxy16.



Scheme 2

Example IV - Study Elucidating Inhibition of PPAR γ Expression by Oxysterols

30 This example demonstrates the anti-adipogenic effects of an osteogenic oxysterol, 20(S)-hydroxycholesterol, which are mediated through a hedgehog-dependent mechanism(s) and are associated with inhibition of PPAR γ expression. The M2-10B4

(M2) murine multipotent bone MSC line was used to assess the inhibitory effects of 20(S)-hydroxycholesterol (20S) and sonic hedgehog (Shh) on peroxisome proliferator-activated receptor γ (PPAR γ) and adipogenic differentiation. All results were analyzed for statistical significance using ANOVA.

5 Treatment of M2 cells with the osteogenic oxysterol 20S completely inhibited adipocyte formation induced by troglitazone after 10 days. PPAR γ mRNA expression assessed by RT-qPCR was significantly induced by Tro after 48 (5-fold) and 96 h (130-fold), and this induction was completely inhibited by 20S. In contrast, 20S did not inhibit PPAR γ transcriptional activity in M2 cells overexpressing PPAR γ and retinoid X receptor
10 (RXR). The hedgehog signaling inhibitor, cyclopamine, reversed the inhibitory effects of 20S and Shh on troglitazone-induced adipocyte formation in 10-day cultures of M2 cells by 70% and 100%, respectively, and the inhibitory effect of 20S and Shh on troglitazone-induced PPAR γ expression was fully reversed at 48 h by cyclopamine. Furthermore, 20S and Shh greatly inhibited PPAR γ 2 promoter activity induced by CCAAT/enhancer-
15 binding protein α overexpression. These studies show that the inhibition of adipogenesis in murine MSCs by the osteogenic oxysterol, 20S, is mediated through a hedgehog-dependent mechanism(s).

20S was purchased from Sigma-Aldrich (St Louis, MO, USA), recombinant mouse sonic hedgehog, amino-terminal peptide from R&D Systems (Minneapolis, MN, USA), troglitazone from BioMol Research Laboratories (Plymouth Meeting, PA, USA), cyclopamine and PD98059 from Calbiochem (La Jolla, CA, USA), RPMI 1640 from Irvine Scientific (Santa Ana, CA, USA), and FBS from Hyclone (Logan, UT, USA).

Mouse M2 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were maintained in growth medium consisting of
25 RPMI 1640 with 10% heat-inactivated FBS and supplemented with 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 U/ml streptomycin. Cell culture was performed in 24- and 6-well plates for adipogenic differentiation and gene expression studies, respectively, and treatment with test agents was done in growth medium.

Oil red O staining for detection of adipocytes was performed as previously
30 described. See Parhami F et al. 1999, Atherogenic diet and minimally oxidized low density lipoprotein inhibit osteogenic and promote adipogenic differentiation of marrow stromal cells, J Bone Miner Res 14:2067-2078. The number of adipocytes was quantitated by counting Oil red O-positive cells in five separate fields per well, in three

wells per experimental condition. The results are reported as the mean of triplicate determination \pm SD.

Total RNA was extracted with the RNA isolation kit from Stratagene (La Jolla, CA, USA) according to the manufacturer's instructions. RNA was DNase-treated using DNA-free kit from Ambion (Austin, TX, USA). Three micrograms of RNA was reverse-transcribed using reverse transcriptase from Stratagene to make single-stranded cDNA. The cDNA was mixed with Qi SYBR Green Supermix (Bio-Rad) for quantitative RT-PCR assay using a Bio-Rad I-cycler IQ quantitative thermocycler. All PCR samples were prepared in triplicate wells of a 96-well plate. After 40 cycles of PCR, melt curves were examined to ensure primer specificity. Fold changes in gene expression were calculated using the $\Delta\Delta C_t$ method. See Tichopad A et al. 2003, Standardized determination of real-time PCR efficiency from a single reaction set-up, *Nucleic Acids Res* **31**:e122. Primers used are as follows: *PPAR γ 2* (5'-TGAACTCTGGGAGATTCTCCTG-3' and 5'-CCATGGTAATTTCTTGTGAAGTGC-3'), *C/EBP α* (5'-GGACAAGAACAGCAACGAGTACC-3' and 5'-GGCGGTCATTGTCACTGGTC-3'), *ap2* (5'-GRCACCATCCGGTCAGAGAGTAC-3' and 5'-TCGTCTGCGGTGATTTTCATC-3'), *LPL* (5'-GTGGCCGAGAGCGAGAAC-3' and 5'-AAGAAGGAGTAGGTTTTATTTGTGGAA-5'), *GATA2* (5'-ATCCACCCTTCCTCCAGTCT-3' and 5'-CTCTCCAAGTGCATGCAAGA-3'), *GATA3* (5'-AGAAGGCATCCAGACCCGAAAC-3' and 5'-AACTTGGAGACTCCTCACGCATGTG-3'), *GILZ* (5'-GCTGCACAATTTCTCCACCT-3' and 5'-GCTCACGAATCTGCTCCTTT-3'), and *pref-1* (5'-CTGTGTCAATGGAGTCTGCAAG-3' and 5'-CTACGATCTCACAGAAGTTGC-3'). See Spinella-Jaegle S et al. 2001, Sonic hedgehog increases the commitment of multipotent mesenchymal cells into the osteoblastic lineage and abolishes adipocytic differentiation, *J Cell Sci* **114**:2085-2094; Suh JM et al. 2006, Hedgehog signaling plays a conserved role in inhibiting fat formation, *Cell Metab* **3**:25-34; Lay SL et al. 2002, Insulin and sterol-regulatory element-binding protein-1c (SREBP-1c) regulation of gene expression in 3T3-L1 adipocytes, *J Biol Chem* **277**:35625-35634; Shi X et al. 2003 A glucocorticoid-induced leucine-zipper protein, *GILZ*, inhibits adipogenesis of mesenchymal cells, *EMBO Rep* **4**:374-380; Phan J et al. 2004, Lipin expression preceding peroxisome proliferators-activated receptor-gamma is critical for adipogenesis in vivo and in vitro, *J Biol Chem* **279**:29558-29564.

M2 cells at 70% confluency in a 24-well plate were transiently transfected with: a plasmid containing three tandem repeats of the PPAR response element (PPRE) upstream of the basic thymidine kinase promoter (p3xPPRE-TK-Luciferase), a control pTK-Luciferase plasmid devoid of PPRE, a CMX-PPAR γ expression plasmid (all kind gifts of
5 Dr Peter Tontonoz), a CMX-RXR α expression plasmid (kind gift of Dr Sotirios Tetradis), and a pTK-Renilla-Luciferase plasmid (Promega, Madison, WI, USA) using Fugene 6 Transfection Reagents from Roche (Indianapolis, IN, USA). Luciferase activity assay was performed using Dual-Luciferase Reporter 1000 Assay System (Promega, Madison, WI, USA). Luciferase reporter activity was normalized to Renilla Luciferase activity.
10 Transfection efficiency was monitored by co-transfecting with a plasmid expressing green fluorescent protein and found to be >30%. For GATA reporter assays, M2 cells were transfected with a GATA Luciferase reporter vector or a control reporter vector (both from Panomics, Fremont, CA, USA), and pTK-Renilla-Luciferase plasmid.

For PPAR γ promoter activity assays, M2 cells were transiently transfected with a
15 murine PPAR γ 2 promoter construct luciferase plasmid (p19-PPAR γ 2; (kind gift of Dr Steven McKnight), along with MSV-C/EBP α overexpression plasmid (kind gift of Dr. Sophia Tsai) and pTK-Renilla-Luciferase plasmid. Luciferase activity was measured after 24 h and normalized for transfection efficiency using the Renilla Luciferase activity.

Statistical analyses were performed using the StatView 5 program. All *p* values
20 were calculated using ANOVA and Fisher's projected least significant difference (PL-SD) significance test. A value of *p* < 0.05 was considered significant.

Results

Effects of 20S on adipogenic differentiation and PPAR γ and C/EBP α mRNA expression

25 Consistent with our previous report, treatment of M2 cells with Tro significantly increased adipocyte formation compared with control after 10 days, and this increase was significantly inhibited in the presence of 5 μ M 20S (Figs. 1A and 1B). See Kha HT et al. 2004 Oxysterols regulate differentiation of mesenchymal stem cells: Pro-bone and anti-fat, J Bone Miner Res **19**:830-840. Figure 1A shows results for M2 cells treated at
30 confluence with control vehicle, 10 μ M Tro, or 5 μ M 20S alone or in combination for 10 days. Adipocyte formation was examined by Oil red O staining. Figure 1B shows the number of adipocytes for the conditions in Fig. 1A, quantitated by counting Oil red O-positive cells in five separate fields per well, and in three wells per experimental

condition. The results are reported as the mean of triplicate determination \pm SD ($p < 0.0001$ for control vs. Tro and Tro vs. Tro + 20S).

To elucidate the mechanism(s) by which osteogenic oxysterols inhibit adipogenesis, we examined the effect of 20S on PPAR γ expression in M2 cells. PPAR γ expression was assessed by RT-qPCR after treating M2 cells with Tro in the presence or absence of 20S. Tro caused a significant increase in PPAR γ mRNA expression after 24-96 h of treatment, with the level of expression increasing in a time-dependent manner (Fig. 2). Tro did not cause a detectable significant increase in PPAR γ expression significantly at earlier time-points (data not shown). Tro-induced PPAR γ expression was almost completely blocked by 20S at all time-points examined (Fig. 2). Figure 2 shows the effect of 20S on PPAR γ mRNA expression induced by Tro. M2 cells at confluence were treated with control vehicle, 10 μ M Tro, or 5 μ M 20S, alone or in combination for 24 hours (Fig. 2A), 48 hours (Fig. 2B), and 96 hours (Fig. 2C). PPAR γ mRNA expression was measured by quantitative real-time PCR. Fold changes in gene expression to the control were calculated using the $\Delta\Delta$ Ct method and reported as the mean of triplicate determination \pm SD ($p < 0.0001$ for Tro vs. control and $p < 0.001$ for Tro vs. Tro + 20S at 24 hours (Fig. 2A); $p < 0.0001$ for Tro vs. control and Tro vs. Tro + 20S at 48 hours (Fig. 2B); $p < 0.0001$ for Tro vs. control and Tro vs. Tro + 20S at 96 hours (Fig. 2C).

Furthermore, the expression of C/EBP α mRNA, a key adipogenic gene, was also significantly increased by Tro at 24 hours (Fig. 3A), 48 hours (Fig. 3B), and 96 hours (Fig. 3C) in a time dependent manner, but 20S did not inhibit this increase in C/EBP α expression at the time-points examined. Figure 3 shows the effect of 20S on C/EBP α mRNA expression induced by Tro. M2 cells at confluence were treated with control vehicle, 10 μ M Tro, or 5 μ M 20S, alone or in combination for 24 (Fig. 3A), 48 (Fig. 3B), and 96 (Fig. 3C) hours. C/EBP α mRNA expression was measured by quantitative real-time PCR. Fold changes in gene expression relative to the control were calculated using the $\Delta\Delta$ Ct method and reported as the mean of triplicate determination \pm SD ($p < 0.0001$ for Tro vs. control and $p < 0.001$ for Tro + 20S vs. control at 24 hours (Fig. 3A); $p < 0.0001$ for Tro vs. control and $p < 0.001$ for Tro + 20S vs. control at 48 hours (Fig. 3B); $p < 0.0001$ for Tro or Tro + 20S vs. control at 96 hours (Fig. 3C).

The expression of aP2, a downstream target of PPAR γ , was significantly increased in Tro-treated cells, and this induction was inhibited by 20S (Fig. 4). Figure 4 shows the effect of 20S on aP2 mRNA expression induced by Tro. M2 cells at confluence were

treated with control vehicle, 10 μ M Tro, or 5 μ M 20S, alone or in combination for 24 hours (Fig. 4A), 48 hours (Fig. 4B), and 96 hours (Fig. 4C). aP2 mRNA expression was measured by quantitative real-time PCR. Fold changes in gene expression relative to the control were calculated using the $\Delta\Delta$ Ct method and reported as the mean of triplicate
5 determination \pm SD ($p < 0.0001$ for Tro or Tro + 20S vs. control or 20S and $p < 0.01$ for Tro vs. Tro + 20S at 24 hours (Fig. 4A); $p < 0.0001$ for Tro or Tro + 20S vs. control or 20S and for Tro vs. Tro + 20S at 48 hours (Fig. 4B); $p < 0.0001$ for Tro vs. control, 20S, or Tro + 20S and $p < 0.001$ for Tro + 20S vs. control or 20S at 96 hours (Fig. 4C)).

Next we examined whether, in addition to inhibiting PPAR γ expression, 20S also
10 inhibits transcriptional activity of PPAR γ protein, i.e., its effect in inducing transcription of a reporter gene. M2 cells were transiently transfected with a reporter construct containing three tandem repeats of a PPRE (pTK-3xPPRE-Luciferase) or the control plasmid (pTK-Luciferase). Cells were treated with 10 μ M Tro or control vehicle, and luciferase activity was measured after 24 and 48 h. Results showed that Tro induced a
15 small but significant increase in reporter activity (40%; Fig. 5A). Interestingly, 20S did not inhibit Tro-induced PPRE reporter activity, but instead caused a small but significant increase in the Tro-induced response (Fig. 5A). Because Tro-induced PPRE reporter activity appeared to be low in M2 cells under baseline conditions, we used a PPAR γ over-expression vector to transiently transfect the cells and assessed whether Tro-induced
20 reporter activity was increased. Indeed, we found a more substantial reporter activity in PPAR γ overexpressing cells in response to Tro after 24 (Fig. 5B) and 48 h (data not shown) of treatment. Consistent with the results obtained without PPAR γ overexpression, 20S did not inhibit but rather enhanced Tro-induced PPRE reporter activity in cells overexpressing PPAR γ . Because PPAR γ and retinoid X receptor (RXR) form obligatory
25 heterodimers, we studied whether 20S would have an effect when both PPAR γ and RXR α were overexpressed. Co-transfection of M2 cells with PPAR γ and RXR α overexpression plasmids showed similar results to transfection with only PPAR γ overexpression plasmid, and 20S enhanced PPAR γ -RXR α induced PPRE reported activity (Fig. 5C). Figure 5 shows the effect of 20S on transcriptional activity of PPAR γ . Figure 5A shows results for
30 M2 cells at 70% confluence in a 24-well plate transiently transfected with a PPRE reporter construct (pTK-3xPPRE-Luciferase) plasmid (PPRE-TK-LUC) or pTK-Luciferase plasmid (pTK-LUC) and pTK-Renilla-Luciferase plasmid. Luciferase activity was measured after 24 hours and normalized for transfection efficiency using the Renilla

luciferase activity. Data are reported as the mean of triplicate determination \pm SD ($p < 0.001$ for control vs. Tro and Tro vs. Tro + 20S). Figure 5B shows results for M2 cells transiently transfected with a PPRE reporter plasmid (pTK-3xPPRE-Luciferase) (PPRE-TK-LUC) or pTK-Luciferase plasmid (pTK-LUC), along with CMX-PPAR γ expression plasmid, and pTK-Renilla-Luciferase plasmid. Luciferase activity was normalized for transfection efficiency using the Renilla luciferase activity. Data are reported as the mean of triplicate determination \pm SD ($p < 0.0001$ for control vs. Tro and Tro vs. Tro + 20S). Figure 5C shows results for M2 cells transiently transfected as described for Fig. 5B along with CMX-RXR α expression plasmid. Luciferase activity was measured after 24 hours and normalized to the Renilla luciferase activity. Data are reported as the mean of triplicate determination \pm SD ($p < 0.0001$ for control vs. Tro and Tro vs. Tro + 20S).

Role of Hedgehog signaling in anti-adipogenic effects of 20S

We previously found that osteogenic oxysterols stimulate osteoblastic differentiation of M2 cells by inducing hedgehog pathway activity, and activation of hedgehog pathway is pro-osteogenic and anti-adipogenic. See Dwyer JR et al. 2007, Oxysterols are novel activators of the hedgehog signaling pathway in pluripotent mesenchymal cells, *J Biol Chem* 282:8959-8968; Spinella-Jaegle S et al. 2001, Sonic hedgehog increases the commitment of pluripotent mesenchymal cells into the osteoblastic lineage and abolishes adipocytic differentiation, *J Cell Sci* 114:2085-2094; Suh JM et al. 2006, Hedgehog signaling plays a conserved role in inhibiting fat formation, *Cell Metab* 3:25-34; Richardson JA et al. 2005, Characterization of osteogenic oxysterols and their molecular mechanism(s) of action, *J Bone Miner Res* 20:S1;S414; Amantea CM et al. 2006, Oxysterols are novel activators of hedgehog and Wnt signaling, *J Bone Miner Res* 21:S1;S156. We evaluated whether the anti-adipogenic effects of 20S are mediated through the hedgehog signaling pathway by assessing the effect of hedgehog pathway inhibitor, cyclopamine, on the anti-adipogenic effects of 20S oxysterol. Consistent with previous results, Oil red O staining showed that Tro treatment greatly increased the number of adipocytes compared with control, 20S significantly inhibited adipocyte formation induced by Tro, and pretreatment with cyclopamine (4 μ M) reversed the anti-adipogenic effects of 20S (Fig. 6A). Shh also significantly inhibited Tro-induced adipocyte formation, and cyclopamine pretreatment completely reversed the anti-adipogenic effects of Shh (Fig. 6B). Cyclopamine also reversed the inhibitory effects of

20S on the expression of adipogenic differentiation marker genes, *LPL* and *aP2* (data not shown).

To determine if cyclopamine's effect on the antiadipogenic actions of 20S and Shh is produced at the level of PPAR γ expression, we evaluated the effects of cyclopamine on PPAR γ mRNA expression by RT-qPCR after 48 h of treatment with Tro. Consistent with
5 PPAR γ mRNA expression by RT-qPCR after 48 h of treatment with Tro. Consistent with earlier results, Tro caused a significant increase in PPAR γ expression, which was blocked by 20S and Shh, and pretreatment with cyclopamine completely abolished the inhibitory effect of 20S and Shh (Figs. 6C and 6D).

To determine if 20S and Shh inhibit PPAR γ expression by acting directly on its
10 promoter, we focused on C/EBP α regulated PPAR γ promoter activity. PPAR γ promoter activity assays using a murine PPAR γ 2 promoter construct luciferase plasmid (p19-PPAR γ 2) containing 2x C/EBP α binding sites, transfected into M2 cells along with MSV-C/EBP α over-expression plasmid, showed that C/EBP α overexpression stimulated PPAR γ 2 promoter activity 6-fold, which was inhibited by both 20S and Shh (Fig. 6E).

15 Figure 6 shows that the hedgehog pathway inhibitor, cyclopamine, blocks inhibitory effects of 20S and Shh on Tro-induced adipogenic differentiation and PPAR γ mRNA expression, and 20S and Shh inhibit the PPAR γ promoter activity induced by C/EBP α overexpression. Figures 6A and 6B show results for M2 cells at confluence treated with control vehicle (control), 10 μ M Tro, or a combination of Tro and 5 μ M 20S
20 or 200 ng/mL Shh, with or without a 2-h pretreatment with control vehicle (VEH) or 4 μ M cyclopamine (CYC). After 10 days, adipocyte formation was measured by Oil red O staining. The number of adipocytes was determined by counting Oil red O-positive cells in five separate fields per well, in three wells per experimental condition. The results are reported as the mean of triplicate determination \pm SD (Fig. 6A: $p < 0.0001$ for control, Tro + 20S, or 20S vs. Tro, Tro + Cyclopamine, or Tro + 20S + Cyclopamine and Tro + 20S
25 vs. Tro, Tro + Cyclopamine, or Tro + 20S + Cyclopamine, and Tro + Cyclopamine vs. Tro + 20S + Cyclopamine; Fig. 6B: $p < 0.0001$ for control, Tro + 20S, or 20S vs. Tro, Tro + Cyclopamine, or Tro + 20S + Cyclopamine and Tro + 20S vs. Tro, Tro + Cyclopamine, or Tro + 20S + Cyclopamine). Figures 6C and 6D show results for M2 cells at confluence
30 treated with control vehicle (control), 10 μ M Tro, or 5 μ M 20S, alone or in combination, with or without a 2-h pretreatment with control vehicle (VEH) or 4 μ M cyclopamine (CYC). After 48 h, PPAR γ mRNA expression was measured by quantitative real-time PCR. Fold changes in gene expression relative to the control were calculated using the

$\Delta\Delta\text{Ct}$ method and reported as the mean of triplicate determination \pm SD (Fig. 6C; $p < 0.0001$ for control vs. Tro + Cyclopamine and Tro + 20S + Cyclopamine, Tro vs. Tro + 20S, and Tro + 20S vs. Tro + 20S + Cyclopamine; $p < 0.001$ for Tro vs. Tro + Cyclopamine; Fig. 6D: $p < 0.0001$ for control or Tro + Shh vs. Tro, Tro + Cyclopamine, or Tro + Shh + Cyclopamine; $p < 0.001$ for Tro vs. control or Tro + Shh). Figure E shows results for M2 cells transiently transfected with a murine PPAR γ 2 promoter construct luciferase plasmid (p19-PPAR γ 2), alone (No Vector) or with MSV-C/EBP α overexpression plasmid (C/EBP-Alpha) and pTK-Renilla-Luciferase plasmid. Luciferase activity was measured after 24 h and normalized for transfection efficiency using the Renilla luciferase activity. Data are reported as the mean of triplicate determination \pm SD ($p < 0.001$ for control vs. control + C/EBP α and for control + C/EBP α vs. 20S + C/EBP α or Shh + C/EBP α).

To elucidate the molecular mechanism(s) by which 20S inhibits PPAR γ expression and adipogenic differentiation, the effect of modulating Hedgehog, Wnt, and MAPK signaling in turn on PPAR γ expression was studied. Modulation of Hedgehog, Wnt, and MAPK signaling was found to mediate the osteogenic effects of oxysterols. In studying the effect of modulating a given signaling mechanism, four sets of M2 cells were used: a control set, a set to which only Tro was administered, a set to which both Tro and 20S were administered, and a set to which only 20S was administered. Within a given set of M2 cells, one part was further treated with a given signaling inhibitor, and another part was not treated with a signaling inhibitor.

The canonical Wnt signaling inhibitor, Dkk-1 reversed the inhibitory effect of 20S on Tro-induced PPAR γ expression by 10%. The MAPK signaling inhibitor, PD98059 (PD) reversed the inhibitory effect of 20S on Tro-induced PPAR γ expression by 45%.

The inhibitory effects of 20S on Tro-induced adipocyte formation in cultures of M2 cells was reversed by 70%, 40%, and 50%, by Cyclopamine (administered at 4 μM), Dkk-1 (administered at 1 $\mu\text{g}/\text{mL}$), and PD98059 (administered at 20 μM), respectively. Thus, the inhibition of adipogenesis by the osteogenic oxysterol, 20S, appears to be mediated via a hedgehog-, Wnt-, and MAPK-dependent mechanism(s).

30

Discussion

This study showed that the inhibition of adipogenesis by the osteogenic oxysterol 20S is associated with the inhibition of PPAR γ mRNA expression in MSCs. Because in

this study, adipogenic differentiation was induced with Tro, a ligand for PPAR γ protein, and given the fact that PPAR γ does not seem to induce its own expression, it is likely that the positive feedback loop between C/EBP α and PPAR γ regulates the induction of key adipogenic genes and adipocyte formation. See Wu Z et al. 1999 Crossregulation of C/EBP α and PPAR γ controls the transcriptional pathway of adipogenesis and insulin sensitivity, *Mol Cell* **3**:151-158. 20S specifically inhibited PPAR γ expression, but not C/EBP α expression, in early adipogenic differentiation induced by Tro. Given that 20S did not inhibit C/EBP α expression, we examined whether inhibition was the level of C/EBP α controlled PPAR γ promoter activity. Indeed, the PPAR γ 2 promoter activity assays showed that 20S and Shh inhibit C/EBP α -induced PPAR γ promoter activity. In a non-limiting possible mechanism explaining oxysterol-mediated inhibition of PPAR expression, these data suggest that inhibition of PPAR γ expression may be at the level of PPAR γ promoter. The molecular mechanism for this inhibition remains to be elucidated; however, it may involve 20S- and Shh-induced regulation of co-activators and/or co-repressors that mediate PPAR γ promoter activity.

Our results also showed that the inhibitory effects of 20S on adipogenesis were mediated by hedgehog signaling, which is also involved in mediating the osteogenic effects of 20S. See Dwyer JR et al. 2007, Oxysterols are novel activators of the hedgehog signaling pathway in pluripotent mesenchymal cells, *J Biol Chem* **282**:8959-8968; Richardson JA et al. 2005, Characterization of osteogenic oxysterols and their molecular mechanism(s) of action, *J Bone Miner Res* **20**:S1,S414; Amantea CM et al. 2006, Oxysterols are novel activators of hedgehog and Wnt signaling. *J Bone Miner Res* **21**:S1,S156. This signaling pathway plays a role in the regulation of osteogenic and adipogenic differentiation of progenitor cells. See Spinella-Jaegle S et al. 2001, Sonic hedgehog increases the commitment of pluripotent mesenchymal cells into the osteoblastic lineage and abolishes adipocytic differentiation, *J Cell Sci* **114**:2085-2094; Suh JM et al. 2006, Hedgehog signaling plays a conserved role in inhibiting fat formation, *Cell Metab* **3**:25-34. Cyclopamine significantly blocked the inhibition of adipocyte formation and PPAR γ mRNA expression by 20S, which suggests that activation of the hedgehog signaling pathway is the prominent antiadipogenic mechanism by which 20S regulates adipogenic, as well as osteogenic, differentiation of MSCs. Shh has been shown to inhibit adipogenesis and the expression of PPAR γ in C3H10T1/2 embryonic fibroblasts. See Spinella-Jaegle S et al. 2001, Sonic hedgehog increases the commitment

of pluripotent mesenchymal cells into the osteoblastic lineage and abolishes adipocytic differentiation, *J Cell Sci* 114:2085-2094. In addition, Shh was reported to inhibit adipogenic differentiation and expression of adipogenic genes in 3T3-L1 pre-adipocytes. See Suh JM et al. 2006, Hedgehog signaling plays a conserved role in inhibiting fat formation, *Cell Metab* 3:25-34. Furthermore, a dominant-negative form of Gli (the transcription factor that mediates hedgehog-regulated gene expression) and cyclopamine were shown to inhibit hedgehog signaling while stimulating adipogenic differentiation in 3T3-L1 cells. See Suh JM et al. 2006 Hedgehog signaling plays a conserved role in inhibiting fat formation, *Cell Metab* 3:25-34. One mechanism by which 20S exerts its anti-adipogenic effects through hedgehog signaling may involve the induction of antiadipogenic transcription factors, such as Gilz, GATAs, and pref-1. Shh was shown to increase the levels of Gilz, GATA2, GATA3, or pref-1 in mouse NIH-3T3 fibroblasts, C3H10T1/2 pluripotent mesenchymal cells, and 3T3-L1 pre-adipocytes. See Suh JM et al. 2006; Ingram WJ et al. 2002, Novel genes regulated by sonic hedgehog in pluripotent mesenchymal cells, *Oncogene* 21:8196-8205. GATA-2 and GATA-3 inhibit PPAR γ expression and adipogenic differentiation through direct binding to the PPAR γ promoter, as well as by physically interacting with C/EBP α . See Tong Q et al. 2000, Function of GATA transcription factors in preadipocyte-adipocyte transition, *Science* 290:134-138; Tong Q et al. 2005, Interaction between GATA and the C/EBP family of transcription factors is critical in GATA-mediated suppression of adipocyte differentiation, *Mol Cell Biol* 25:706-715. Gilz and pref-1 also inhibit adipogenic differentiation and keep 3T3-L1 as preadipocytes. See Suh JM et al. 2006; Shi X et al. 2003, A glucocorticoid-induced leucine-zipper protein, GILZ, inhibits adipogenesis of mesenchymal cells, *EMBO Rep* 4:374-380. We examined the effects of 20S on GATA2, GATA3, Gilz, and pref-1 expression in M2 cells using RT-qPCR and on GATA transcriptional activity using a GATA reporter transfected into M2 cells. Results showed that 20S does not increase GATA2/GATA3, Gilz, and pref-1 gene expression nor GATA transcriptional activity assessed after 24 and 48 h of treatments (data not shown). The molecular mechanism(s) by which 20S inhibits PPAR γ expression in a hedgehog signaling-dependent manner remains unclear. Although 4 μ M cyclopamine treatment fully reversed the inhibitory effect of 20S on PPAR γ mRNA expression at 48 h, it did not completely reverse 20S effects on adipocyte formation as measured by Oil red O staining at late stages of cell differentiation, and increasing the dose of cyclopamine from 4 to 8 μ M did not have any

additional effect on adipocyte formation (data not shown). However, the inhibitory effects of Shh on PPAR γ mRNA expression as well as adipocyte formation were completely reversed by 4 pM cyclopamine. This suggests that, in addition to hedgehog signaling, other anti-adipogenic mediators may contribute to the complete inhibition of adipogenesis by 20S in MSCs.

Unlike Shh, 20S activates not only the hedgehog pathway but also liver X receptor (LXR) signaling (Fig. 8). Figure 8 illustrates the regulation of adipogenic differentiation of bone MSCs by 20S. 20S activates LXRs and Hh signaling pathways. LXR activation increases the expression of SREBP-1c/ADD1. LXRs and PPAR γ positively regulate each other's expression. Moreover, SREBP-1c/ADD1 regulates adipogenesis through PPAR γ gene expression and through the production of an endogenous PPAR γ ligand(s). Despite activation of LXRs by 20S oxysterol, the activation of Hh signaling induced by 20S inhibits PPAR γ expression and adipogenic differentiation of M2 cells.

Oxysterols are ligands for LXRs, which regulate cholesterol, lipid, and carbohydrate metabolism. LXR activation increases the expression of sterol regulatory element binding protein-1c (SREBP-1c)/adipogenic differentiation of factor 1 (ADD1), which induces the expression of fatty acid synthase, glycerol-3-phosphate acyltransferase, and stearyl CoA desaturase 2 during adipogenic differentiation. It has been shown that activation of LXRs increases lipid accumulation during adipogenic differentiation of 3T3-L1 and 3T3-F422A pre-adipocytes. LXRs and PPAR γ seem to positively regulate each other's expression. The expression of LXR α is increased directly by PPAR γ activation in 3T3-L1 pre-adipocytes and in a mouse model. Juvet LK et al. 2003, On the role of liver X receptors in lipid accumulation in adipocytes, *Mol Endocrinol* 17:172-182. Furthermore, PPAR γ promoter contains the conserved binding site for LXR, and LXR activation increases PPAR γ expression. See Seo JB et al. 2004, Activated Liver X receptors stimulate adipocyte differentiation through induction of peroxisome proliferators-activated receptor γ expression, *Mol Cell Biol* 74:3430-3444. In addition, SREBP1/ADD1 regulates adipogenesis through PPAR γ gene expression through E-box motifs in the PPAR γ promoter and through the production of an endogenous PPAR γ ligand(s) to increase PPAR γ transcriptional activity. Fajas L et al. 1999, Regulation of peroxisome proliferators-activated receptor γ expression by adipocyte differentiation and determination 1: Implications for adipocyte differentiation and metabolism, *Mol Cell Biol*

19:5495-5503; Kim JB et al. 1998 ADD1/SREBP1 activates PPAR γ through the production of endogenous ligand, Proc Natl Acad Sci USA 95:4333-4337.

In this study, we examined whether 20S, in addition to inhibiting PPAR γ mRNA expression, also inhibits PPAR γ transcriptional activity. When a vector was inserted into the cells that caused the expression of PPAR γ protein, without the normal transcriptional machinery that drives PPAR γ mRNA expression from the cellular genome, we found that troglitazone-induced PPAR γ transcriptional induction activity was not inhibited but rather enhanced by 20S. Given the positive interactions between LXR and PPAR γ in the context of adipogenesis, one potential explanation for this enhancement is that LXR activation by 20S causes further stimulation of troglitazone-induced PPAR γ transcriptional activity. Despite the activation of LXRs by 20S in M2 cells (data not shown), the activation of hedgehog signaling by 20S inhibited PPAR γ mRNA expression and adipogenic differentiation of these cells, suggesting that this level of hedgehog pathway activation is capable of counteracting any LXR-mediated pro-adipogenic effects of 20S. The role of LXRs in regulating adipogenesis in bone MSCs remains unclear.

Altogether, this study shows that specific oxysterols with pro-osteogenic and anti-adipogenic properties serve as regulators of a shift in differentiation of MSCs into osteoblasts and away from adipocytes. Such oxysterols may be used to regulate the lineage-specific differentiation of MSC under physiological and/or pathological conditions.

Example V - Oxysterol Inhibition of PPAR

Synthetic oxysterol compounds were studied for inhibition of adipogenesis and PPAR expression. Figure 7A shows results obtained with M2-10B4 bone marrow stromal cells treated with control vehicle or the PPAR γ activator, troglitazone (Tro, 10 μ M), in the presence or absence of various oxysterols (5 μ M), as indicated. After 10 days of treatment, cells were stained with Oil-red-O to detect adipocytes, and the number of positively stained cells was determined using light microscopy. Data from a representative experiment are reported as the mean of triplicate determination (average of five fields per well, 3 wells per experimental condition) \pm SD. The data indicate that the oxysterol compounds identified as Oxy11, Oxy12, Oxy13, Oxy14, and Oxy16 had an inhibitory effect on Tro-induced adipocyte formation.

Figure 7B shows results obtained with M2-10B4 bone marrow stromal cells treated with control vehicle or the PPAR γ activator, troglitazone (Tro, 10 μ M), in the presence or absence of Oxy13 (5 μ M), as indicated. After 48 hours of treatment, RNA was extracted from cells and analyzed for PPAR γ expression by Q-RT-PCR. Data from a representative experiment, normalized to GAPDH expression, are reported as the mean of triplicate determinations \pm SD. The data indicate that the oxysterol compound identified as Oxy13 inhibits Tro-induced PPAR γ expression.

10 **Example VI - Osteogenic Oxysterols that Activate Hedgehog Signaling in Determined Preadipocytes Also Inhibit Adipogenesis**

We examined whether 20(S)-hydroxycholesterol (20S), an osteogenic oxysterol that activates hedgehog (Hh) signaling and osteogenesis as well as inhibiting adipogenesis in a number of multipotent mesenchymal cells, also inhibits adipogenesis in predetermined adipocytes. In order to test this possibility, we used the 3T3-L1 predetermined preadipocyte line which has been shown in the past to readily undergo adipogenesis. To our best knowledge, this cell line is not multipotent unless additional genes are exogenously introduced and expressed in them in order to artificially induce their differentiation into cell types other than adipocytes.

20 We found that 20S significantly inhibited the expression of Peroxisome Proliferator- Activated Receptor gamma (PPAR γ), the master regulator of adipogenesis, and adipocyte formation in the presence of adipogenic inducers (DMI: dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 15 μ g/ml insulin) in 3T3-L1 cells (Figure 1). Thus it is clear that 20S inhibits adipogenic differentiation in both multipotent mesenchymal cells and in committed preadipocytic cells. Furthermore, we found that anti-adipogenic effects of 20S are correlated with the activation of Hh pathway in 3T3-L1 cells as demonstrated by the induced expression of Hh target genes Gli1 and Ptch1 (Figure 2).

In the studies shown herein, adipogenesis in 3T3-L1 cells was induced by the classic adipogenic cocktail, DMI, which has been shown to stimulate the adipogenic differentiation program in these cells. Adipogenesis can also be triggered by ligands that directly activate PPAR γ such as troglitazone and other members of the thiazolidinedione family of drugs.

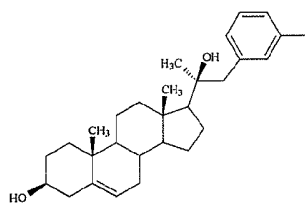
Studies such as those described in this Example are also performed with other oxysterols, including the novel synthetic oxysterols described herein. It is expected that the novel synthetic oxysterols will elicit effects that are even stronger than those obtained with 20S.

5

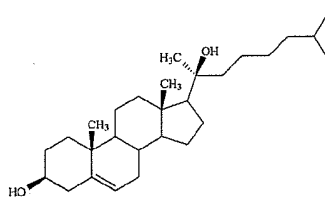
From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make changes and modifications of the invention to adapt it to various usage and conditions and to utilize the present invention to its fullest extent. The preceding preferred specific embodiments are to be construed as merely illustrative, and not limiting 10 of the scope of the invention in any way whatsoever. The entire disclosure of all applications, patents, and publications cited above, including U.S. provisional patent application 61/224,656, filed July 10, 2009, are hereby incorporated by reference in their entirety.

WE CLAIM:

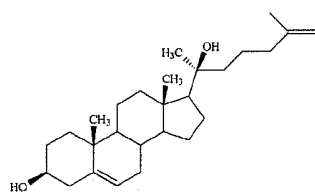
1. A method for inhibiting expression of a peroxisome proliferator activated receptor (PPAR) in a preadipocyte cell, comprising:
 - 5 contacting the cell with at least one oxysterol compound in an amount effective to inhibit expression of the PPAR in the cell; and
 - measuring inhibition of expression of the PPAR.
2. The method of claim 1, wherein the inhibited expression is of PPAR gamma.
- 10 3. The method of claim 1, wherein the at least one oxysterol compound inhibits adipogenesis of the cell.
4. The method of claim 1, wherein the at least one oxysterol compound is an
15 activator of the hedgehog signaling pathway.
5. The method of claim 1, wherein the at least one oxysterol compound is 20(S)-hydroxycholesterol.
- 20 6. The method of claim 1, wherein the at least one oxysterol compound is 20(S)-hydroxycholesterol, and wherein the at least one oxysterol contacts the cell at a concentration of at least about 5 μ M.
7. The method of claim 1, wherein the at least one oxysterol compound is selected
25 from the group consisting of 20(S)-hydroxycholesterol,



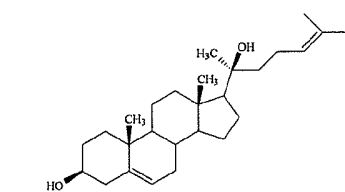
Oxy 11



Oxy 12

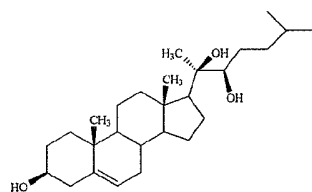


Oxy13



Oxy14

, and

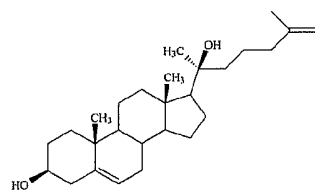


Oxy16

, and combinations thereof.

5

8. The method of claim 1, wherein the at least one oxysterol compound is selected from the group consisting of 20(S)-hydroxycholesterol and



Oxy13

10

and combinations thereof.

9. The method of claim 1, wherein the at least one oxysterol compound is selected from the group consisting of 22(R)-hydroxycholesterol, 22(S)-hydroxycholesterol, 25-hydroxycholesterol, 25(S)-hydroxycholesterol, 5-cholesten-3-beta-20-alpha-diol-3-acetate, 24-hydroxycholesterol, 24(S)-hydroxycholesterol, 24(S),25-epoxycholesterol, 26-hydroxycholesterol, 4-beta-hydroxycholesterol, and pregnenolone and combinations thereof.

20 10. The method of claim 1, wherein the expression of PPAR in the cell is inhibited relative to a baseline value.

11. The method of claim 1, wherein contacting the cell with the at least one oxysterol compound inhibits the mRNA expression of PPAR in the cell relative to a baseline value.

12. The method of claim 1, wherein contacting the cell with at least one oxysterol compound inhibits the dysregulated differentiation of the cell into an adipocyte.
- 5 13. The method of claim 1, wherein contacting the cell with at least one oxysterol compound inhibits expression and/or activity of a PPAR target gene in the cell; and further comprising measuring inhibition of expression of the PPAR target gene.
14. The method of claim 13, wherein the PPAR target gene is a gene selected from the
10 group consisting of adipocyte protein 2 (aP2) and lipoprotein lipase (LPL).
15. The method of claim 1, wherein the oxysterol compound reduces the expression of PPAR in a 3T3-L1 predetermined preadipocyte cell stimulated with 20(S)-hydroxycholesterol.
- 15 16. The method of claim 1, wherein the inhibition of PPAR expression in the cell is sufficient to inhibit a PPAR mediated response in the cell.
17. The method of any of claims 1-16, wherein the cell is *in vitro* or *ex vivo*.
- 20 18. The method of any of claims 1-16, wherein the cell is in a mammalian subject.
19. The method of any of claims 1-16, wherein the cell is in a human.
- 25 20. A method of treating or preventing a condition associated with increased adipogenesis in a subject, comprising administering to the subject an amount of an oxysterol compound effective to treat the condition, wherein the oxysterol compound has been shown to be in an amount that is effective to inhibit or prevent PPAR expression in a preadipocyte.
- 30 21. The method of claim 20, wherein the oxysterol compound is administered systemically.

22. The method of claim 20, wherein the oxysterol compound is administered locally to a target tissue of interest.
23. The method of claim 20, wherein the condition is obesity, osteoporosis, diabetes,
5 or aging.
24. A method of treating or preventing a condition associated with excessive accumulation of intracellular and/or extracellular fats and/or lipids in a subject, comprising administering to the subject an amount of an oxysterol compound effective to
10 treat or prevent the condition, wherein the oxysterol compound has been shown to be in an amount that is effective to inhibit PPAR expression in a preadipocyte.
25. The method of claim 24, wherein the oxysterol compound is administered systemically.
15
26. The method of claim 24, wherein the oxysterol compound is administered locally to a target tissue of interest.
27. The method of claim 24, wherein the condition is xanthoma formation.
20
28. A kit, comprising
a dosage form of a pharmaceutical composition comprising an oxysterol compound effective to inhibit expression of PPAR in a preadipocyte, optionally in a container.
25
29. An *in vitro* method for identifying an oxysterol compound that inhibits expression of PPAR, comprising:
screening a candidate oxysterol compound for the ability to inhibit expression of PPAR in a preadipocyte contacted with a PPAR-stimulatory agent, comprising contacting
30 the preadipocyte with the candidate oxysterol and measuring the ability of the candidate oxysterol to reduce the stimulation of PPAR expression in the preadipocyte, and
selecting a candidate oxysterol compound that measurably inhibits the PPAR expression.

30. The method of claim 29, wherein the PPAR-stimulatory agent is a PPAR agonist, a DMI cocktail, or thiazolidinedione.
- 5 31. The method of claim 29,
wherein the amount of expression of PPAR is at least 3-fold less when measured in the presence of the candidate oxysterol than when measured in the absence of the candidate oxysterol.
- 10 32. The method of claim 29,
wherein the amount of expression of PPAR is at least 10-fold less when measured in the presence of the candidate oxysterol than when measured in the absence of the candidate oxysterol.
- 15 33. The method of claim 29,
wherein the preadipocyte is contacted with the PPAR stimulatory agent in the presence and in the absence of the candidate oxysterol; and the amount of expression of PPAR is at least 100-fold less when measured in the presence of the candidate oxysterol than when measured in the absence of the candidate oxysterol.
- 20 34. The method of claim 29,
wherein the PPAR expression is PPAR gamma expression.

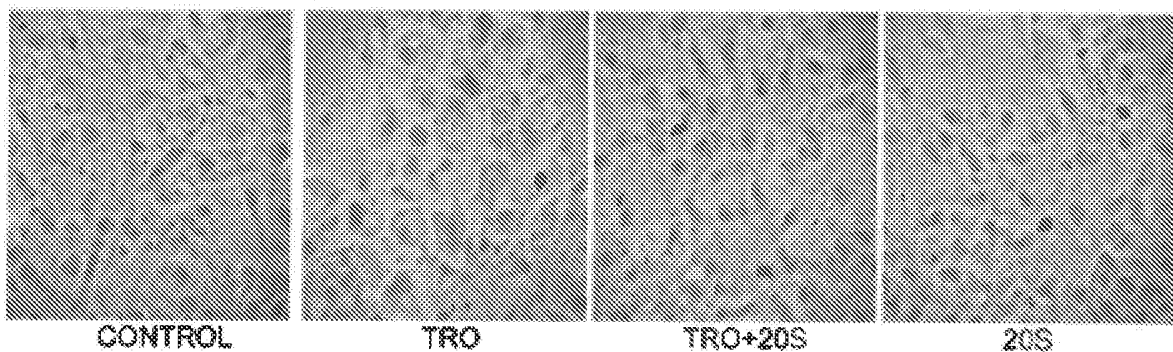


FIG. 1A

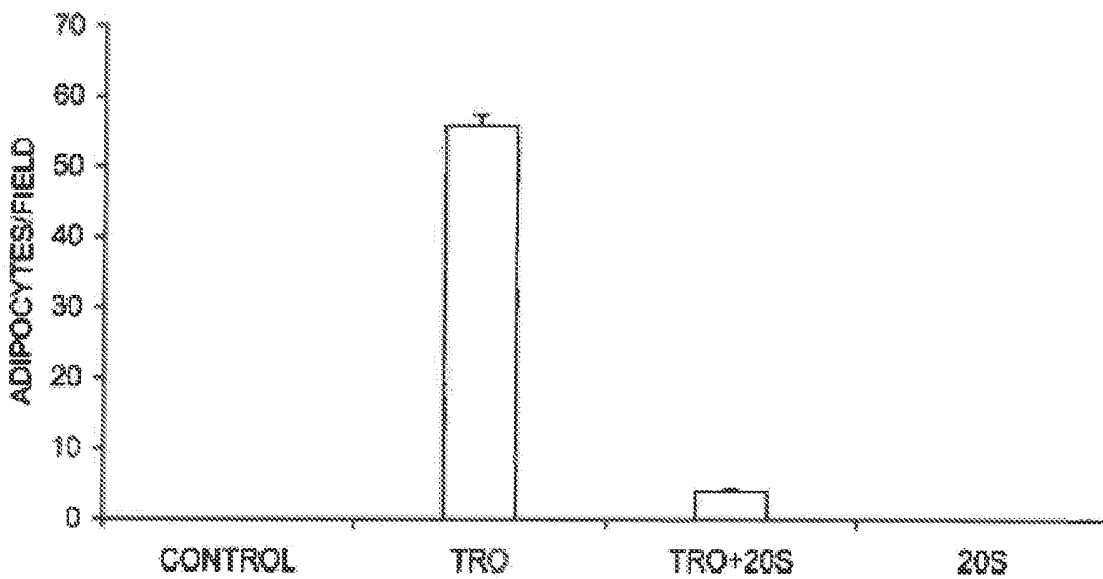
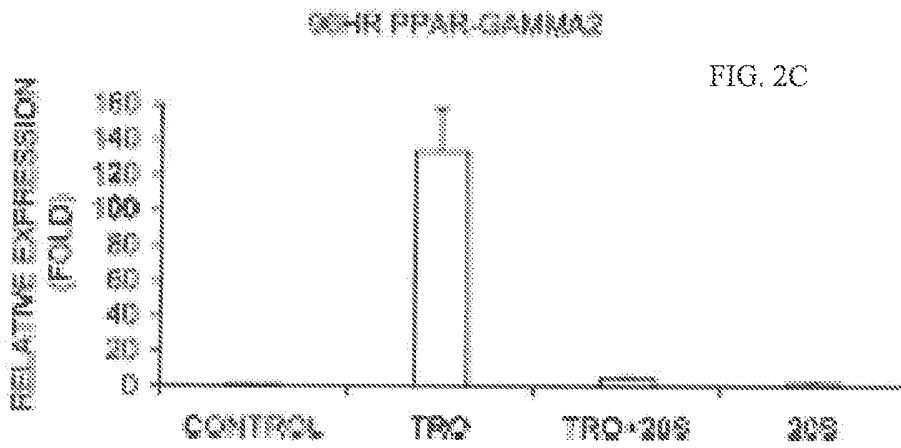
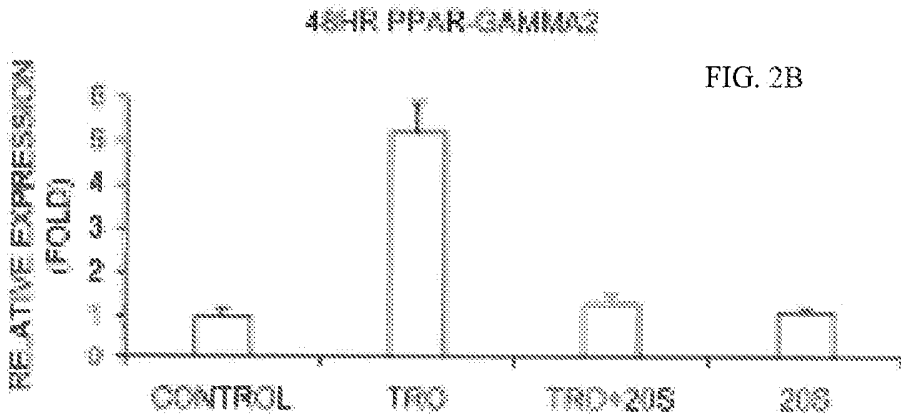
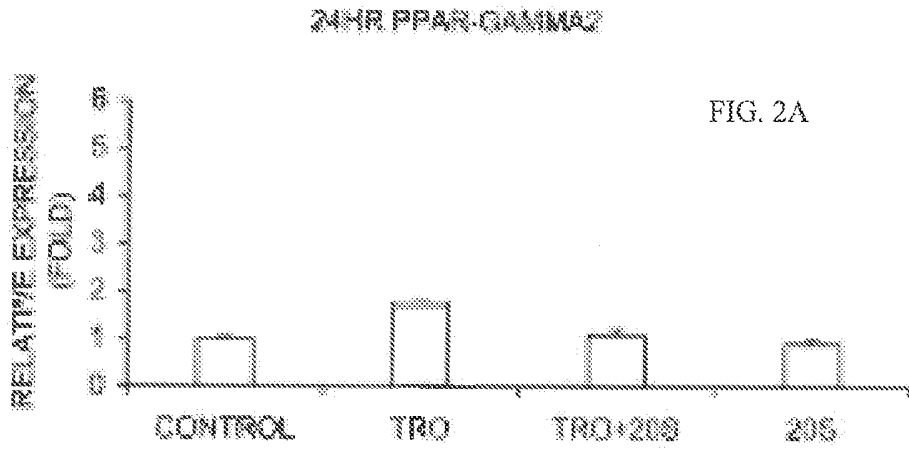
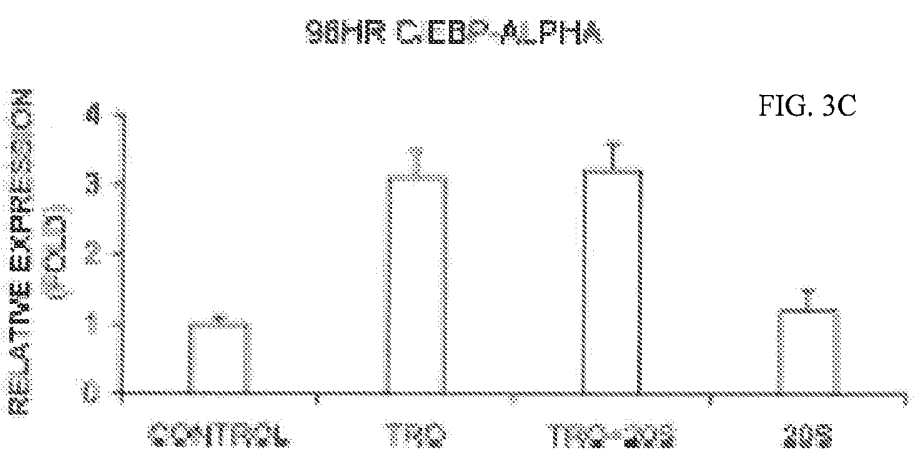
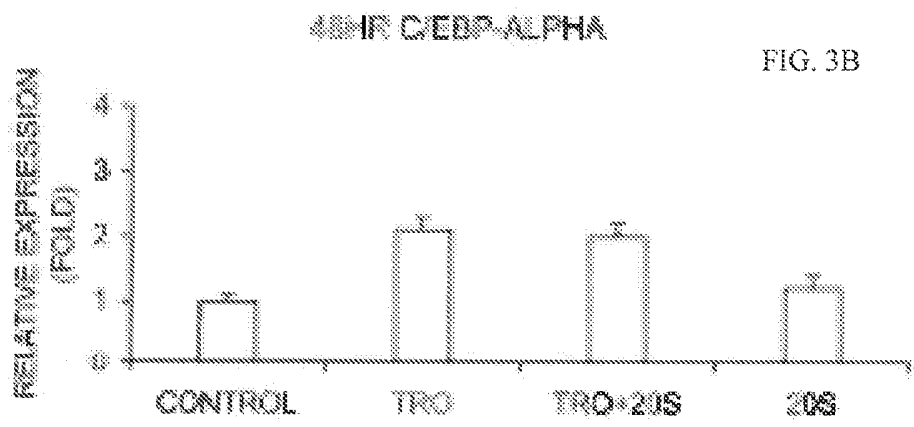
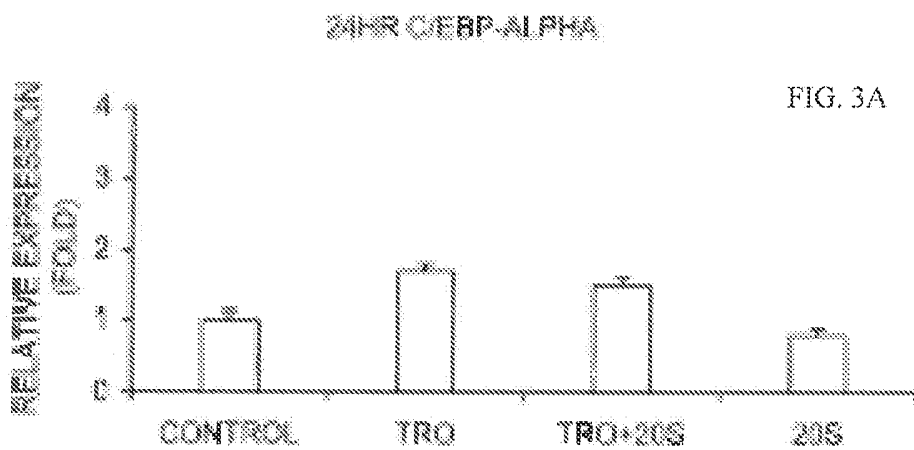


FIG. 1B





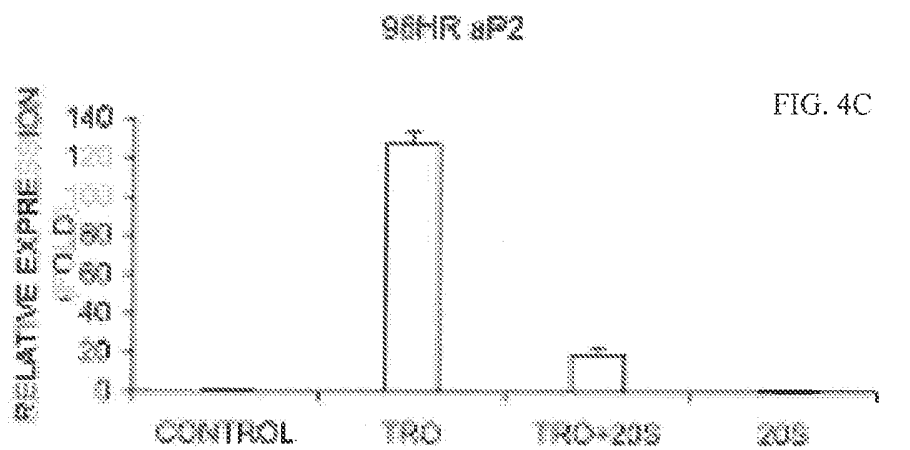
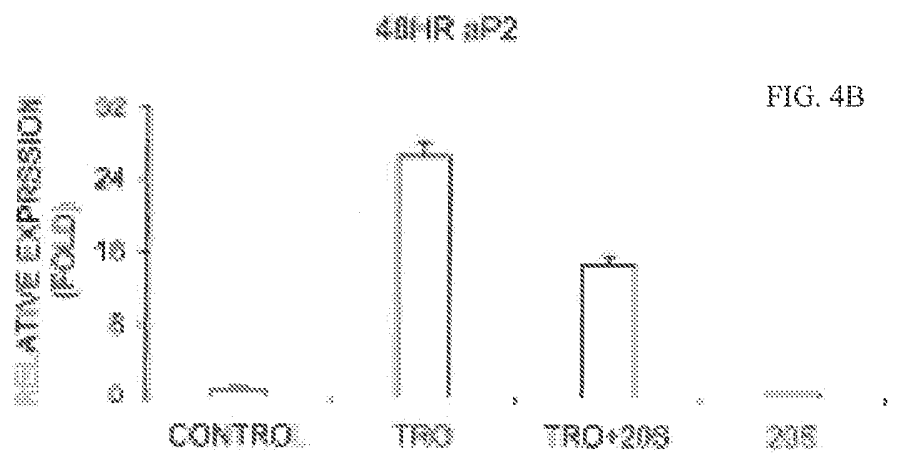
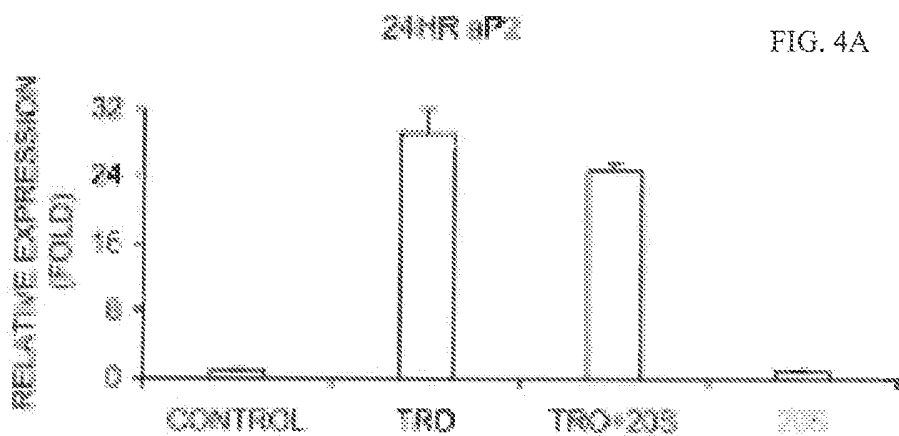


FIG. 5A

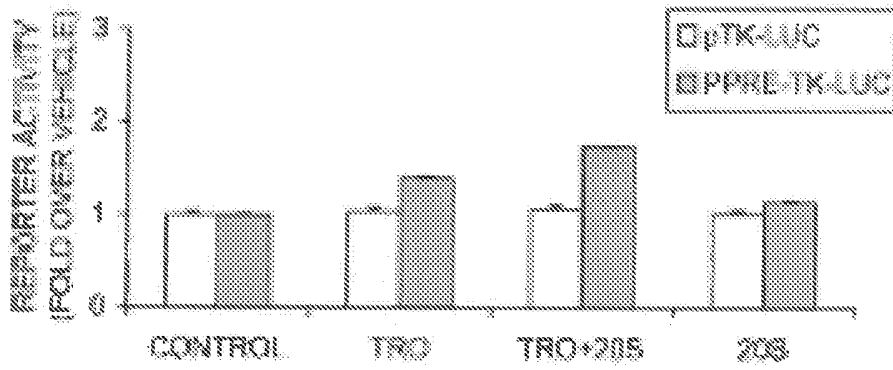


FIG. 5B

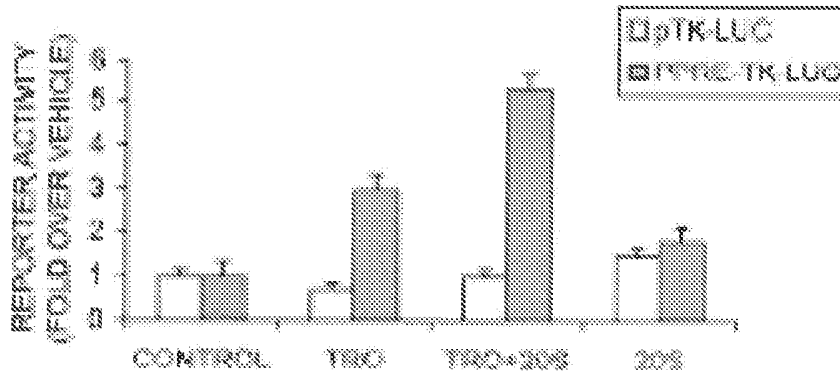
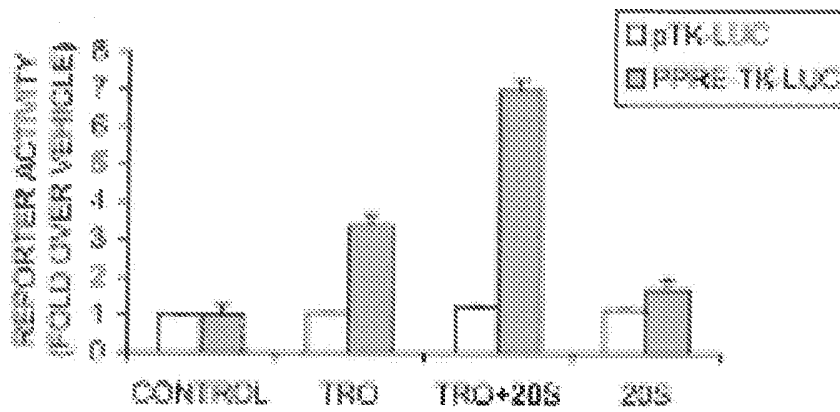


FIG. 5C



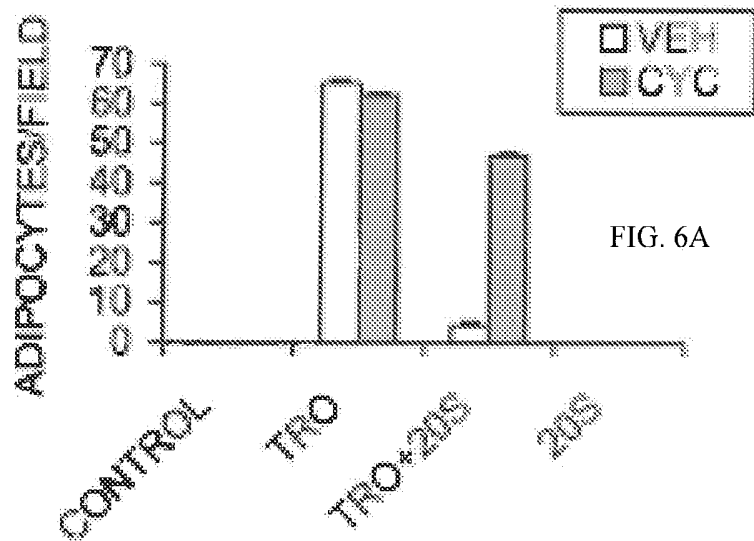


FIG. 6A

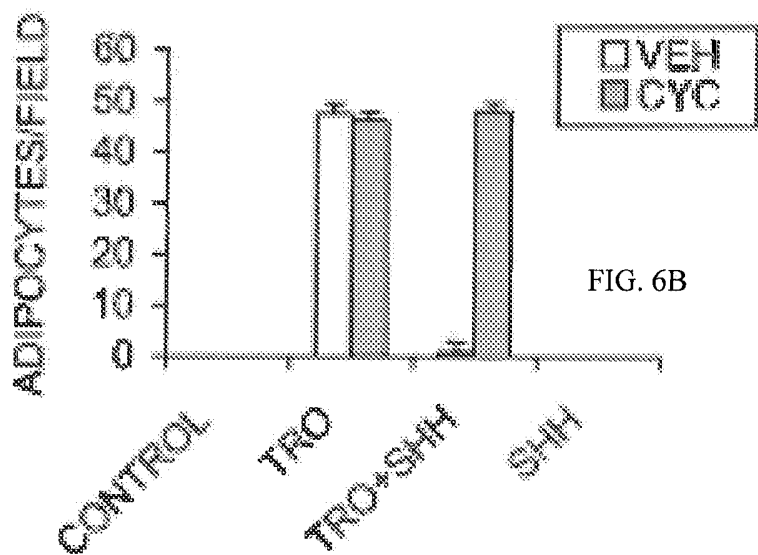


FIG. 6B

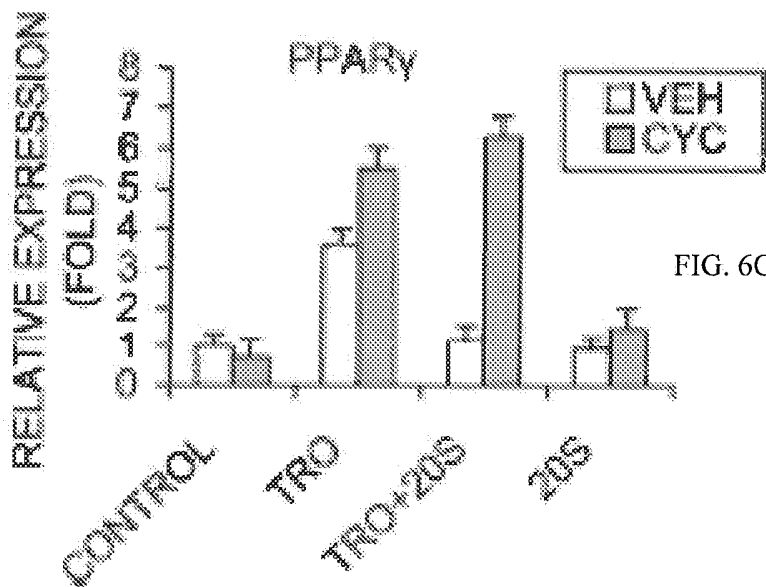


FIG. 6C

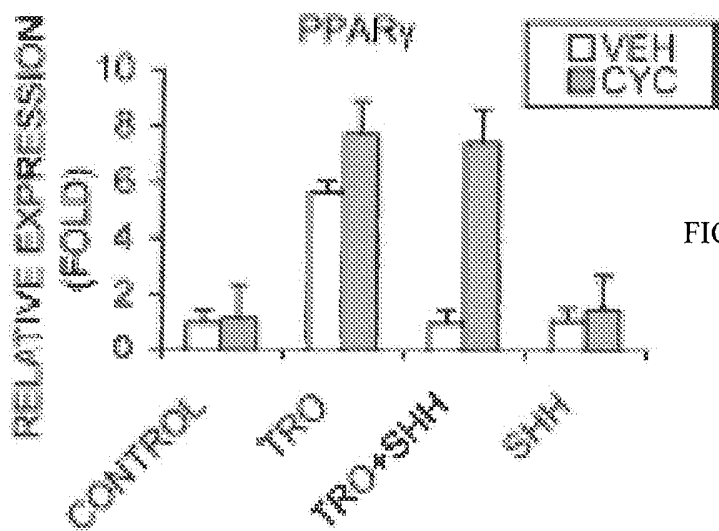


FIG. 6D

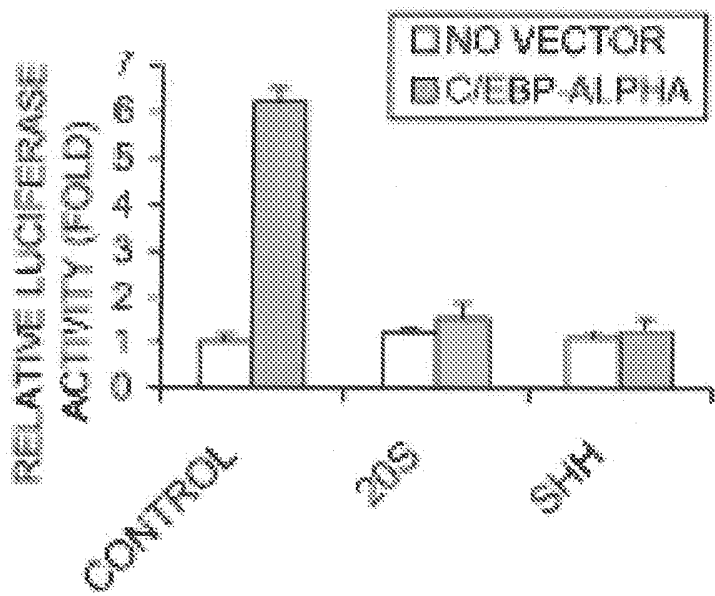


FIG. 6E

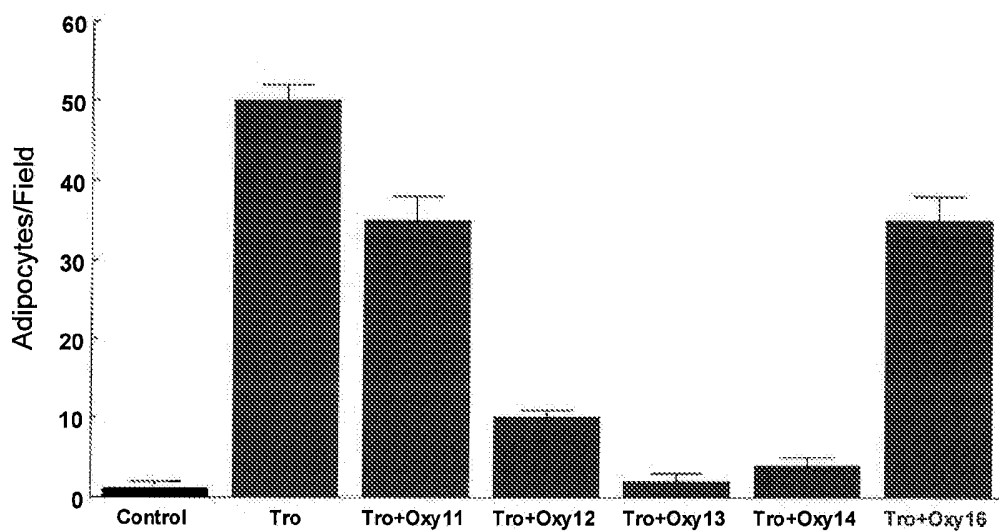


FIG. 7A

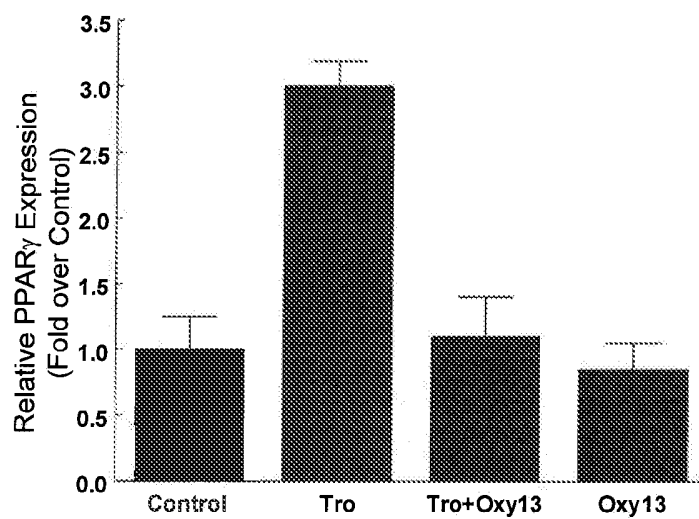


FIG. 7B

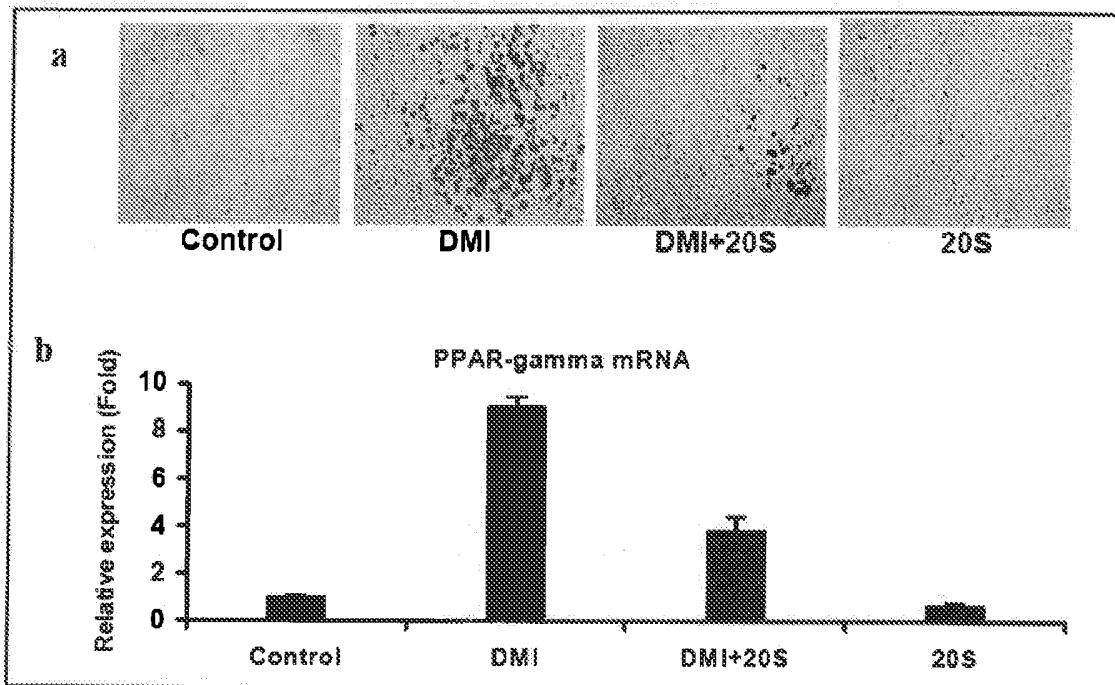


FIG. 8

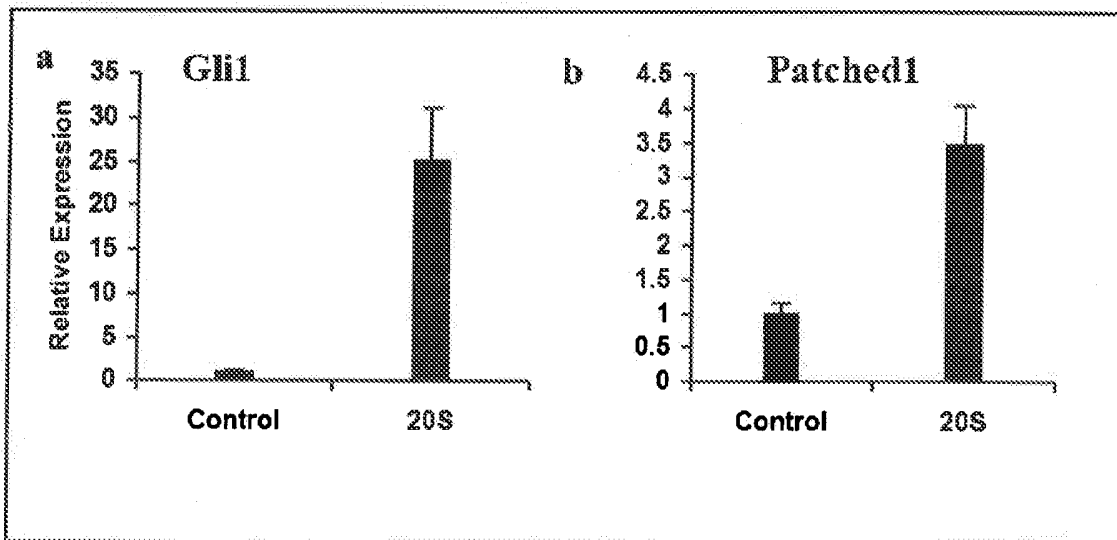


FIG. 9

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 10/41560

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A01N 45/00 (2010.01)

USPC - 514/26

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)

USPC: 514/26

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 514/102, 107-108, 114, 365, 369, 519, 530, 538 (see search terms below)

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

PubWEST (PGPB,USPT,EPAB,JPAB), Google Scholar, Patentscope

PPAR\$, express\$, inhibit\$, modulat\$, oxysterol, 20(S)-hydroxycholesterol, formulation, composition, administ\$, preadipocyte, adipogenesis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	WO 2008/082520 A2 (PARHAMI et al.) 10 July 2008 (10.07.2008) pg 3, ln 15-23; pg 4, ln 12-14; ln 16-26; ln 19-20; pg 5, ln 1-2; ln 6-8, pg 6, ln 1-4; pg 9, ln 30 to pg 10, ln 6; pg 11, ln 6-9; pg 14, ln 7 to pg 15, ln 4; pg 33, ln 3-10; pg 48, ln 9-19; pg 49, ln 1-12; Fig 1A, Fig 1B; Fig 2A, Fig 2B, Fig 2C; Fig 4A, Fig 4B; Fig 7B	1-14, 16-34 ----- 15
Y	SEO et al. Activated Liver X Receptors Stimulate Adipocyte Differentiation through Induction of Peroxisome Proliferator-Activated Receptor Gamma Expression. MOLECULAR AND CELLULAR BIOLOGY, 2004, Vol 24, No 8, pp 3430-3444; pg 3432, para 7; pg 3433, para 3; Fig 4	15
Y	US 2008/0070883 A1 (NAGPAL) 20 March 2008 (20.03.2008) para [0028], [0042]-[0043]	15

 Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier 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 priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

25 August 2010 (25.08.2010)

Date of mailing of the international search report

31 AUG 2010

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Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

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