US 20230233580A1

(19) **United States**

(12) **Patent Application Publication**

Ren et al.

(10) **Pub. No.: US 2023/0233580 A1**

(43) **Pub. Date: Jul. 27, 2023**

(54) **USE OF OXYGENATED CHOLESTEROL SULFATES FOR CANCERS AND NON-CANCEROUS TRANSFORMATIONS RELATED TO EPSTEIN-BARR VIRUS**

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(21) Appl. No.: **18/008,585**

(22) PCT Filed: **Jun. 25, 2021**

(86) PCT No.: **PCT/US2021/039207**

§ 371 (c)(1),
(2) Date: **Dec. 6, 2022**

Related U.S. Application Data

(60) Provisional application No. 63/044,631, filed on Jun. 26, 2020, provisional application No. 63/127,905, filed on Dec. 18, 2020, provisional application No. 63/141,382, filed on Jan. 25, 2021, provisional application No. 63/146,559, filed on Feb. 5, 2021, provisional application No. 63/146,563, filed on Feb. 5, 2021, provisional application No. 63/146,565, filed on Feb. 5, 2021, provisional application No. 63/146,566, filed on Feb. 5, 2021, provisional application No. 63/146,568, filed on Feb. 5, 2021, provisional application No. 63/149,977, filed on Feb. 16, 2021, provisional application No. 63/149,993, filed on Feb. 16, 2021.

Publication Classification

(51) **Int. Cl.**
A61K 31/575 (2006.01)
A61P 35/00 (2006.01)

(52) **U.S. Cl.**
CPC ***A61K 31/575*** (2013.01); ***A61P 35/00*** (2018.01)

(57) **ABSTRACT**

Aspects of the present disclosure include methods for treating at least one of cancer and non-cancerous transformation related to Epstein-Barr virus, such as at least one of Hodgkin's lymphoma, soft tissue sarcoma, leiomyosarcoma, nasopharyngeal carcinoma, Burkitt's lymphoma, T-cell lymphoma, gastric carcinoma, invasive breast cancer, and hierarchically organized carcinoma. Hierarchically organized carcinomas include, but are not limited to, pancreatic ductal adenocarcinoma, urothelial cancer, colorectal cancer, head and neck cancer, non-small cell lung cancer, esophagus cancer, breast cancer, thyroid cancer, oral cancer, cervical cancer, ovarian cancer, and liver cancer. In practicing the subject methods, an effective amount of at least one compound selected from 25-hydroxycholesterol-3-sulfate (25HC3S), 25-hydroxycholesterol-disulfate (25HCDS), 27-hydroxycholesterol-3-sulfate (27HC3S), 27-hydroxycholesterol-disulfate (27HCDS), 24-hydroxycholesterol-3-sulfate (24HC3S), 24-hydroxycholesterol-disulfate (24HCDS), and 24,25-epoxycholesterol-3-sulfate, or salt thereof is administered to the subject.

Specification includes a Sequence Listing.

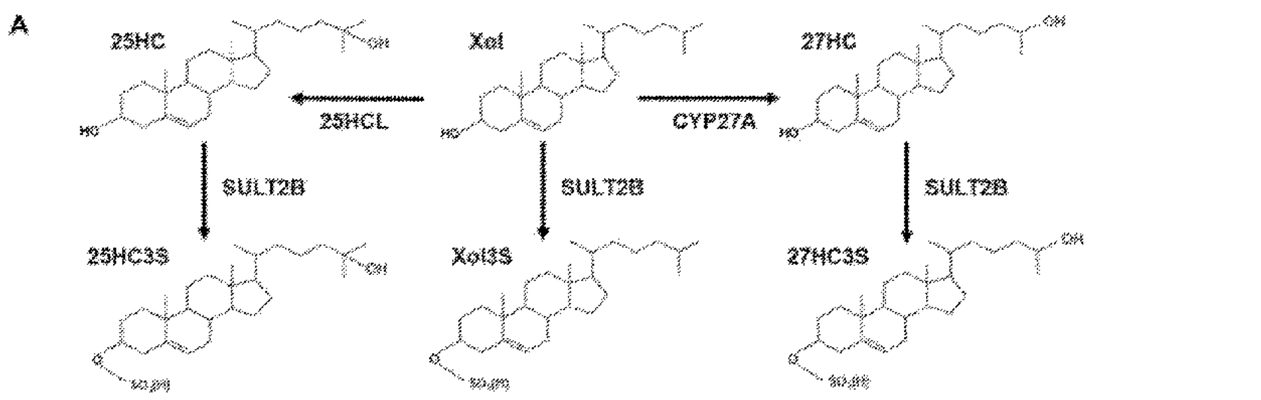
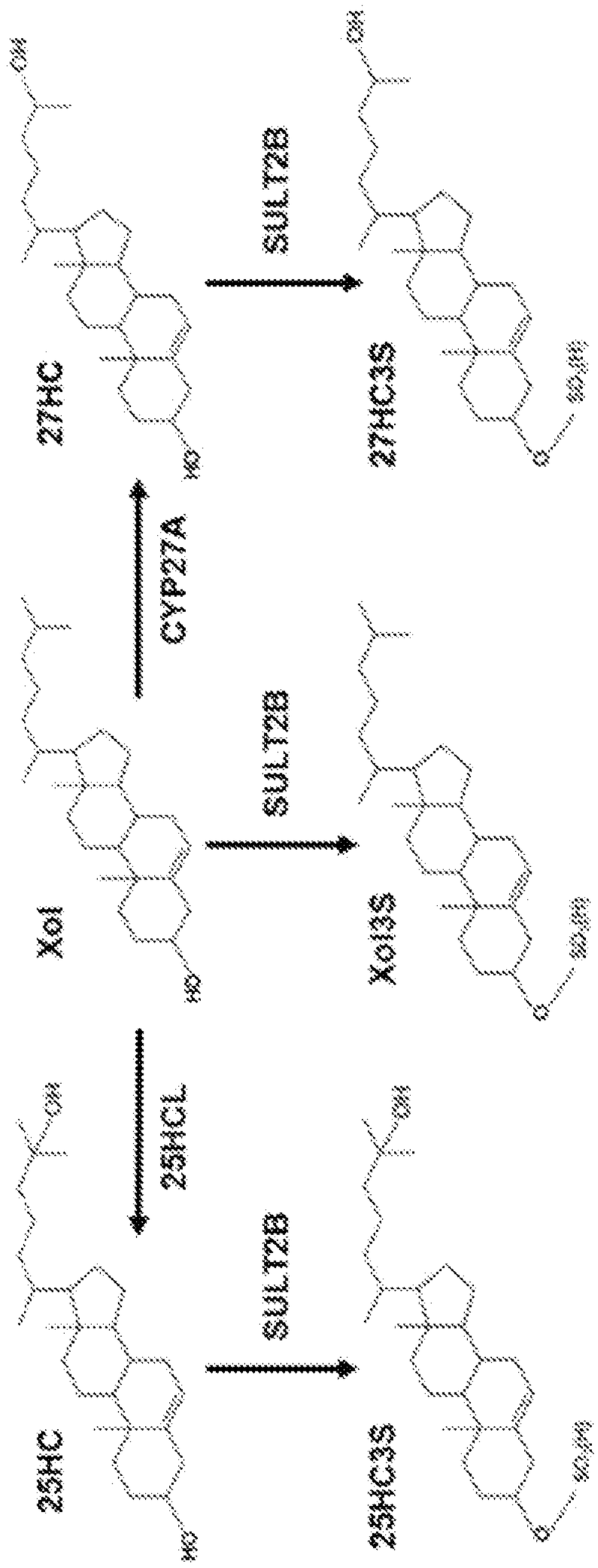


Figure 1


A
25HC
25HCL
SULT2B
25HC3S
27HC
CYP27A
SULT2B
27HC3S
Xol
SULT2B
Xol3S

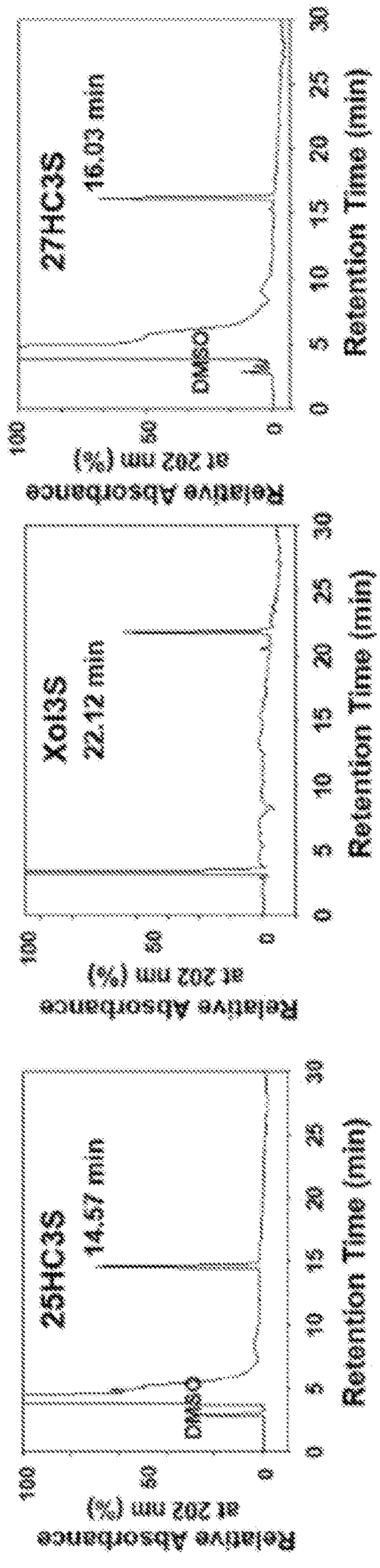
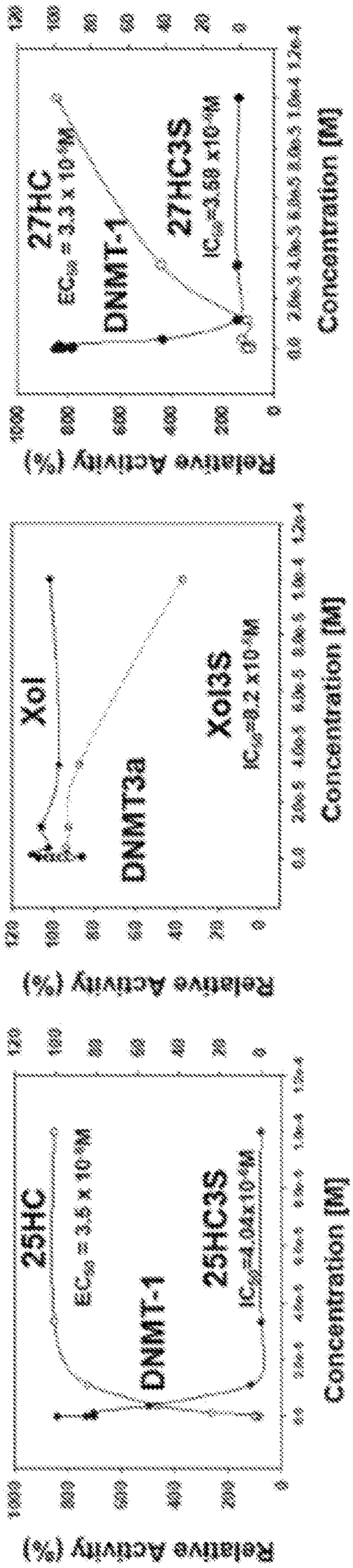


Figure 1 cont.



D

Figure 1 cont.

Figure 2

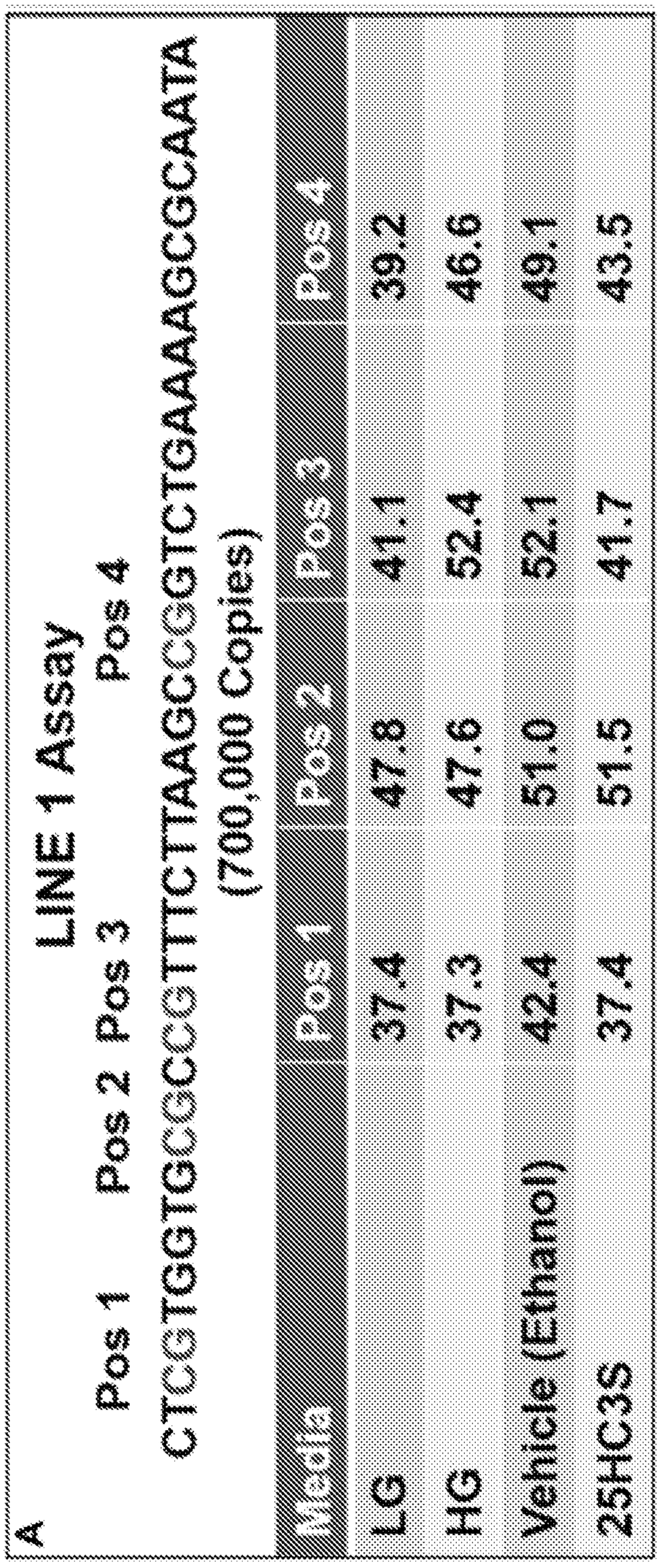

A
LINE 1 Assay
Pos 1 Pos 2 Pos 3 Pos 4
CTCGTGGTGCGGTTCTTAAGCCGGTCTGAAAATAAGGCAATA
(700,000 Copies)
Micelle
Pos 1 Pos 2 Pos 3 Pos 4
LG 37.4 47.8 41.1 39.2
HG 37.3 47.6 52.4 46.6
Vehicle (Ethanol) 42.4 51.0 52.1 49.1
25HC3S 37.4 51.5 41.7 43.5

Figure 2 cont.

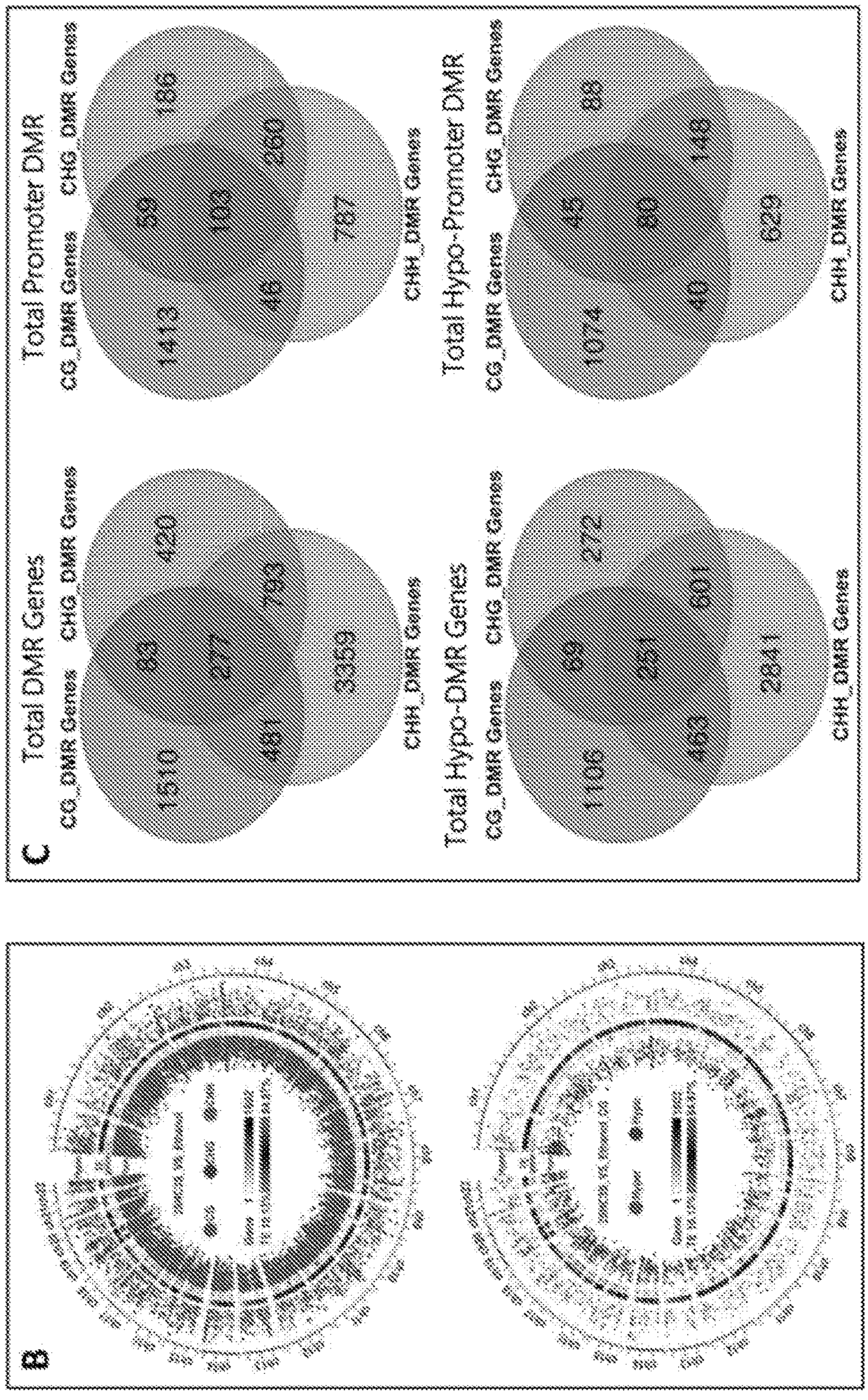


Figure 2 cont.

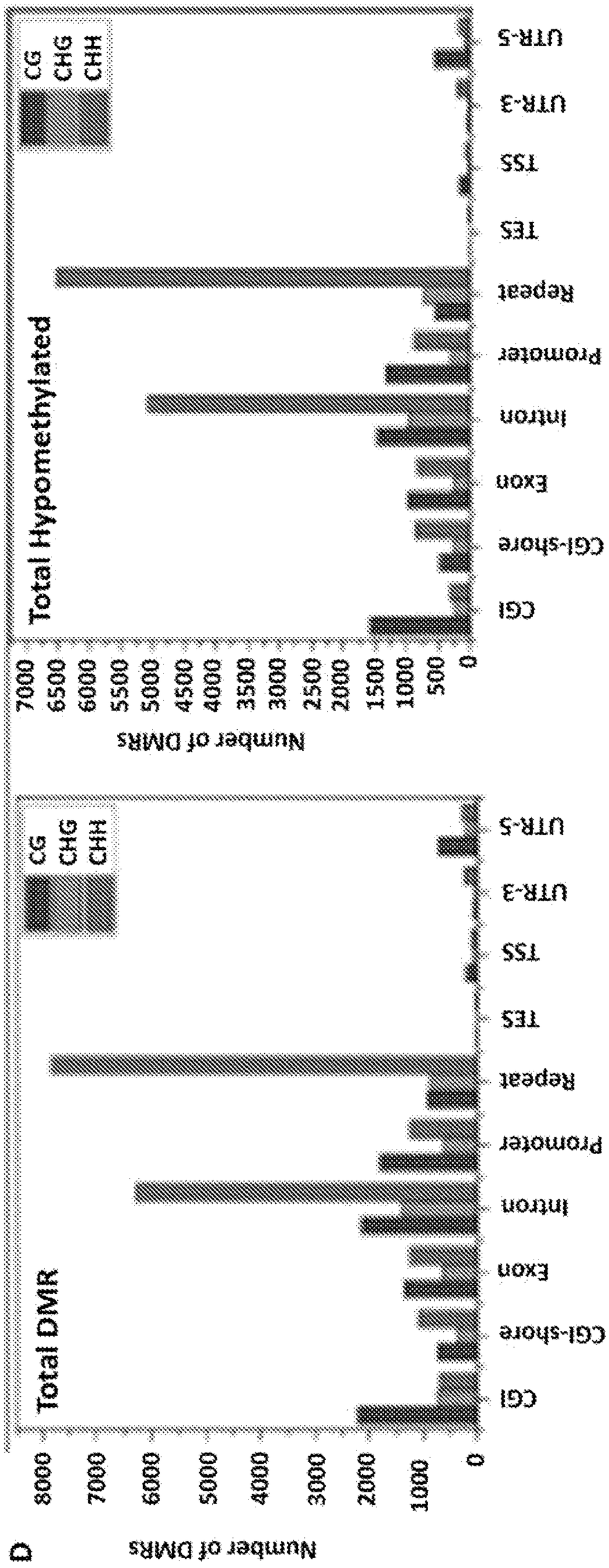
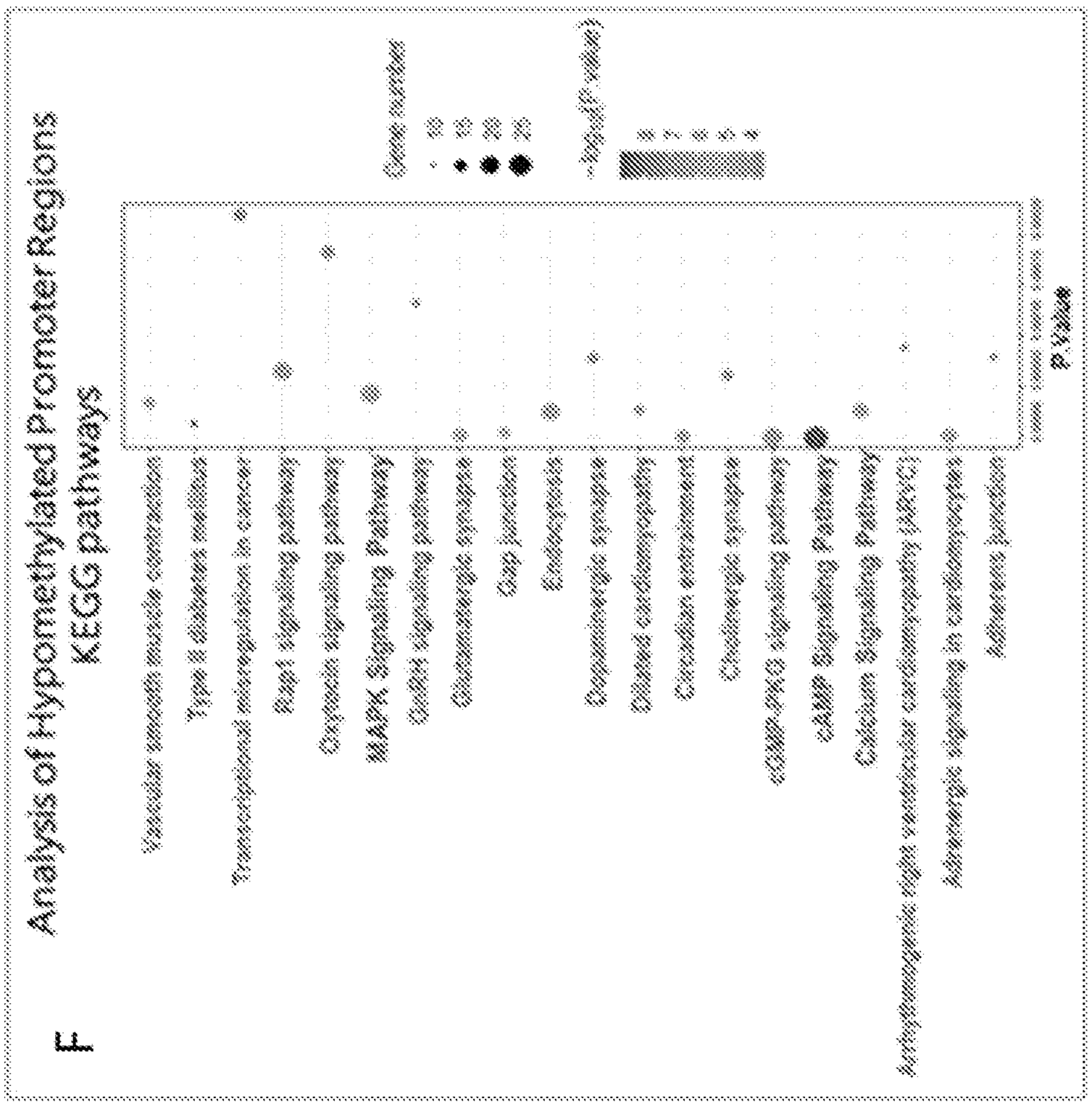


Figure 2 cont.

Figure 2 cont.



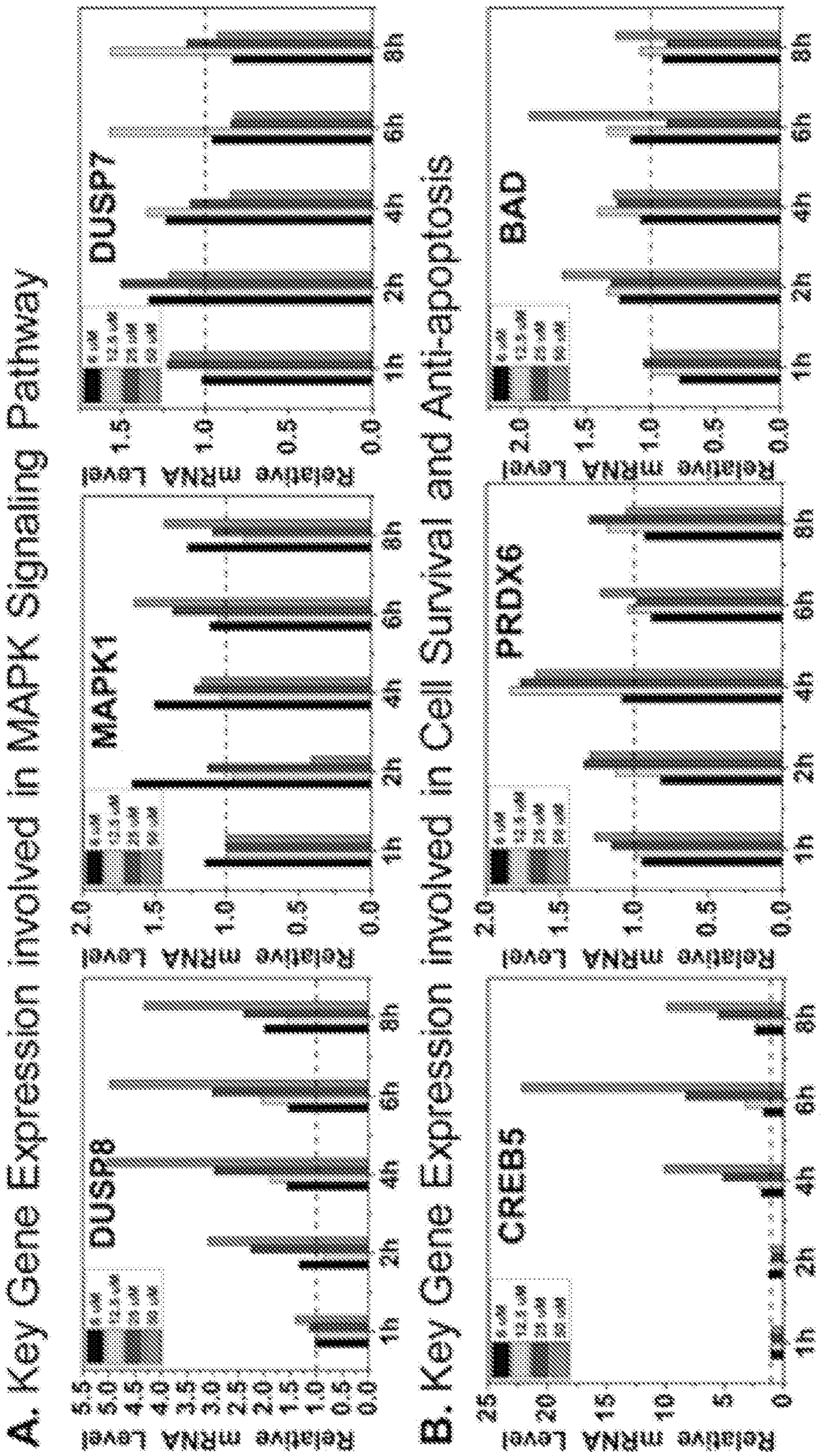

A. Key Gene Expression involved in MAPK Signaling Pathway
B. Key Gene Expression involved in Cell Survival and Anti-apoptosis
DUSP8
MAPK1
DUSP7
BAD
CREB5
PRDX6
Relative mRNA Level
1h
2h
4h
6h
8h

Figure 3

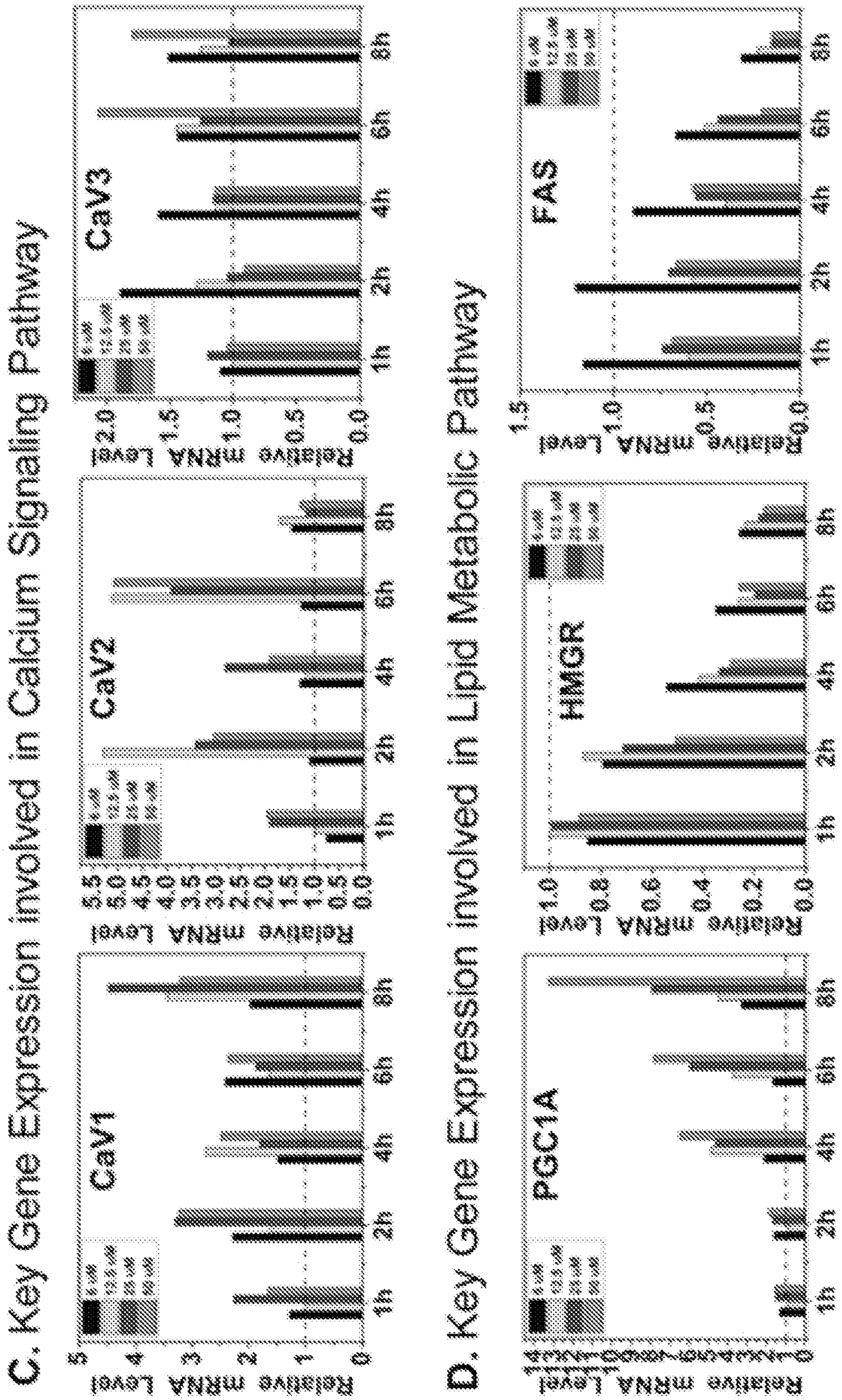


Figure 3 cont.

Figure 4

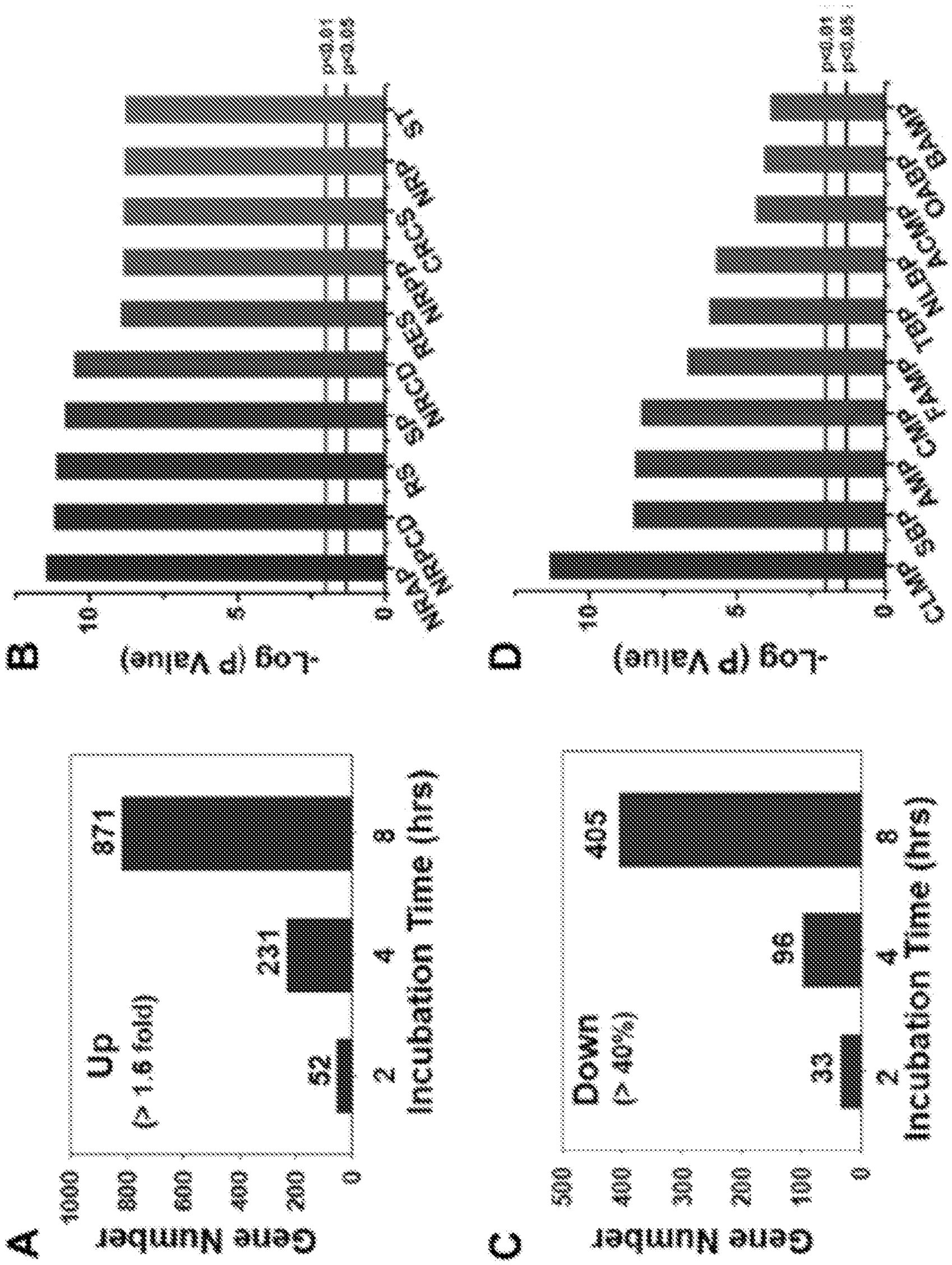

A
Up
(> 1.6 fold)
871
231
52
Gene Number
1000
800
600
400
200
0
2
4
8
Incubation Time (hrs)
C
Down
(> 40%)
405
96
33
Gene Number
500
400
300
200
100
0
2
4
8
Incubation Time (hrs)
B
-Log (P Value)
10
5
0
MRAP
MRPCD
RS
SP
NRCD
RES
NRBP
CRCS
MRP
D
-Log (P Value)
10
5
0
CLMP
SBP
AMP
CMP
FAMP
TBP
NLBP
ACMP
OABP
BAMP

Figure 4 cont.

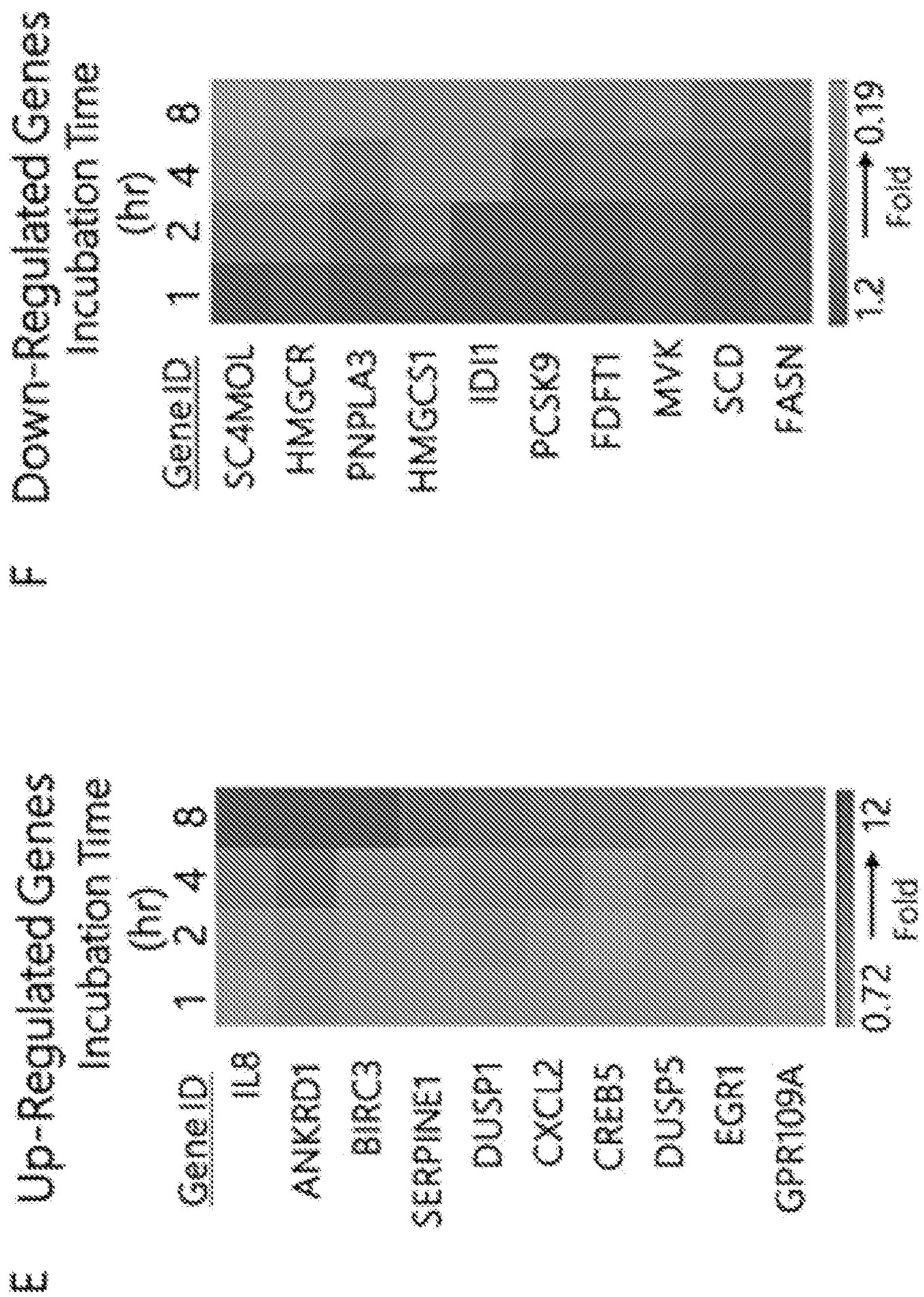


Figure 5

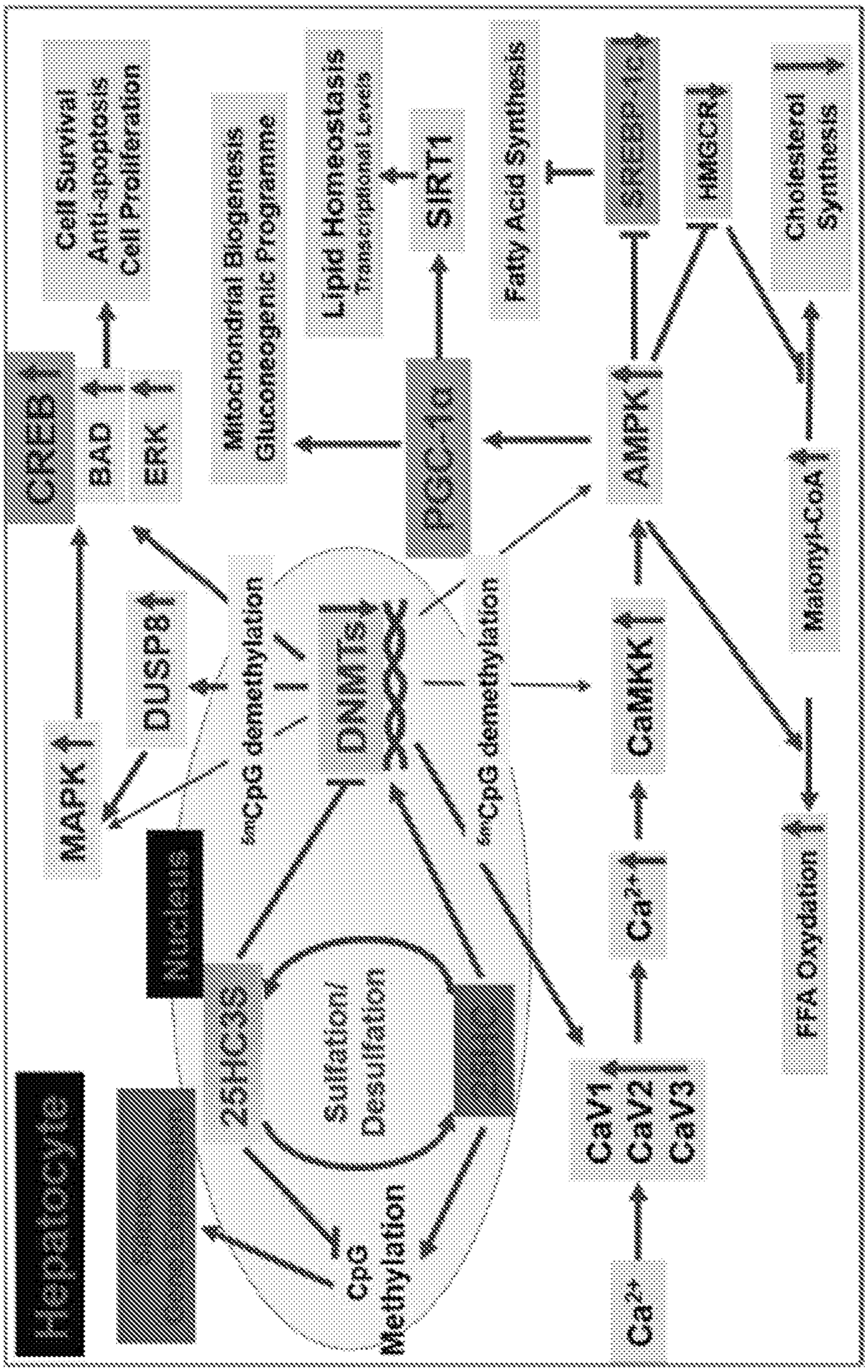

Cell Survival
Anti-apoptosis
Cell Proliferation
CREB
BAD
ERK
MAPK
DUSP8
Mitochondrial Biogenesis
Gluconeogenic Programme
PGC-1α
Lipid Homeostasis
Transcriptional Level
SIRT1
Fatty Acid Synthesis
AMPK
CaMKK
HMGCR
Cholesterol Synthesis
Malonyl-CoA
FFA Oxydation
Ca2+
CaV1
CaV2
CaV3
Ca2+
Ca2+
DNMTs
mCpG demethylation
mCpG demethylation
Nucleus
Hepatocyte
25HC3S
Sulfation/
Desulfation
CpG
Methylation

Figure 6

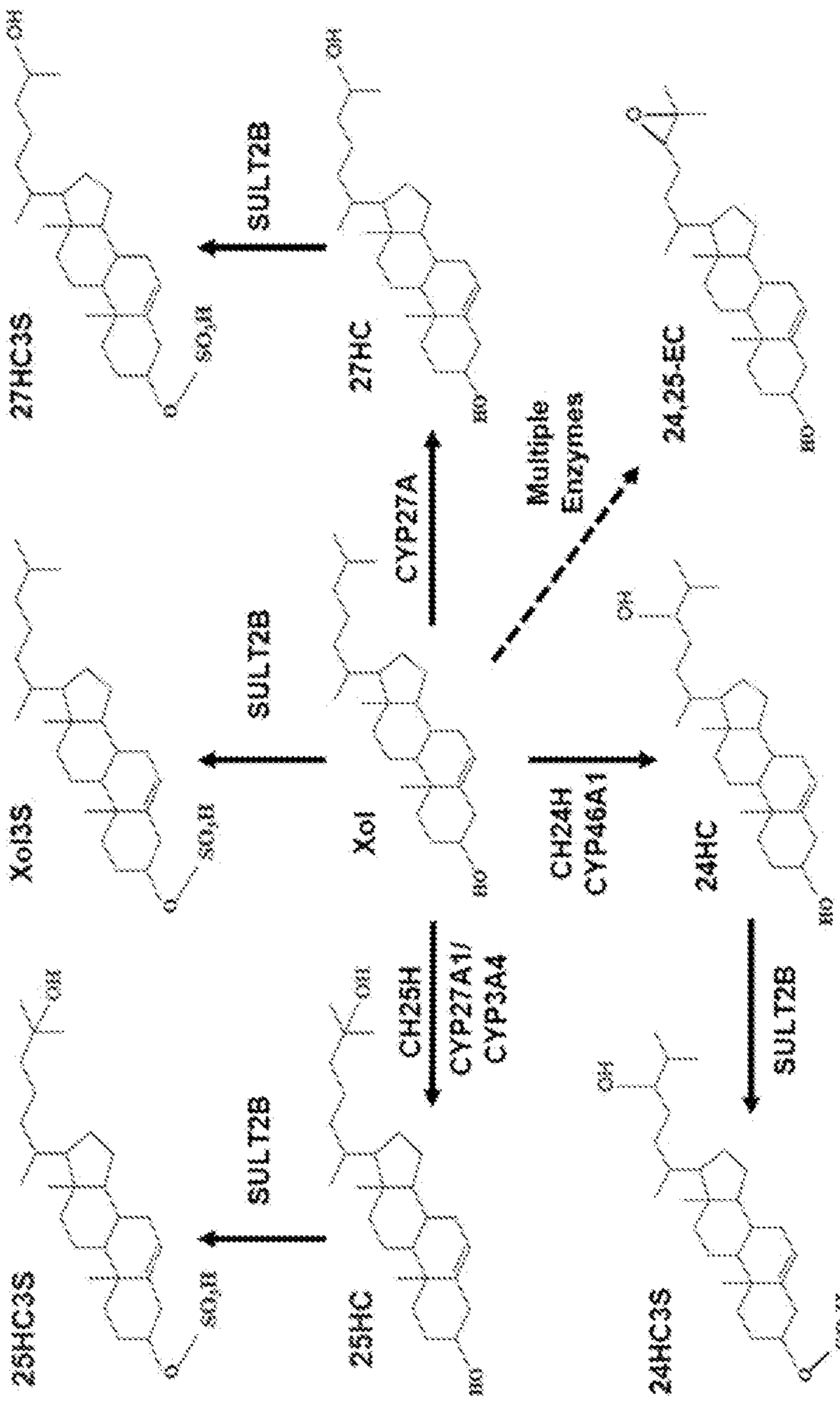
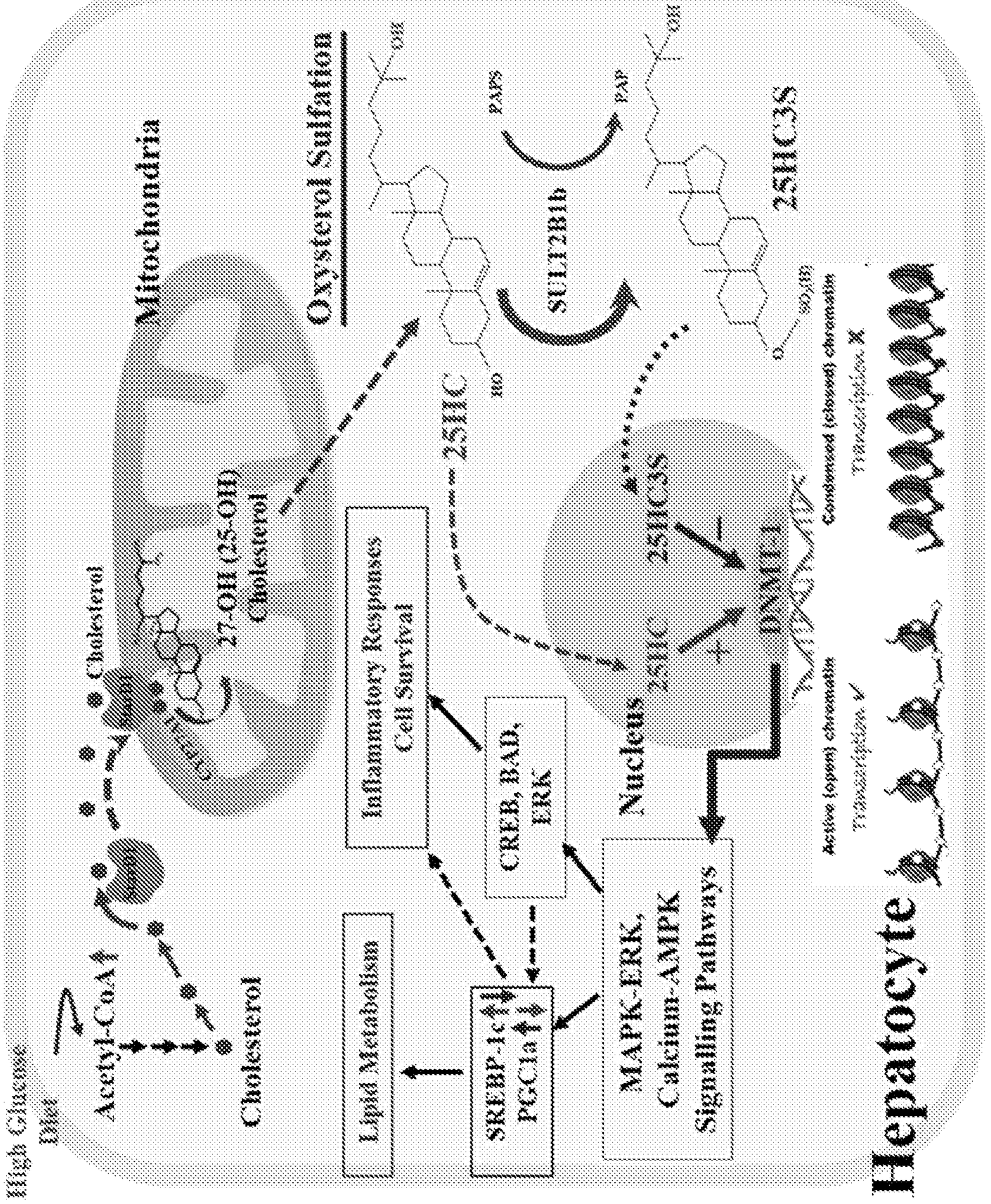

Xol3S
SULT2B
Xol
CYP27A
27HC3S
SULT2B
27HC
Multiple Enzymes
24,25-EC
CH24H
CYP46A1
24HC
25HC3S
SULT2B
25HC
CH25H
CYP27A1/
CYP3A4
24HC3S
SULT2B

Figure 7


Hepatocyte
High Glucose Diet
Acetyl-CoA
Cholesterol
Lipid Metabolism
SREBP-1c
PGC1a
MAPK-ERK, Calcium-AMPK Signalling Pathways
Inflammatory Responses
Cell Survival
CREB, BAD, ERK
Nucleus
25HC
25HC3S
DNMT-1
Mitochondria
27-OH (25-OH) Cholesterol
Oxysterol Sulfation
SULT2B1b
PAPS
PAP
25HC3S

USE OF OXYGENATED CHOLESTEROL SULFATES FOR CANCERS AND NON-CANCEROUS TRANSFORMATIONS RELATED TO EPSTEIN-BARR VIRUS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims priority under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 63/044,631, filed Jun. 26, 2020, Provisional Application No. 63/127,905, filed Dec. 18, 2020, Provisional Application No. 63/141,382, filed Jan. 25, 2021, Provisional Application No. 63/146,559, filed Feb. 5, 2021, Provisional Application No. 63/146,563, filed Feb. 5, 2021, Provisional Application No. 63/146,565, filed Feb. 5, 2021, Provisional Application No. 63/146,566, filed Feb. 5, 2021, Provisional Application No. 63/146,568, filed Feb. 5, 2021, Provisional Application No. 63/149,977, filed Feb. 16, 2021, Provisional Application No. 63/149,993, filed Feb. 16, 2021, the disclosures of which are expressly incorporated by reference herein in their entireties.

STATEMENT OF FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

[0002] The invention was made, in part, with government support under VA Merit Review Grant, Grant No. 1I01BX003656 awarded by Veterans Affairs. The government has certain rights in the invention.

INTRODUCTION

[0003] Oxysterols have long been believed to be ligands of nuclear receptors such as liver x receptor (LXR), and they play an important role in lipids homeostasis and immune system, where they are involved in both transcriptional and post-transcriptional mechanisms. Oxysterols are the oxidized form of cholesterol. In vivo, enzymatic transformation of sterols to oxysterols is for biosynthesis of important biological products such as steroid hormones, bile acids, and vitamin D in cells, blood, and tissues. Oxysterols participate in many biological processes including cholesterol homeostasis, triglyceride metabolism, inflammatory responses, cell proliferation, platelet aggregation, and apoptosis. The oxysterols have also been implicated in many diseases such as metabolic syndrome and neurodegenerative diseases. Oxysterols can be sulfated by sulfotransferase 2B1b (SULT2B1b) at the 3-position of the ring A of cholesterol to be oxysterol 3-sulfates including 5-cholesten-3β-25-diol-3-sulphate (25HC3S), 5-cholesten-3β-24-diol-3-sulphate (24HC3S), 5-cholesten-3β-27-diol-3-sulphate (27HC3S) as well as Xol3S (cholesterol 3-sulfate).

[0004] It has been shown previously that cholesterol metabolite, 5-cholesten-3β-25-diol-3-sulphate, decreases lipid biosynthesis and increases cholesterol secretion and degradation, and may be useful for the treatment and prevention of hypercholesterolemia, hypertriglyceridemia, and conditions related to fat accumulation and inflammation (e.g., non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), alcoholic hepatitis, acute kidney injury (AKI), psoriasis, and atherosclerosis). Oxysterols have also been implicated in several diseases such as metabolic syndrome. Oxysterols can be sulfated, and the sulfated oxysterols act in different direction: they decrease lipid biosynthesis, suppress inflammatory responses, and promote cell survival.

SUMMARY

[0005] The present disclosure provides methods for treating at least one of cancer and non-cancerous transformation related to Epstein-Barr virus, such as at least one of Hodgkin's lymphoma, soft tissue sarcoma, leiomyosarcoma, nasopharyngeal carcinoma, Burkitt's lymphoma, T-cell lymphoma, gastric carcinoma, invasive breast cancer, and hierarchically organized carcinoma. Hierarchically organized carcinomas include, but are not limited to, pancreatic ductal adenocarcinoma, urothelial cancer, colorectal cancer, head and neck cancer, non-small cell lung cancer, esophagus cancer, breast cancer, thyroid cancer, oral cancer, cervical cancer, ovarian cancer, and liver cancer. In practicing the subject methods, an effective amount of at least one oxysterol active agent compound selected from 25-hydroxycholesterol-3-sulfate (25HC3S), 25-hydroxycholesterol-disulfate (25HCDS), 27-hydroxycholesterol-3-sulfate (27HC3S), 27-hydroxycholesterol-disulfate (27HCDS), 24-hydroxycholesterol-3-sulfate (24HC3S), 24-hydroxycholesterol-disulfate (24HCDS), and 24,25-epoxycholesterol-3-sulfate, or salt thereof is administered to the subject.

[0006] Aspects of the disclosure include:

1. A method of treating at least one of cancer related to Epstein-Barr virus and non-cancerous transformation related to Epstein-Barr virus in a subject in need thereof, comprising:

[0007] administering to the subject an effective amount of at least one compound selected from 25-hydroxycholesterol-3-sulfate (25HC3S), 25-hydroxycholesterol-disulfate (25HCDS), 27-hydroxycholesterol-3-sulfate (27HC3S), 27-hydroxycholesterol-disulfate (27HCDS), 24-hydroxycholesterol-3-sulfate (24HC3S), 24-hydroxycholesterol-disulfate (24HCDS), and 24,25-epoxycholesterol-3-sulfate, or salt thereof.

2. The method of aspect 1, wherein the at least one of cancer related to Epstein-Barr virus and non-cancerous transformation related to Epstein-Barr virus is at least one cancer related to Epstein-Barr virus.

3. The method of aspect 2, wherein the at least one cancer comprises at least one of Hodgkin's lymphoma, soft tissue sarcoma, leiomyosarcoma, nasopharyngeal carcinoma, Burkitt's lymphoma, T-cell lymphoma, gastric carcinoma, invasive breast cancer, and hierarchically organized carcinoma.

4. The method of aspect 3, wherein the hierarchically organized carcinoma comprises at least one of pancreatic ductal adenocarcinoma, urothelial cancer, colorectal cancer, head and neck cancer, non-small cell lung cancer, esophagus cancer, breast cancer, thyroid cancer, oral cancer, cervical cancer, ovarian cancer, and liver cancer.

5. The method of aspect 2, wherein the at least one cancer comprises Hodgkin's lymphoma.

6. The method of aspect 2, wherein the at least one cancer comprises soft tissue sarcoma.

7. The method of aspect 2, wherein the at least one cancer comprises leiomyosarcoma.

8. The method of aspect 2, wherein the at least one cancer comprises nasopharyngeal carcinoma.

9. The method of aspect 2, wherein the at least one cancer comprises Burkitt's lymphoma.

10. The method of aspect 2, wherein the at least one cancer comprises T-cell lymphoma.

11. The method of aspect 2, wherein the at least one cancer comprises gastric carcinoma.

12. The method of aspect 2, wherein the at least one cancer comprises invasive breast cancer.
13. The method of aspect 2, wherein the at least one cancer comprises hierarchically organized carcinoma.
14. The method of aspect 13, wherein the hierarchically organized carcinoma comprises pancreatic ductal adenocarcinoma.
15. The method of aspect 13, wherein the hierarchically organized carcinoma comprises urothelial cancer.
16. The method of aspect 13, wherein the hierarchically organized carcinoma comprises colorectal cancer.
17. The method of aspect 13, wherein the hierarchically organized carcinoma comprises head and neck cancer.
18. The method of aspect 13, wherein the hierarchically organized carcinoma comprises non-small cell lung cancer.
19. The method of aspect 13, wherein the hierarchically organized carcinoma comprises esophagus cancer.
20. The method of aspect 13, wherein the hierarchically organized carcinoma comprises breast cancer.
21. The method of aspect 13, wherein the hierarchically organized carcinoma comprises thyroid cancer.
22. The method of aspect 13, wherein the hierarchically organized carcinoma comprises oral cancer.
23. The method of aspect 13, wherein the hierarchically organized carcinoma comprises cervical cancer.
24. The method of aspect 13, wherein the hierarchically organized carcinoma comprises ovarian cancer.
25. The method of aspect 13, wherein the hierarchically organized carcinoma comprises liver cancer.
26. The method of any one of aspects 1 to 25, wherein the method comprises administering to the subject an effective amount of 25-hydroxycholesterol-3-sulfate (25HC3S) or salt thereof.
27. The method of any one of aspects 1 to 25, wherein the method comprises administering to the subject an effective amount of 25-hydroxycholesterol-disulfate (25HCDS) or salt thereof.
28. The method of any one of aspects 1 to 25, wherein the method comprises administering to the subject an effective amount of 27-hydroxycholesterol-3-sulfate (27HC3S) or salt thereof.
29. The method of any one of aspects 1 to 25, wherein the method comprises administering to the subject an effective amount of 27-hydroxycholesterol-disulfate (27HCDS) or salt thereof.
30. The method of any one of aspects 1 to 25, wherein the method comprises administering to the subject an effective amount of 24-hydroxycholesterol-3-sulfate (24HC3S) or salt thereof.
31. The method of any one of aspects 1 to 25, wherein the method comprises administering to the subject an effective amount of 24-hydroxycholesterol-disulfate (24HCDS) or salt thereof.
32. The method of any one of aspects 1 to 25, wherein the method comprises administering to the subject an effective amount of 24,25-epoxycholesterol-3-sulfate or salt thereof.
33. The method of any one of aspects 1 to 32, wherein the at least one compound is administered in an amount ranging from 0.001 mg/kg/day to 100 mg/kg/day.
34. The method of any one of aspects 1 to 33, wherein the at least one compound is administered in an amount ranging from 0.1 mg/kg to 100 mg/kg, based on body mass of the subject.
35. The method of any one of aspects 1 to 33, wherein the at least one compound is administered in an amount ranging from 1 mg/kg to 10 mg/kg, based on body mass of the subject.
36. The method of any one of aspects 1 to 35, wherein the administering is performed from once to 3 times per day.
37. The method of any one of aspects 1 to 36, wherein the administering comprises at least one of oral administration, enteric administration, sublingual administration, transdermal administration, intravenous administration, peritoneal administration, parenteral administration, administration by injection, subcutaneous injection, and intramuscular injection.
38. The method of any one of aspects 1 to 37, wherein the administering comprises administering a pharmaceutical composition comprising the at least one compound and a physiologically acceptable excipient, diluent, or carrier.
39. The method of aspect 38, wherein the pharmaceutical composition is formulated in unit dosage form.
40. The method of aspect 38 or 39, wherein the pharmaceutical composition is in solid form.
41. The method of any one of aspects 38 to 40, wherein the pharmaceutical composition is in the form of a powder, a tablet, a capsule, or a lozenge.
42. The method of any one of aspects 38 to 41, wherein the pharmaceutical composition comprises the at least one compound in freeze-dried form together with a bulking agent.
43. The method of any one of aspects 38 to 42, wherein the pharmaceutical composition is in a sealed vial, ampoule, syringe, or bag.
44. The method of aspect 38 or 43, wherein the pharmaceutical composition comprises a carrier that is a liquid.
45. The method of aspect 44, wherein the at least one compound is solubilized in the liquid or dispersed in the liquid.
46. The method of any one of aspects 44 and 45, wherein the liquid is aqueous.
47. The method of any one of aspects 44 to 46, wherein the liquid is sterile water for injections or phosphate-buffered saline.
48. The method of any one of aspects 38 and 44 to 47, wherein the pharmaceutical composition is in a sealed vial, ampoule, syringe, or bag.
49. Use of at least one compound selected from 25-hydroxycholesterol-3-sulfate (25HC3S), 25-hydroxycholesterol-disulfate (25HCDS), 27-hydroxycholesterol-3-sulfate (27HC3S), 27-hydroxycholesterol-disulfate (27HCDS), 24-hydroxycholesterol-3-sulfate (24HC3S), 24-hydroxycholesterol-disulfate (24HCDS), and 24,25-epoxycholesterol-3-sulfate, or salt thereof, for the manufacture of a medicament for treating at least one of cancer related to Epstein-Barr virus and non-cancerous transformation related to Epstein-Barr virus in a subject in need thereof.
50. Use according to aspect 49, wherein said treating is a method of treating according to any one of aspects 1 to 48.
51. At least one compound selected from 25-hydroxycholesterol-3-sulfate (25HC3S), 25-hydroxycholesterol-disulfate (25HCDS), 27-hydroxycholesterol-3-sulfate (27HC3S), 27-hydroxycholesterol-disulfate (27HCDS), 24-hydroxycholesterol-3-sulfate (24HC3S), 24-hydroxycholesterol-disulfate (24HCDS), and 24,25-epoxycholesterol-3-sulfate, or salt thereof, for use in a method for treating at least one of cancer related to Epstein-Barr virus and non-cancerous transformation related to Epstein-Barr virus.

52. At least one compound for use of aspect 51, wherein the method is a method according to any one of aspects 1 to 48.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] FIGS. **1**A-**1**D. Synthesis and enzyme kinetic studies of Xol3S, 25HC3S, and 27HC3S. The biosynthesis of Xol3S, 25HC3S, and 27HC3S in the cells is shown in FIG. **1**A. HPLC profiles of purified 25HC3S; Xol3S; and 27HC3S are shown in FIG. **1**B. Effects of 25HC3S, 27HC3S, Xol3S, and their precursors, 25HC, 27HC, and cholesterol, on DNMT1/3a/3b activities. The concentration dependent, 0-0.001 M (10 points), effects of 25HC3S, Xol3S, and 27HC3S on the enzyme activities is shown in FIG. **1**C. Comparison of 25HC with 25HC3S, cholesterol with Xol3S, and 27HC with 27HC3S is shown in FIG. **1**D.

[0009] FIGS. **2**A-**2**F. Effects of 25HC3S on DNA methylation in hepatocytes by global methylation sequencing analysis. Huh-7 cells were cultured in HG media for 72 hours and treated with ethanol (vehicle) and 25 mM 25HC3S in ethanol for 4 hours. The levels of global methylation were estimated by LINE-1 assay. Four CpG sites in promoter regions of LINE-1 element were chosen as the target positions as shown in FIG. **2**A. Detailed global methylation was measured by WGBS. Circos maps of DMR distribution in chromosomes is shown in FIG. **2**B: the first circle shows the distribution of hypermethylation DMRs; the second shows transposable element (TE) density; and the third shows the distribution of hypomethylation DMRs. Venn diagrams of hypomethylated DMR-associated genes (DMGs) in 25HC3S and Vehicle libraries under CG, CHG, and CHH contexts of whole genome (Up) and promoter regions (Low) are shown in FIG. **2**C. KOBAS software was used to test the statistical enrichment of DMR related genes in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. DNA methylation levels in different genomic functional regions of the whole genome in FIG. **2**D, where the x-axis represents the different genomic regions (CGI, CGI-shore, promoter, UTR 5, exon, intron, UTR 3, and repeat), and the y-axis represents the methylation levels in 25HC3S and vehicle libraries under CG, CHG, and CHH contexts. High enrichment of hypomethylated DMRs in whole genome in KEGG pathways is shown in FIG. **2**E. High enrichment of hypomethylated DMRs in promoter regions in KEGG pathways is shown in FIG. **2**F. The detailed KEGG pathways are shown in Table 1.3.

[0010] FIGS. **3**A-**3**D. Expression of key genes related to signaling pathways. Huh-7 cells were cultured in HG media for 72 hours and treated with 25HC3S at 6.25 μM, 12.5 M, 25 μM, and 50 μM for 1 hour, 2 hours, 4 hours, 6 hours, and 8 hours. Key Genes and their targeting genes expression were determined by RT-PCR analysis. The expressions of DUSP8 (Dual Specificity Phosphatases 8), DUSP7 (Dual Specificity Phosphatases 7), and MAPK1 (Mitogen-activated protein kinase 1) in MAPK signaling pathway are shown in FIG. **3**A; their target genes, CREB (cAMP responsive element binding protein), PRDX6 (peroxiredoxin 6), and BAD (BCL2 Associated Agonist Of Cell Death) are shown in FIG. **3**B; Key genes, CACNA family (calcium voltage-gated channel subunits), in calcium-AMK pathway are shown in FIG. **3**C; their targeting genes PGC1A (PPARG co-activator 1 alpha), HMGR (3-hydroxy-3-methylglutaryl-CoA reductase), and FAS (fatty acid synthase) are shown in FIG. **3**D.

[0011] FIGS. **4**A-**4**F. Effect of 25HC3S on transcription levels in hepatocytes. HepG-2 cells were cultured in HG media and treated with 25 μM of 25HC3S for 2 hours, 4 hours, and 8 hours. The up-regulated genes (>1.6 fold) are shown in FIG. **4**A. Enrichment of up-regulated genes (8 hours) to Gene ontological (GO) groups are shown in FIG. **4**B (NRAP: negative regulation of apoptotic process; NRPCD: negative regulation of programmed cell death; RS: regulation of signaling; SP: regulation of phosphorylation; NRCD negative regulation of cell death; RES: response to stress; NRPP: negative regulation of protein phosphorylation; CRCS: cellular response to chemical stimulus; NRP: negative regulation of phosphorylation; ST: signal transduction). Down-regulated genes (reduction>40%) are shown in FIG. **4**C. Enrichment of down-regulated genes (8 hours) to GO groups are shown in FIG. **4**D (CLMP: cellular lipid metabolic process; SBP: steroid biosynthetic process; AMP: alcohol metabolic process; CMP: cholesterol metabolic process; FAMP: fatty acid metabolic process; TBP: triglyceride biosynthetic process; NLBP: neutral lipid biosynthetic process; ACMP: acyl-CoA metabolic process; OABP: organic acid biosynthetic process; BAMP: bile acid metabolic process). Heatmap of up-regulated genes related to this study is shown in FIG. **4**E; down-regulated genes are shown in FIG. **4**F.

[0012] FIG. **5**. Sulfation of 25HC as an epigenetic regulatory pathway. 25HC is an endogenous agonist of DNMT-1 that methylates CpG in promoter regions and subsequently silences gene expression, resulting in cell death and lipogenesis. 25HC can be sulfated to 25HC3S, which acts as an endogenous ligand and inhibits activities of DNMTs. 25HC3S demethylates ^{5m}CpG in promoter regions, and successively increases gene expression. The eminent pathways regulated by the sulfation of oxysterol are involved in energy and lipids metabolisms, MAPK-ERK, and calcium-AMPK. 25HC3S significantly increases Dual-specificity phosphatases (DUSPs) and CREB expression, which activate MAPK/ERK pathway, including CREB, BAD, and ERK, and subsequently regulate cell survival and death. 25HC3S decreases lipid biosynthesis and reduces lipid accumulation by demethylating ^{5m}CpG in promoter regions, increasing expression of key genes involved in calcium channels and AMPK, and activating corresponding signaling pathways, which result in increased oxidation of free fatty acids (FFA), and decreased biosynthesis of cholesterol and FFA. The global regulation by sulfation of oxysterol suggests the physiological and pathophysiological significance of this regulatory mechanism.

[0013] FIG. **6**. Mechanisms of sulfation and metabolic pathways of oxysterol sulfates.

[0014] FIG. **7**. Regulatory pathway of oxysterol sulfation.

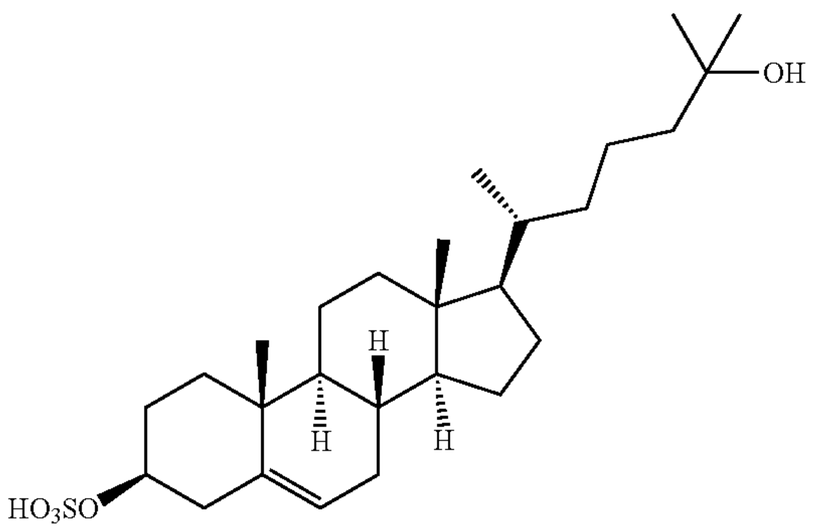
DETAILED DESCRIPTION OF THE INVENTION

[0015] The present disclosure provides methods for treating at least one of cancer and non-cancerous transformation related to Epstein-Barr virus, such as at least one of Hodgkin's lymphoma, soft tissue sarcoma, leiomyosarcoma, nasopharyngeal carcinoma, Burkitt's lymphoma, T-cell lymphoma, gastric carcinoma, invasive breast cancer, and hierarchically organized carcinoma. Hierarchically organized carcinomas include, but are not limited to, pancreatic ductal adenocarcinoma, urothelial cancer, colorectal cancer, head and neck cancer, non-small cell lung cancer, esophagus

cancer, breast cancer, thyroid cancer, oral cancer, cervical cancer, ovarian cancer, and liver cancer.

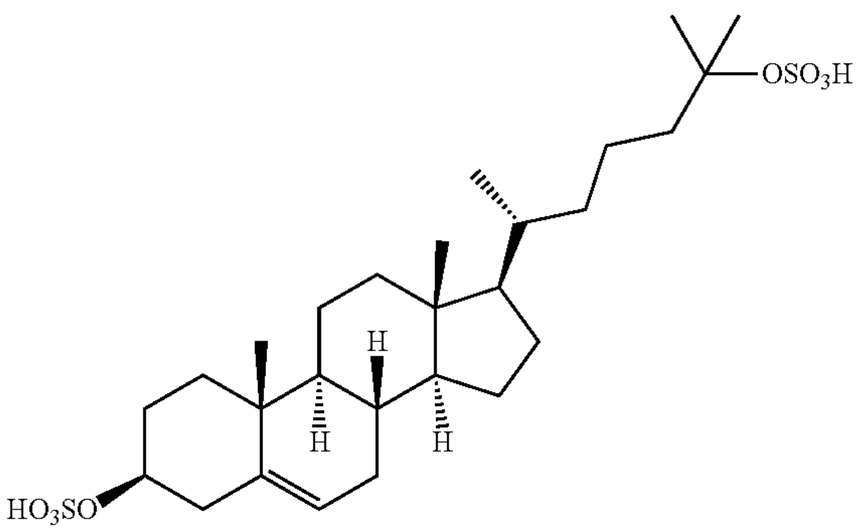
[0016] As used herein, non-cancerous transformation means a change that is not cancerous, benign, and/or non-malignant. Examples of non-cancerous transformations include, but are not limited to, infectious mononucleosis, chronic active EBV infection, EBV-associated hemophagocytic lymphohistiocytosis (EBV-HLH), and other systemic autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, and Sjogren's syndrome.

[0017] In practicing the subject methods, one or more oxysterol active agent compound selected from 25-hydroxycholesterol-3-sulfate (25HC3S), 25-hydroxycholesterol-disulfate (25HCDS), 27-hydroxycholesterol-3-sulfate (27HC3S), 27-hydroxycholesterol-disulfate (27HCDS), 24-hydroxycholesterol-3-sulfate (24HC3S), 24-hydroxycholesterol-disulfate (24HCDS), and 24,25-epoxycholesterol-3-sulfate (24,25-EC3S), or salt thereof is administered to a subject (e.g., human subject). As described herein, the compound 25-hydroxycholesterol-3-sulfate (25HC3S) refers to a compound having the chemical structure:



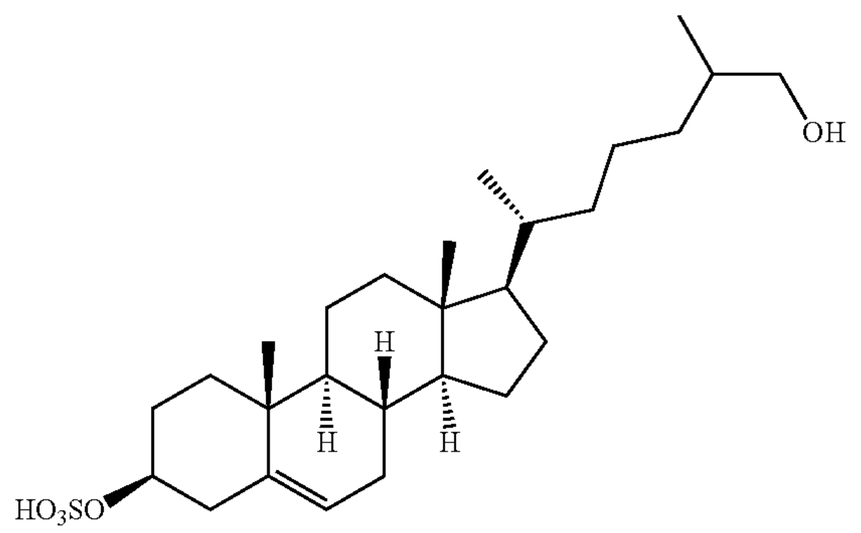
25HC3S

The compound 25-hydroxycholesterol-disulfate (25HCDS) refers to a compound having the chemical structure:



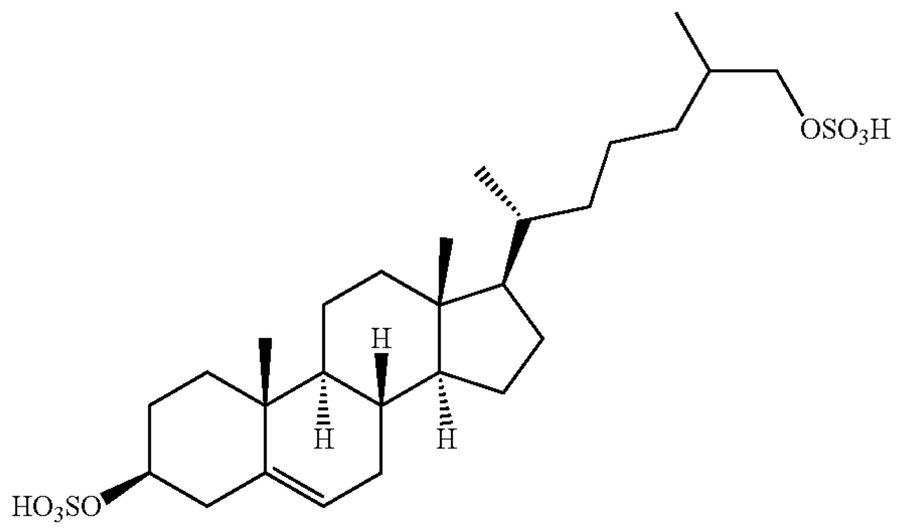
25HCDS

The compound 27-hydroxycholesterol-3-sulfate (27HC3S) refers to a compound having the chemical structure:



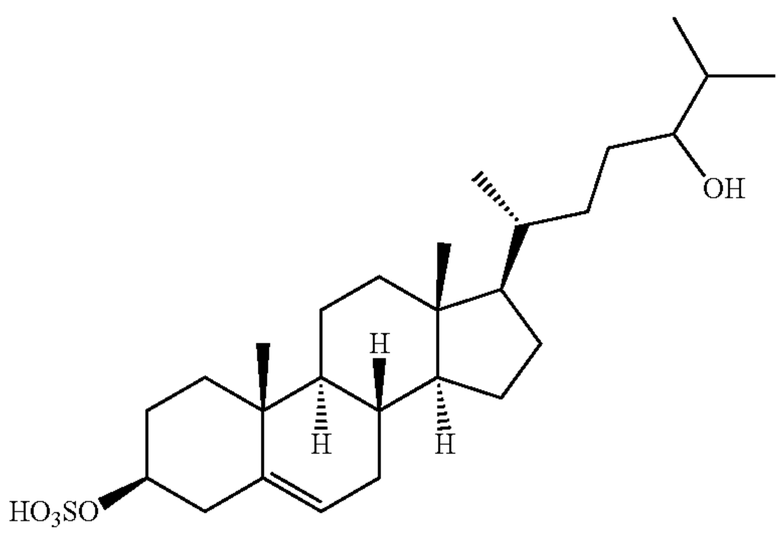
27HC3S

[0018] The compound 27-hydroxycholesterol-disulfate (27HCDS) refers to a compound having the chemical structure:



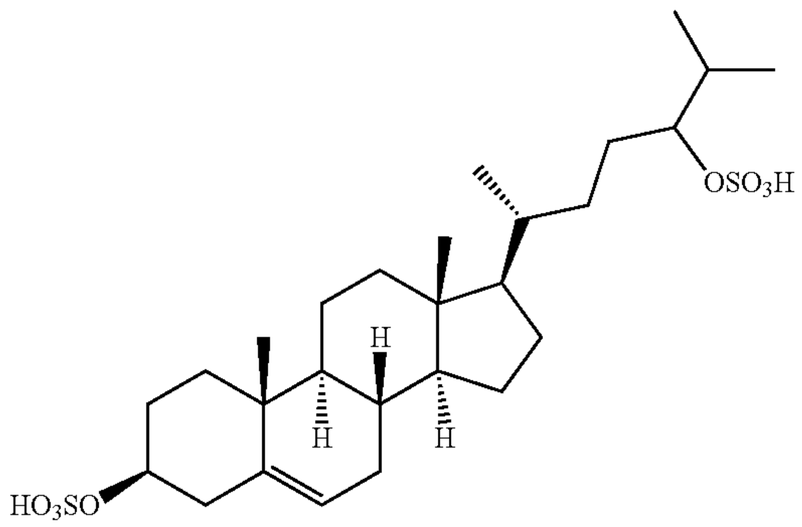
27HCDS

[0019] The compound 24-hydroxycholesterol-3-sulfate (24HC3S) refers to a compound having the chemical structure:



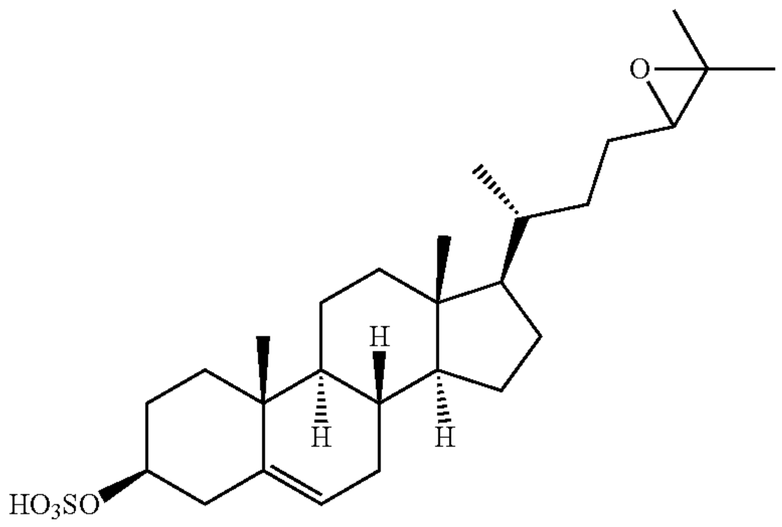
24HC3S

[0020] The compound 24-hydroxycholesterol-disulfate (24HCDS) refers to a compound having the chemical structure:



24HCDS

[0021] The compound 24,25-epoxycholesterol-3-sulfate (24,25-EC3S) refers to a compound having the chemical structure:



24,25-EC3S

[0022] Oxysterols according to certain instances, can be sulfated by sulfotransferase 2B1b (SULT2B1b) at the 3-position of the ring A of cholesterol to be oxysterol 3-sulfates including 25HC3S, 24HC3S, 27HC3S as well as Xol3S (cholesterol 3-sulfate) as summarized in FIG. **6**. The oxysterol sulfate can be further sulfated by sulfotransferase 2A1 (SULT2A1) to be oxysterol disulfates. For example, 25-hydroxycholesterol 3-sulfate (25HC3S) can be further sulfated by SULT2A1 to 5-cholesten-3β, 25-diol-disulfate (25HCDS). 25HC3S and 25HCDS are the only oxysterol sulfates that have been identified in vivo in hepatocyte nuclei while 27HC3S in human sera and 24HC3S in urine. 25HC3S and 25HCDS are also potent regulators but function in a different direction from their precursor 25HC.

[0023] Cholesterol can be hydroxylated by CYP27A1 to 25HC or 27HC in the mitochondria, and hydroxylated to 25HC by CYP3A4, or by cholesterol 25-hydroxylase (CH25HL) in endoplasmic reticulum. Cholesterol can also be hydroxylated by cholesterol 24-hydroxylase to 24HC in brain tissue. This cholesterol precursor can also be used for synthesis of desmosterol via a shunt of the mevalonate pathway. The desmosterol can be oxygenated by CYP46A1 to form 24, 25-epoxycholesterol (24,25EC). 25HC, 27HC, 24HC, and cholesterol can be subsequently sulfated at the 30-position by SULT2B1b to form 25HC3S, 27HC3S, 24HC3S, and Xol3S, respectively. 24, 25EC can be sulfated to be 24, 25EC3S.

[0024] While not being bound by theory, the function of 25HC and 25HC3S in global regulation indicates that they are epigenetic regulators. Methylation at position 5 of cytosine (5-methylcytosine, ^{5m}C) in DNA promoter regions is an important epigenetic modification that regulates gene expression and other functions of the genome. Cytosine methylation of CpG in promoter regions is inversely correlated with transcriptional activity of associated genes as it causes chromatin condensation and gene silencing. Dysregulation of CpG methylation and gene expression affect metabolism, tissue function, and the metabolic state. Cytosine methylation is catalyzed by DNA methyltransferases (DNMT-1, 3a/3b), which in some cases play a role in the regulation of DNA methylation/demethylation. 25HC and 25HC3S are ligands of DNA methyltranferase-1 (DNMT-1). In some cases, the oxysterol active agent compounds described herein are cellular regulatory molecules that epigenetically regulate lipid metabolism, cell survival/death, and inflammatory responses via DNA CpG methylation and ^{5m}CpG demethylation. In some cases, high glucose incubation increases CpG methylation in promoter regions via increasing nuclear 25HC levels, which silences key gene expressions involved in PI3K-Akt, cAMP, NAFLD, Type II Diabetes Mellitus, and Insulin Secretion signaling pathways. In certain cases, oxysterol active agent compounds disclosed herein (e.g., 25HC3S) de-methylates ^{5m}CpG in these promoter regions, increases gene expression, and up-regulates these signaling pathways. In some cases, the oxysterol active agent compound regulates the signaling pathways in an opposite direction from precursor 25HC. In some cases, the one or more oxysterol active agent compounds regulate cell signaling pathways in response to stress responses. In certain cases, the one or more oxysterol active agent compounds affect protein phosphorylation, inositol phosphorylation, and sphingosine phosphorylation in regulating cellular functions. In certain cases, the one or more oxysterol active agent compounds regulate gene expression at transcriptional levels. An illustrative mechanism is depicted in FIG. **7**. The one or more oxysterol active agent compounds (e.g., 25HC3S) in certain cases decrease lipid accumulation, anti-inflammatory response, and anti-apoptosis by increasing gene expression through demethylation of ^{5m}CpG in promoter regions of the key genes involved in MAPK-ERK and Calcium-AMPK signaling pathways, such as CREB5 (CAMP Responsive Element Binding Protein 5), BAD (BCL2 Associated Agonist of Cell Death), and ERK (Mitogen-activated protein kinase 1).

[0025] LMP1 is expressed in most EBV-associated various non-cancerous, pre-cancerous, and cancerous lymphoproliferative or non-lymphoproliferative disorders or diseases (LPD or non-LPD), and it critically contributes to pathogenesis and disease phenotypes, such as but not limited to Hodgkin's lymphoma, soft tissue sarcoma, leiomyosarcoma, nasopharyngeal carcinoma, Burkitt's lymphoma, T-cell lymphoma, gastric carcinoma, invasive breast cancer, and hierarchically organized carcinoma. EBV LMP1 directly induces promoter activity of DNMT1, resulting in hypermethylation and silencing of E-cadherin gene expression. LMP1 also upregulates the expression of PD-L1 via activation of MAPK/NF-kB pathway. Transformation from normal cells to precancerous cells to cancer cells involves

epigenetic dysregulation (DNA hypermethylation) of stem cells to cancer stem cells. While not being bound by theory, inhibition of DNMT by the compounds of the present application may be useful in treating EBV-associated various non-cancerous, pre-cancerous, and cancerous lymphoproliferative or non-lymphoproliferative disorders.

[0026] In some cases, the term "treat" is used herein to refer to administering at least one oxysterol active agent compound selected from 25-hydroxycholesterol-3-sulfate (25HC3S), 25-hydroxycholesterol-disulfate (25HCDS), 27-hydroxycholesterol-3-sulfate (27HC3S), 27-hydroxycholesterol-disulfate (27HCDS), 24-hydroxycholesterol-3-sulfate (24HC3S), 24-hydroxycholesterol-disulfate (24HCDS), and 24,25-epoxycholesterol-3-sulfate, or salt thereof to a human subject that: (1) already exhibits at least one symptom of at least one of cancer and non-cancerous transformation related to Epstein-Barr virus, such as at least one of Hodgkin's lymphoma, soft tissue sarcoma, leiomyosarcoma, nasopharyngeal carcinoma, Burkitt's lymphoma, T-cell lymphoma, gastric carcinoma, invasive breast cancer, and hierarchically organized carcinoma. Hierarchically organized carcinomas include, but are not limited to, pancreatic ductal adenocarcinoma, urothelial cancer, colorectal cancer, head and neck cancer, non-small cell lung cancer, esophagus cancer, breast cancer, thyroid cancer, oral cancer, cervical cancer, ovarian cancer, and liver cancer; and/or (2) is diagnosed as having at least one of cancer and non-cancerous transformation related to Epstein-Barr virus, such as at least one of Hodgkin's lymphoma, soft tissue sarcoma, leiomyosarcoma, nasopharyngeal carcinoma, Burkitt's lymphoma, T-cell lymphoma, gastric carcinoma, invasive breast cancer, and hierarchically organized carcinoma. Hierarchically organized carcinomas include, but are not limited to, pancreatic ductal adenocarcinoma, urothelial cancer, colorectal cancer, head and neck cancer, non-small cell lung cancer, esophagus cancer, breast cancer, thyroid cancer, oral cancer, cervical cancer, ovarian cancer, and liver cancer, such as by a trained clinical professional. In some cases, "treatment" involves the lessening or attenuation, or in some instances, the complete eradication, of at least one symptom of the at least one of cancer and non-cancerous transformation related to Epstein-Barr virus, such as at least one of Hodgkin's lymphoma, soft tissue sarcoma, leiomyosarcoma, nasopharyngeal carcinoma, Burkitt's lymphoma, T-cell lymphoma, gastric carcinoma, invasive breast cancer, and hierarchically organized carcinoma. Hierarchically organized carcinomas include, but are not limited to, pancreatic ductal adenocarcinoma, urothelial cancer, colorectal cancer, head and neck cancer, non-small cell lung cancer, esophagus cancer, breast cancer, thyroid cancer, oral cancer, cervical cancer, ovarian cancer, and liver cancer that was present prior to or at the time of administration of the at least one oxysterol active agent compound selected from 25-hydroxycholesterol-3-sulfate (25HC3S), 25-hydroxycholesterol-disulfate (25HCDS), 27-hydroxycholesterol-3-sulfate (27HC3S), 27-hydroxycholesterol-disulfate (27HCDS), 24-hydroxycholesterol-3-sulfate (24HC3S), 24-hydroxycholesterol-disulfate (24HCDS), and 24,25-epoxycholesterol-3-sulfate, or salt thereof. In some cases, treatment according to the present disclosure is sufficient to improve clinical indicators in the subject. In certain instances, the improvement in the clinical indicators in the subject is such that the subject is considered to no longer have the at least one of cancer and non-cancerous transformation related to Epstein-Barr virus, such as at least one of Hodgkin's lymphoma, soft tissue sarcoma, leiomyosarcoma, nasopharyngeal carcinoma, Burkitt's lymphoma, T-cell lymphoma, gastric carcinoma, invasive breast cancer, and hierarchically organized carcinoma. Hierarchically organized carcinomas include, but are not limited to, pancreatic ductal adenocarcinoma, urothelial cancer, colorectal cancer, head and neck cancer, non-small cell lung cancer, esophagus cancer, breast cancer, thyroid cancer, oral cancer, cervical cancer, ovarian cancer, and liver cancer.

[0027] In practicing the subject methods, an effective amount of at least one oxysterol active agent compound selected from 25-hydroxycholesterol-3-sulfate (25HC3S), 25-hydroxycholesterol-disulfate (25HCDS), 27-hydroxycholesterol-3-sulfate (27HC3S), 27-hydroxycholesterol-disulfate (27HCDS), 24-hydroxycholesterol-3-sulfate (24HC3S), 24-hydroxycholesterol-disulfate (24HCDS), and 24,25-epoxycholesterol-3-sulfate, or salt thereof is administered to the subject. In some cases, the oxysterol active agent compound is administered to the subject at a dosage of from 0.00001 mg/kg/day to 500 mg/kg/day, such as from 0.00005 mg/kg/day to 450 mg/kg/day, such as from 0.0001 mg/kg/day to 400 mg/kg/day, such as from 0.0005 mg/kg/day to 350 mg/kg/day, such as from 0.001 mg/kg/day to 300 mg/kg/day, such as from 0.005 mg/kg/day to 250 mg/kg/day, such as from 0.01 mg/kg/day to 200 mg/kg/day, such as from 0.05 mg/kg/day to 150 mg/kg/day, and including from 0.001 mg/kg/day to 100 mg/kg/day. In certain cases, the oxysterol active agent compound is administered to the subject at a dosage of from 0.001 mg/kg/day to 100 mg/kg/day. In certain cases, the oxysterol active agent compound is administered to the subject at a dosage of from 0.1 mg/kg/day to 100 mg/kg/day. In certain cases, the oxysterol active agent compound is administered to the subject at a dosage of from 1 mg/kg/day to 100 mg/kg/day.

[0028] In some cases, the amount of each daily dose of the at least one oxysterol active agent compound, such as 25-hydroxycholesterol-3-sulfate or 25-hydroxycholesterol-3-sulfate sodium, administered to the individual is from 0.5 mg to 5 mg, 5 mg to 10 mg, 10 mg to 15 mg, 15 mg to 20 mg, 20 mg to 25 mg, 20 mg to 50 mg, 25 mg to 50 mg, 50 mg to 75 mg, 50 mg to 100 mg, 75 mg to 100 mg, 100 mg to 125 mg, 125 mg to 150 mg, 150 mg to 175 mg, 175 mg to 200 mg, 200 mg to 225 mg, 225 mg to 250 mg, 250 mg to 300 mg, 300 mg to 350 mg, 350 mg to 400 mg, 400 mg to 450 mg, or 450 mg to 500 mg. In some cases, the amount of oxysterol active agent compound in the effective amount administered to the individual (e.g., a unit dosage form) is in the range of from 0.5 mg to 500 mg, such as from 1 mg to 450 mg, such as from 2 mg to 400 mg, such as from 5 mg to 300 mg, such as from 10 mg to 200 mg, or such as from 20 mg to 100 mg.

[0029] The oxysterol active agent compound may be administered to the subject once per day or more, such as twice per day or more, such as three times per day or more, and including four times per day or more. For instance, the oxysterol active agent compound may be administered twice a day, once a day, once every other day, once every three days, once a week, or once a month. In some cases, the oxysterol active agent compound is administered to the subject once per day. In some cases, the oxysterol active agent compound is administered to the subject twice per day. In some instances, the oxysterol active agent compound is administered to the subject once or twice per day in a cycle

for a duration of ranging from 1 day to 10 days, 1 day to 30 days, 7 days to 30 days, 7 days to 90 days, 10 days to 180 days, or 30 days to 1 year, 30 days to 5 years, 90 days to 5 years, or 1 year to 10 years. In some cases, the oxysterol active agent compound is administered to the subject once per day for a duration of from 1 day to 30 days, such as once per day for a duration of from 1 day to 28 days, from 1 day to 21 days, from 7 days to 14 days. In some cases, the oxysterol active agent compound is administered to the subject twice per day for a duration of from 1 day to 30 days, such as twice per day for a duration of from 1 day to 28 days, from 1 day to 21 days, from 7 days to 14 days. In some cases, the oxysterol active agent compound is administered to the subject three times per day for a duration of from 1 day to 30 days, such as three times per day for a duration of from 1 day to 28 days, from 1 day to 21 days, from 7 days to 14 days.

[0030] In some cases, the dosing is administered in cycles of administration of the oxysterol active agent compound. In some cases, the cycle is 1 day or more, such as 2 days or more, such as 3 days or more, such as 4, days or more, such as 5 days or more, such as 6 days or more, such as 7 days or more, such as 14 days or more, such as 21 days or more, such as 28 days or more, and in some instances the cycle is 30 days or more. The cycles of drug administration may be repeated for 1, 2, 3, 4, 5, 6, 7, 8, or more than 8 dosage cycles, for a total period of 6 months, 1 year, 2 years, 3 years, or 4 years or more. The administration of each pharmaceutical composition can be extended over an extended period of time (such as during maintenance therapy), such as from a month up to seven years. In some cases, the oxysterol active agent compound may be administered over a period of about any of 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 18, 24, 30, 36, 48, 60, 72, or 84 months. In other cases, the oxysterol active agent compound is administered for the rest of the subject's lifetime.

[0031] Implementation of the methods generally involves identifying (e.g., diagnosing) patients suffering from or at risk of at least one of cancer and non-cancerous transformation related to Epstein-Barr virus, such as at least one of Hodgkin's lymphoma, soft tissue sarcoma, leiomyosarcoma, nasopharyngeal carcinoma, Burkitt's lymphoma, T-cell lymphoma, gastric carcinoma, invasive breast cancer, and hierarchically organized carcinoma. Hierarchically organized carcinomas include, but are not limited to, pancreatic ductal adenocarcinoma, urothelial cancer, colorectal cancer, head and neck cancer, non-small cell lung cancer, esophagus cancer, breast cancer, thyroid cancer, oral cancer, cervical cancer, ovarian cancer, and liver cancer. The exact dosage to be administered may vary depending on the age, gender, weight, and overall health status of the individual patient, as well as the precise etiology of the disease. The dose will vary with the route of administration, the bioavailability, and the particular formulation that is administered, as well as according to the nature of the malady that is being prevented or treated. Further, the effective dose can vary depending upon factors such as gender, age, and other conditions of the patient, as well as the extent or progression of the disease condition being treated. In some cases, each dosage of the oxysterol active agent compound is administered to the subject over duration of from 0.1 hours to 12 hours (e.g., by intravenous administration) such as from 0.5 hours to 10 hours, such as from 1 hour to 8 hours, and including over a duration of from 2 hours to 6 hours.

[0032] Administration may be oral or parenteral, including intravenously, intramuscularly, subcutaneously, intradermal injection, intraperitoneal injection, etc., or by other routes (e.g., transdermal, sublingual, rectal and buccal delivery, inhalation of an aerosol, intravaginally, intranasally, topically, as eye drops, via sprays, etc.). In certain cases, the oxysterol active agent compound is administered to the subject by one or more of oral administration, enteric administration, sublingual administration, transdermal administration, intravenous administration, peritoneal administration, parenteral administration, administration by injection, subcutaneous injection, and intramuscular injection. The route of administration will depend on the nature or the condition that is treated, e.g., on the type or degree of the disease, and whether the treatment is prophylactic or intended to effect a cure. Further, administration of the compound by any means may be carried out as a single mode of therapy, or in conjunction with other therapies and treatment modalities, e.g., diet regimens, etc.

[0033] In some cases, the compositions are administered in conjunction with other treatment modalities such as various pain relief medications, anti-arthritis agents, chemotherapeutic agents, antibiotic agents, anti-neurodegeneration agents, anti-addiction agents, anti-virals, antibodies, radiation, resection, and the like, depending on the malady that is afflicting the subject. "In conjunction with" refers to both administration of a separate preparation of the one or more additional agents, and also to inclusion of the one or more additional agents in a composition of the present disclosure.

[0034] For example, the oxysterol active agent may be administered in conjunction with at least one antiviral, such as at least one of acyclovir, desciclovir, ganciclovir, valgancyclovir, omaciclovir, valomaciclovir, naribavir, cidofovir, thymidine derivatives, interferon-alfa, interferon-gamma, adenine arabinoside, and phosphonoacetic acid, and salts thereof. For example, the oxysterol active agent may be administered in conjunction with at least one anticancer agent and/or cancer therapy, such as at least one of MEK inhibitors (e.g., MEK1, MEK2, or MEK1 and MEK2) (e.g., XL518, CI-1040, PD035901, selumetinib/AZD6244, GSK1120212/trametinib, GDC-0973, ARRY-162, ARRY-300, AZD8330, PD0325901, U0126, PD98059, TAK-733, PD318088, AS703026, BAY 869766), alkylating agents (e.g., cyclophosphamide, ifosfamide, chlorambucil, busulfan, melphalan, mechlorethamine, uramustine, Thiotepa, nitrosoureas, nitrogen mustards (e.g., mechlorethamine, cyclophosphamide, chlorambucil, meiphalan), ethyleneimine and methylmelamines (e.g., hexamethlymelamine, thiotepa), alkylsulfonates (e.g., busulfan), nitrosoureas (e.g., carmustine, lomustine, Semustin, streptozocin), triazenes (decarbazine)), antimetabolites (e.g., 5-azathioprine, leucovorin, capecitabine, fludarabine, gemcitabine, pemetrexed, raltitrexed, folic acid analogs (e.g., methotrexate) or pyrimidine analogues (e.g., fluorine analogs), Floxouridine, cytarabine), purine analogues (e.g., mercaptopurine, thioguanine, Pentostatin), etc.), vegetable alkaloids (e.g., vincristine, vinblastine, vinorelbine, vindesine, podophyllotoxin, paclitaxel, docetaxel, etc.), topoisomerase inhibitors (e.g., irinotecan, topotecan, amsacrine, etoposide (VP16), etoposide phosphate, teniposicin (e.g., Adriamycin, daunorubicin, epirubicin, actinomycin, bleomycin, mitomycin, mitoxantrone, plicamycin, etc.), platinum-based compounds (e.g., cisplatin, Oxaloplatin, carboplatin), anthracendione (e.g., mitoxantrone), substituted urea (e.g., hydroxyurea),

methylhydrazine derivative (e.g., procarbazine), adrenocortical suppressant (e.g., mitotane, aminoglutethimide), epipodophyllotoxins (e.g., Etoposide), antibiotics (e.g., daunorubicin, doxorubicin, bleomycin), enzymes (e.g., L-asparaginase), inhibitors of mitogen-activated protein kinase signal transmission (e.g., U0126, PD98059, PD184352, PD0325901, ARRY-142886, SB239063, SP600125, BAY 43-9006, Wortmannin or LY294002, Syk inhibitors, mTOR inhibitors, antibodies (e.g., Rituxan rituximab), Gossyphol, Genasense, Polyphenol E, chlorofusin, all-trans retinoic acid (ATRA), bryostatin, the apoptosis-inducing ligand (TRAIL) related to the tumor necrosis factor, 5-aza-2'-deoxycytidine, all-trans-retinoic acid, doxorubicin, vincristine, etoposide, gemcitabine, imatinib (Gleevec®), geldanamycin, 17-N-Allylamino-17-demethoxygeldanamycin (17-AAG), Flavopiridol LY294002, Bortezomib, Trastuzumab, BAY 11-7082, PKC412, PD184352, 20-epi-1, 25-dihydroxyvitamin D3; 5-ethynyluracil; Abiraterone; Aclarubicin; Acylfulvene; Adecypenol; Adozelesin; Aldesleukin; ALL-TK antagonists; Altretamine; Ambamustine; Amidox; Amifostine; Aminolevulinic acid; Amrubicin; Amsacrine; Anagrelide; Anastrozole; Andrographolide; Angiogenesis inhibitors; Antagonist D; Antagonist G; Antarelix; antidorsal morphogenic protein-1; Antiandrogens, prostate cancer; Antiestrogen; Antineoplaston; Antisense oligonucleotides; Aphidicol glycinate; Apoptosis gene modulators; Apoptosis regulators; Apuric acid; ara-CDP-DL-PTBA; Arginine deaminase; Asulacrine; Atamestane; Atezolizumab; Atrimustine; Axinastatin 1; Axinastatin 2; Axinastatin 3; Azasetron; Azatoxin; Azatyrosine; Baccatin III derivatives; Balanol; Batimastat; BCR/ABL antagonists; Benzochlorine; Benzoylstaurosporine; Beta-lactam derivatives; Beta alethine; Betaclamycin B; Betulinic acid; Bevacizumab; bFGF inhibitor; Bicalutamide; Bisantren; Bisaziridinylspermine; Bisnafid; Bistrates A; Bizelesin; Breflat; Bropirimine; Budotitan; Buthionine sulfoximine; Calcipotriol; Calphostin C; Camptothecin derivatives; Canarypox IL-2; Capecitabine; Carboxamidaminotriazole; Carboxyamidotriazole; CaRest M3; CARN 700; cartilage derived inhibitor; Carzelesin; Casein kinase inhibitors (ICOS); Castanospermine; Cecropin B; Cetrorelix; Chlorins; Chloroquinoxalinesulfonamide; Cicaprost; Cisporphyrin; Cladribine; Clomiphene analogs; Clotrimazole; Collismycin A; Collismycin B; Combretastatin A4; Combretastatin analog; Conagenin; Crambescidin 816; Crisnatol; Cryptophycin 8; Cryptophycin A derivatives; Curacin A; Cyclopentanthraquinones; Cycloplatam; Cypemycin; Cytarabinocfosfate; cytolytic factor; Cytostatin; Dacliximab; Daratumumab; Decitabine; Dehydrodidemnin B; Deslorelin; Dexamethasone; Dexifosfamide; Dexrazoxane; Dexverapamil; Diaziquon; Didemnin B; Didox; Diethylnorspermin; Dihydro-5-azacytidine; 9-dioxamycin; Diphenylspiromustine; Docosanol; Dolasetron; Doxifluridine; Droloxifene; Dronabinol; Duocarmycin SA; Ebselen; Ecomustine; Noble fosine; Edrecolomab; Eflornithine; Elements; Emitefur; Enzalutamide; Epirubicin; Epristerid; Estramustine analog; Estrogen agonists; Estrogen antagonists; Etanidazole; Etoposide phosphate; Exemestane; Fadrozole; Fazarabine; Fenretinide; Filgrastim; Finasteride; Flavopiridol; Flezelastine; Fluasterone; Fludarabine; Fluorodaunorubicin hydrochloride; Forfenimex; Formestane; Fostriecin; Fotemustin; Gadolinium texaphyrin; Gallium nitrate; Galocitabine; Ganirelix; Gelatinase inhibitors; Gemcitabine; Glutathione inhibitors; Hepsulfame; Heregulin; Hexamethylene bisacetamide; Hypericin; Ibandronic acid; Ibrutinib; Idarubicin; Idoxifene; Idramanton; Ilmofosin; Ilomastat; Imidazoacridones; Imiquimod; immune stimulating peptides; insulin-like growth factor-1 receptor inhibitor; Interferon agonists; Interferons; Interleukins; lobenguan; lododoxorubicin; Ipomeanol, 4-; Iroplact; Irsogladin; Isobengazole; Isohomohalicondrin B; Itasetron; Jasplakinolide; Kahalalid F; Lamellarin-N-triacetate; Lanreotide; Leinamycin; Lenalidomide; Lenograstim; Lentinan sulfate; Leptolstatin; Letrozole; Leukemia inhibiting factor; Leukocyte alpha interferon; Leuprolide+estrogen+progesterone; Leuprorelin; Levamisole; Liarozole; linear polyamine analog; lipophilic disaccharide peptide; lipophilic platinum compounds; Lissoclinamide 7; Lobaplatin; Lombricine; Lometrexol; Lonidamine; Losoxantrone; Lovastatin; Loxoribine; Lurtotecan; Lutetium texaphyrin; Lysofylline; lytic peptides; Maitansine; Mannostatin A; Marimastat; Masoprocol; Maspin; Matrilysin inhibitors; Matrix metalloproteinase inhibitors; Menogaril; Merbaron; Meterelin; Methioninase; Metoclopramide; MIF inhibitor; Mifepristone; Miltefosine; Mirimostim; mismatched double-stranded RNA; Mitoguazone; Mitolactol; Mitomycin analogs; Mitonafid; Mitotoxin fibroblast growth factor saporin; Mitoxantrone; Mofarotes; Molgramostim; monoclonal antibody; human chorionic gonadotropin; Monophosphoryl lipid A+myobacterial cell wall SK; Mopidamol; Gene inhibitor for multiple drug resistance; Therapy with multiple tumor suppressor 1; Mustard anticancer agents; Mycaperoxide B; mycobacterial cell wall extract; Myriaporone; N-acetyldinaline; N-substituted benzamides; Nafarelin; Nagrestip; Naloxone+pentazocine; Napavin; Naphterpine; Nartograstim; Nedaplatin; Nemorubicin; Neridronic acid; neutral endopeptidase; Nilutamide; Nisamycin; Nitric oxide modulators; Nitroxide antioxidant; Nitrullyn; Nivolumab; 06-benzylguanine; Obinutuzumab; Octreotide; Okicenone; Oligonucleotides; Onapristone; Ondansetron; Ondansetron; Oracin; oral cytokine inducer; Ormaplatin; Osaterone; Osimertinib; Oxaliplatin; Oxaunomycin; Palauamine; Palbocicib; Palmitoyl rhizoxin; Pamidronic acid; Panaxytriol; Panomiphene; Parabactin; Pacelliptin; Pegaspargase; Peldesin; Pembrolizumab; Pentosan polysulfate sodium; Pentostatin; Pentrozole; Perflubron; Perfosfamide; Perillyl alcohol; Pertuzumab; Phenazinomycin; Phenyl acetate; Phosphatase inhibitors; Picibanil; Pilocarpine hydrochloride; Pirarubicin; Piritrexim; Placetin A; Placetin B; Plasminogen activator inhibitor; Platinum complex; Platinum compounds; Platinum triamine complex; Porfimer sodium; Porfiromycin; Prednisone; Propyl bisacridone; Prostaglandin J2; Proteasome inhibitors; Protein A-based immunomodulator; Protein kinase C inhibitor; Protein kinase C inhibitors from microalgae; Protein tyrosine phosphatase inhibitors; Purine nucleoside phosphorylase inhibitors; Purpurins; Pyrazoloacridine; pyridoxylated hemoglobin-polyoxyethylene conjugate; Raf antagonists; Raltitrexed; Ramosetron; Ras farnesyl protein transferase inhibitors; Ras inhibitors; Ras-GAP inhibitor; Retelliptin, demethylated; Rhenium Re-186 etidronate; Rhizoxin; Ribozymes; RII retinamide; Rogletimide; Rohitukin; Romurtide; Roquinimex; Rubiginone B1; Ruboxyl; Ruxolitinib; Safingol; Saintopin; SarCNU; Sarcophytol A; Sargramostim; Sdi-1 mimetics; Semustine; senescence derived inhibitor 1; Sense oligonucleotides; Signal transduction inhibitors; Signal transduction modulators; single chain antigen binding protein; Sizofuran; Sobuzoxan; Sodium boro-

captate; Sodium phenyl acetate; Solverol; Somatomedin binding protein; Sonermin; Sparfosinic acid; Spicamycin D; Spiromustine; Splenopentine; Spongistatin 1; Squalamine; Stem cell inhibitor; Inhibitors of stem cell division; Stipiamide; Stromelysin inhibitors; Sulfinosine; superactive vasoactive gut peptide antagonists; Suradista; Suramin; Swainsonine; synthetic glycosaminoglycans; Tallimustin; Tamoxifen methiodide; Tauromustine; Tazarotene; Tecogalan sodium; Tegafur; Tellurapyrylium; Telomerase inhibitors; Temoporfin; Temozolomide; Teniposide; Tetrachlorododeoxide; Tetrazomine; Thaliblastine; Thiocoralin; Thrombopoietin; Thrombopoietin mimetic; Thymalfasin; Thymopoietin receptor agonists; Thymotrinan; Thyroid stimulating hormone; Tin ethyletiopurpurine; Tirapazamine; Titanocene dichloride; Topsentine; Toremifene; totipotent stem cell factor; Translation inhibitors; Tretinoin; Triacetyluridine; Triciribine; Trimetrexate; Triptorelin; Tropisetron; Turosteride; Tyrosine kinase inhibitors; Tyrphostins; UBC inhibitors; Ubenimex; growth inhibiting factor derived from the genitourinary sinus; Urokinase receptor antagonists; Vapreotide; Variolin B; Vector system, erythrocyte gene therapy; Velaresol; Venetoclax; Veramin; Verdins; Verteporfin; Vinorelbine; Vinxaltine; Vitaxin; Vorozol; Zanoterone; Zeniplatin; Zilascorb; Zinostatin-stimalamer, adriamycin, dactinomycin, bleomycin, vinblastine, cisplatin, acivicin; Aclarubicin; Acodazole hydrochloride; Acronine; Adozelesin; Aldesleukin; Altretamine; Ambomycin; Ametantrone acetate; Aminoglutethimide; Amsacrine; Anastrozole; Anthramycin; Asparaginase; Asperline; Azacitidine; Azetepa; Azotomycin; Batimastat; Benzodepa; Bicalutamide; Bisantrene hydrochloride; Bisnafid dimesylate; Bizelesin; Bleomycin sulfate; Brequina sodium; Bropirimine; Busulfan; Cactinomycin; Calusteron; Caracemid; Carbetimer; Carboplatin; Carmustine; Carubicin hydrochloride; Carzelesin; Cedefingol; Chlorambucil; Cirolemycin; Cladribine; Crisnatol mesylate; Cyclophosphamide; Cytarabine; Dacarbazine; Daunorubicin hydrochloride; Decitabine; Dexormaplatin; Deazaguanine; Deazaguanine mesylate; Diaziquon; Doxorubicin; Doxorubicin hydrochloride; Droloxifene; Droloxifene citrate; Dromostanolone propionate; Duazomycin; Edatrexate; Eflornithine hydrochloride; Elsamitrucine; Enloplatin; Enpromat; Epipropidin; Epirubicin hydrochloride; Erbulozole; Esorubicin hydrochloride; Estramustine; Estramustine phosphate sodium; Etanidazole; Etoposide; Etoposide phosphate; Etoprine; Fadrozole hydrochloride; Fazarabine; Fenretinide; Floxuridine; Fludarabine phosphate; Fluorouracil; Fluorocitabine; Fosquidone; Fostriecin sodium; Gemcitabine; Gemcitabine hydrochloride; Hydroxyurea; Idarubicin hydrochloride; Ifosfamide; limofosin; Interleukin I1 (including recombinant interleukin II or rIL.sub.2), interferon alfa-2a; Interferon alfa-2b; Interferon alfa-n1; Interferon alfa-n3; Interferon beta-1a; Interferon gamma-1b; Iproplatin; Irinotecan hydrochloride; Lanreotide acetate; Letrozole; Leuprolide acetate; Liarozole hydrochloride; Lometrexol sodium; Lomustine; Losoxantrone hydrochloride; Masoprocol; Maytansine; Mechlorethamine hydrochloride; Megestrol acetate; Melengestrol acetate; Melphalan; Menogaril; Mercaptopurine; Methotrexate; Methotrexate sodium; Metoprine; Meturedepa; Mitindomide; Mitocarcin; Mitocromine; Mitogillin; Mitomalcin; Mitomycin; Mitosper; Mitotane; Mitoxantrone hydrochloride; Mycophenolic acid; Nocodazole; Nogalamycin; Ormaplatin; Oxisuran; Pegaspargase; Peliomycin; Pentamustine; Peplomycin sulfate; Perfosfamide; Pipobroman; Piposulfan; Piroxantrone hydrochloride; Plicamycin; Plomestane; Porfimer sodium; Porfiromycin; Prednimustine; Procarbazine hydrochloride; Puromycin; Puromycin hydrochloride; Pyrazofurin; Riboprin; Rogletimide; Safingol; Safingol hydrochloride; Semustine; Simtrazen; Sparfosate sodium; Sparsomycin; Spirogermanium hydrochloride; Spiromustine; Spiroplatin; Streptonigrin; Streptozocin; Sulofenur; Talisomycin; Tecogalan sodium; Tegafur; Teloxantrone hydrochloride; Temoporfin; Teniposide; Teroxirone; Testolactone; Thiamiprine; Thioguanine; Thiotepa; Tiazofurin; Tirapazamine; Toremifene citrate; Trestolone acetate; Triciribine phosphate; Trimetrexate; Trimetrexate glucuronate; Triptorelin; Tubulozole hydrochloride; Uracil mustard; Uredepa; Vapreotide; Verteporfin; Vinblastine sulfate; Vincristine sulfate; Vindesine; Vindesine sulfate; Vinepidine sulfate; Vinglycinate sulfate; Vinleurosine sulfate; Vinorelbine tartrate; Vinrosidine sulfate; Vinzolidine sulfate; Vorozol; Zeniplatin; Zinostatin; Zorubicin hydrochloride, agents that arrest cells in the G2-M phases and/or modulate the formation or stability of microtubules (e.g., Taxol™ (i.e., paclitaxel), Taxotere™, compounds comprising the taxane skeleton, erbulozol (i.e., R-55104), dolastatin 10 (i.e., DLS-10 and NSC-376128), Mivobulin Isethionate (i.e., as CI-980), Vincristine, NSC-639829, Discodermolide (i.e., as NVP-XX-A-296), ABT-751 (i.e., Abbott, i.e., E-7010), Altorhyrtine (e.g., Altorhyrtin A and Altorhyrtin C), Spongistatin (e.g., Spongistatin 1, Spongistatin 2, Spongistatin 3, Spongistatin 4, Spongistatin 5, Spongistatin 6, Spongistatin 7, Spongistatin 8 and Spongistatin 9) (i.e., LU-103793 and NSC-D-669356), epothilones (e.g., epothilone A, epothilone B, epothilone C (i.e., desoxyepothilone A or dEpoA), epothilone D (i.e., KOS-862, dEpoB and desoxyepothilone B), epothilone E, epothilone F, epothilone BN oxide, epothilone AN oxide, 16-azaepothilone B, 21-aminoepothilone B (i.e., BMS-310705), 21-hydroxyepothilone D (i.e., deoxyepothilone F and dEpoF), 26-fluorepothilone, auristatin PE (i.e., NSC-654663), Soblidotin (i.e., TZT-1027), LS-4559-P (Pharmacia, i.e., LS-4577), LS-4578 (Pharmacia, i.e., LS-477-P), LS-4477 (Pharmacia), LS-4559 (Pharmacia), RPR-112378 (Aventis), vincristine sulfate, DZ-3358 (Daiichi), FR-182877 (Fujisawa, d h WS-9885B), GS-164 (Takeda), GS-198 (Takeda), KAR-2 (Hungarian Academy of Sciences), BSF-223651 (BASF, i.e., ILX-651 and LU-223651), SAH-49960 (Lilly/Novartis), SDZ-268970 (Lilly/Novartis), AM-97 (Armad/Kyowa Hakko), AM-132 (Armad), AM-138 (Armad/Kyowa Hakko), IDN-5005 (Indena), Cryptophycin 52 (i.e., LY-355703), AC-7739 (Ajinomoto, i.e., AVE-8063A and CS-39.HCl), AC-7700 (Ajinomoto, i.e., AVE-8062, AVE-8062A, CS-39-L-Ser.HCl and RPR-258062A), Vitilevuamid, Tubulysin A, Canadensol, Centaureidin (i.e., NSC-106969), T-138067 (Tularik, i.e., T-67, TL-138067, and TI-138067), COBRA-1 (Parker Hughes Institute, i.e., DDE-261 and WHI-261), H10 (Kansas State University), H16 (Kansas State University), Oncocidin A1 (i.e., BTO-956 and DIME), DDE-313 (Parker Hughes Institute), Fijianolid B, Laulimalid, SPA-2 (Parker Hughes Institute), SPA-1 (Parker Hughes Institute, i.e., SPIKET-P), 3-IAABU (Cytoskeleton/Mt. Sinai School of Medicine, i.e., MF-569), Narcosine (also known as NSC-5366), Nascapin, D-24851 (Asta *Medica*), A-105972 (Abbott), Hemiasterlin, 3-BAABU (Cytoskeleton/Mt. Sinai School of Medicine, i.e., MF-191), TMPN (Arizona State University), Vanadocene acetylacetonate, T-138026 (Tularik), Monsatrol, Inanocin (i.e., NSC-698666), 3-IAABE

(Cytoskeleton/Mt. Sinai School of Medicine), A-204197 (Abbott), T-607 (Tuiarik, i.e., T-900607), RPR-115781 (Aventis), eleutherobins (such as desmethyleleutherobin, desaetyleleutherobin, isoeleutherobin A and Z-eleutherobin), caribaeoside, Halichondrin B, D-64131 (Asta Medica), D-68144 (Asta Medica), Diazonamid A, A-293620 (Abbott), NPI-2350 (Nereus), Taccalonolide A, TUB-245 (Aventis), A-259754 (Abbott), Diozostatin, (−)-Phenylahistin (i.e., NSCL-96F037), D-68838 (Asta Medica), D-68836 (Asta Medica), Myoseverin B, D-43411 (Zentaris, i.e., D-81862) A-289099 (Abbott), A-318315 (Abbott), HTI-286 (i.e., SPA-110, trifluoroacetate salt) (Wyeth), D-82317 (Zentaris), D-82318 (Zentaris), SC-12983 (NCI), resverastatin phosphate sodium, BPR-OY-007 (National Health Research Institutes) and SSR-250411 (Sanofi)), steroids (e.g., dexamethasone), finasteride, aromatase inhibitors, gonadotropin releasing hormone agonists (GnRH) such as goserelin or leuprolide, adrenocorticosteroids (e.g., Prednisone), progestins (e.g., hydroxyprogest eroncaproate, megestrol acetate, medroxyprogesterone acetate), estrogens (e.g., B. Diethylstilbestrol, Ethinylestradiol), antiestrogens (e.g., tamoxifen), androgens (e.g., testosterone propionate, fluoxymesterone), antiandrogens (e.g., flutamide), immunostimulants (e.g., *Bacillus* Calmette-Guerin (BCG), Levamisole, interleukin-2, alpha interferon, etc.), monoclonal antibodies (e.g., anti-CD20, anti-HER2, anti-CD52, anti-HLA-DR, and anti-VEGF monoclonal antibodies), Immunotoxins (e.g., conjugate of anti-CD33 monoclonal antibody and calicheamicin, conjugate of anti-CD22 monoclonal antibody and *Pseudomonas* exotoxin, etc.), radioimmunotherapy (e.g., anti-CD20 monoclonal antibody conjugated to 111 In, 90 Y or 1311 etc.), triptolide, homoharringtonine, dactinomycin, doxorubicin, epirubicin, topotecan, itraconazole, vindesine, cerivastatin, vincristine, deoxyadenosine, sertraline, pitavastatin, irinottryibamine, donyotecan, vindesine, disinfectiburafenib, irinotecan, donyoaburafenib, Gefitinib, EGFR inhibitors, T targeted to the epidermal growth factor receptor (EGFR) therapy or targeted therapeutic agent (e.g., B. Gefitinib (Iressa™), Erlotinib (Tarceva™) Cetuximab (Erbitux™), Lapatinb (Tykerb™), Panitumumab (Vectibix™), Vandetanib (Caprelsa™), Afatinib/BIBW2992, CI-1033/Canertinib, Neratinib/HKI-272, CP-724714, TAK-285, AST-1306, ARRY334543, ARRY-380, AG-1478, Dacomitinib/PF299804, OSI-420/Desmethyl-Erlotinib, AZD8931, AEE788, Pelitinib/EKB-569, CUDC-101, WZ8040, WZ4002, WZ3146, AG-490, XL647, PD153035, BMS-599626), sorafenib, imatinib, sunitinib, and dasatinib, and combinations of the above anticancer agents and therapies.

[0035] The oxysterol active agent compounds may be administered in the pure form or in a pharmaceutically acceptable formulation including suitable elixirs, binders, and the like (generally referred to a "carriers") or as pharmaceutically acceptable salts (e.g., alkali metal salts such as sodium, potassium, calcium, or lithium salts, ammonium, etc.) or other complexes. It should be understood that the pharmaceutically acceptable formulations include liquid and solid materials conventionally utilized to prepare both injectable dosage forms and solid dosage forms such as tablets and capsules and aerosolized dosage forms. In addition, the oxysterol active agent compounds may be formulated with aqueous or oil-based vehicles. Water may be used as the carrier for the preparation of compositions (e.g., injectable compositions), which may also include conventional buffers and agents to render the composition isotonic. Other potential additives and other materials (preferably those which are generally regarded as safe [GRAS]) include: colorants; flavorings; surfactants (TWEEN®, oleic acid, etc.); solvents, stabilizers, elixirs, and binders or encapsulants (lactose, liposomes, etc). Solid diluents and excipients include lactose, starch, conventional disintegrating agents, coatings, and the like. Preservatives such as methyl paraben or benzalkium chloride may also be used. Depending on the formulation, it is expected that the active component (at least one oxysterol active agent) will be present at 1% to 99% of the composition and the vehicular "carrier" will constitute 1% to 99% of the composition. The pharmaceutical compositions of the present disclosure may include any suitable pharmaceutically acceptable additives or adjuncts to the extent that they do not hinder or interfere with the therapeutic effect of the at least one oxysterol active agent compound. Additional suitable agents that may be co-administered or co-formulated also include other agents, including but not limited to: metabolites of the methionine and/or glutathione biosynthetic pathways such as S-adenosylhomocysteine (SAH), S-methylmethionine (SMM), cystine, betaine, etc., or various forms and/or salts thereof, e.g., acetylcysteine (e.g., intravenous N-acetylcysteine), various neutraceuticals, etc.

[0036] Pharmaceutical compositions may include one or more pharmaceutically acceptable carriers. Pharmaceutically acceptable excipients have been amply described in a variety of publications, including, for example, A. Gennaro (2000) "Remington: The Science and Practice of Pharmacy", 20th edition, Lippincott, Williams, & Wilkins; Pharmaceutical Dosage Forms and Drug Delivery Systems (1999) H. C. Ansel et al., eds 7th ed., Lippincott, Williams, & Wilkins; and Handbook of Pharmaceutical Excipients (2000) A. H. Kibbe et al., eds., 3rd ed. Amer. Pharmaceutical Assoc. For example, the one or more excipients may include sucrose, starch, mannitol, sorbitol, lactose, glucose, cellulose, talc, calcium phosphate, or calcium carbonate, a binder (e.g., cellulose, methylcellulose, hydroxymethylcellulose, polypropylpyrrolidone, polyvinylpyrrolidone, gelatin, gum arabic, poly(ethylene glycol), sucrose or starch), a disintegrator (e.g., starch, carboxymethylcellulose, hydroxypropyl starch, low substituted hydroxypropylcellulose, sodium bicarbonate, calcium phosphate, or calcium citrate), a lubricant (e.g., magnesium stearate, light anhydrous silicic acid, talc, or sodium lauryl sulfate), a flavoring agent (e.g., citric acid, menthol, glycine, or orange powder), a preservative (e.g., sodium benzoate, sodium bisulfite, methylparaben, or propylparaben), a stabilizer (e.g., citric acid, sodium citrate, or acetic acid), a suspending agent (e.g., methylcellulose, polyvinylpyrrolidone, or aluminum stearate), a dispersing agent (e.g., hydroxypropylmethylcellulose), a diluent (e.g., water), and base wax (e.g., cocoa butter, white petrolatum, or polyethylene glycol).

[0037] In some cases, compositions of interest include an aqueous buffer. Suitable aqueous buffers include, but are not limited to, acetate, succinate, citrate, and phosphate buffers varying in strengths from 5 mM to 100 mM. In some cases, the aqueous buffer includes reagents that provide for an isotonic solution. Such reagents include, but are not limited to, sodium chloride; and sugars, e.g., mannitol, dextrose, sucrose, and the like. In some cases, the aqueous buffer further includes a non-ionic surfactant such as polysorbate 20 or 80. In some instances, compositions of interest further include a preservative. Suitable preservatives include, but

are not limited to, a benzyl alcohol, phenol, chlorobutanol, benzalkonium chloride, and the like. In many cases, the composition is stored at about 4° C. Formulations may also be lyophilized, in which case they generally include cryoprotectants such as sucrose, trehalose, lactose, maltose, mannitol, and the like. Lyophilized formulations can be stored over extended periods of time, even at ambient temperatures.

[0038] In some cases, compositions include other additives, such as lactose, mannitol, corn starch, or potato starch; with binders, such as crystalline cellulose, cellulose derivatives, acacia, corn starch, or gelatins; with disintegrators, such as corn starch, potato starch, or sodium carboxymethylcellulose; with lubricants, such as talc or magnesium stearate; and if desired, with diluents, buffering agents, moistening agents, preservatives, and flavoring agents.

[0039] Where the composition is formulated for injection, the compositions may be formulated by dissolving, suspending, or emulsifying the oxysterol active agent compound in an aqueous or nonaqueous solvent, such as vegetable or other similar oils, synthetic aliphatic acid glycerides, esters of higher aliphatic acids, or propylene glycol; and if desired, with conventional additives such as solubilizers, isotonic agents, suspending agents, emulsifying agents, stabilizers, and preservatives.

[0040] In some cases, methods according to the present disclosure are directed to treatment of a subject based on a cellular response when the subject is administered the oxysterol active agent compound. In some instances of the present disclosure, epigenetic modification plays a role in the regulation and coordination of gene expression. Methylation at position 5 of cytosine (5-methylcytosine, 5mC) in DNA is an important epigenetic modification that regulates gene expression among other functions of the genome. While not being bound by theory, cytosine methylation of CpG in the promoter region is inversely correlated with transcriptional activity of associated genes as it causes chromatin condensation and thus gene silencing. Dysregulation of CpG methylation and gene expression can affect tissue function and metabolic state. Cytosine methylation is catalyzed by DNA methyltransferase (DNMT-1, 3a/3b), which also functions in the regulation of DNA methylation.

[0041] The major epigenetic regulation includes DNA and histone methylation, demethylation, acetylation, and deacetylation. The enzymes involved in the process are DNA and histone methyltransferases/demethylases, and acetyltransferases/deacetylases. In some cases, administering one or more of the oxysterol active agent compounds is sufficient to act as an epigenetic modulator of one or more of DNMT1, DNMT3a, DNMT3b, GCN3 (Giant congenital nevi), p300 (histone acetyl transferase), Pcaf (KAT2B lysine acetyltransferase 2B), HDAC1 (histone deacetylase 1), HDAC2 (histone deacetylase 2), HDAC3 (histone deacetylase 3), HDAC6 (histone deacetylase 6), HDAC10 (histone deacetylase 10), and KDM6B-JMJD3 (lysine demethylase 6B), such as where 25HC3S, 27HC, and 27HC3S, or cholesterol (Xol) and cholesterol-3-sulfate (Xol3S) are their endogenous ligand(s) to one or more of DNMT1, DNMT3a, DNMT3b, GCN3 (Giant congenital nevi), p300 (histone acetyl transferase), Pcaf (KAT2B lysine acetyltransferase 2B), HDAC1 (histone deacetylase 1), HDAC2 (histone deacetylase 2), HDAC3 (histone deacetylase 3), HDAC6 (histone deacetylase 6), HDAC10 (histone deacetylase 10), and KDM6B-JMJD3 (lysine demethylase 6B).

[0042] While not being bound by theory, in some cases the one or more administered oxysterol active agent compounds inhibits DNMT-1, 3a, and 3b, which demethylated ^{5m}CpG in promoter regions, increased gene expression and up-regulated master signaling pathways such as MAPK, Calcium, AMPK, and CREB signaling pathways. In certain cases, the one or more oxysterol active agent compounds regulate cell signaling pathways at transcriptional levels in nuclei. In some cases, the one or more oxysterol active agent compounds are administered in an amount sufficient to affect protein phosphorylation, inositol phosphorylation, and/or sphingosine phosphorylation in regulating cellular functions.

[0043] In some cases, the addition of one or more of the subject oxysterol active agent compound to human hepatocytes is sufficient to reverse methylation induced by HG, increase hypomethylated CpG in promoter regions of the key genes and increase targeting gene expression. In some cases, while not being bound by theory, CpG demethylation by the oxysterol active agent compound is the mechanism for its function of global regulation: decreasing lipid accumulation, anti-inflammatory responses, anti-oxidants, and anti-cell death.

[0044] The DUSP family is a subset of protein tyrosine phosphatases, many of which dephosphorylate mitogen-activated protein kinases (MAPKs) and hence are referred to as MAPK phosphatase. DUSP8, a unique member of DUSP family, plays an important role in signal transduction of the phosphorylation-mediated MAPK pathway, which regulates responses to oxidative stress and cell death signals in various human diseases. In some cases, administering the one or more oxysterol active agent compounds is sufficient to demethylate ^{5m}CpG in promoter regions of DUSP genes, including DUSP8, DUSP1, and DUSP7, and their downstream genes, CREB5, PRDX, BAD, and ERK, and increase their expression. While not being bound by theory, the transcribed proteins from these genes are responsible for cell survival and proliferation. In certain cases, the effects of the at least one oxysterol active agent compound on promoting cell survival/proliferation and alleviating oxidative stress occur through inhibiting DNMTs and increasing expression of the DUSP family, especially DUSP8 and their downstream elements.

[0045] In some cases, a method of treatment involves modulating at least one gene selected from ABCC4, AC005264.2, ADCY1, ADCY4, ADCY5, ADH6, ADRB, ADRB1, AFDN, AGTR1, AKAP12, AL671762.1, ALAD, ANKRD1, ANKRD43, ATF3, ATP1A3, BAD, BIRC3, C11orf96, CACNA1A, CACNA1C-AS1, CACNA1D, CACNA1H, CACNB2, CACNG8, CELSR2, CREB5, CTB-186G2.1, CXCL2, CYB5B, CYP24A1, CYP51A1, CYR61, DDIT3, DRD5P2, DUSP genes, DUSP8, DUSP1, DUSP7, CREB5, EDNRB, EDN1, EHHADH, ELOVL6, ERK, FABP1, FDFT1, FRMD3, FMC1, FSTL3, GABBR1, GABBR2, GADD45B, GIPR, GLI3, GNA11, GNAQ, GNAS, GRIN2A, GRIN2C, GRIN3B, HBEGF, HMGCR, HMGCS1, HRAS, HRH1, HSPA6, ICAM1, ID3, ID4, IDI1, IL8, IL11, ITPKB, KANK4, KLB, KLF5, KLLN, KRTAP3-1, MAP2K6, MAP4K1, MAP4K4, MAPK1, MAPK8, MAT1A, MAX, METTL7A, MVK, NAP1L5, NCMAP, NTF3, P2RY8, PAQR8, PAQR9, PCSK9, PDE4D, PDGFB, PLA2G12B, PLCD1, PLPPR1, PMAIP1, PNPLA3, POU2AF1, PPP1CB, PRDX, PRLR, PTCH1, RAB11FIP4, RALGPS1, RAPGEF2, RELA, RHOBTB1, ROCK2,

SC4MOL, SCN1A, SEC16B, SERPINEl, SKIL, SLC8A3, SLCO2B1, SLCO4C1, SLC2A14, SOCS2, SORBS2, SPHK1, SPTLC3, SQLE, TAB3, TCIM, TGFB3, THBS1, TMEM170B, TNS1, TNFSF10, TUBB8, UBASH3B, VAV2, VAV3 and ZNF385B.

[0046] In some cases, a method of treatment involves modulating at least one pathway selected from cAMP signaling pathway, cGMP-PKG signaling pathway, circadian entrainment, glutamatergic synapse, adrenergic signaling in cardiomyocytes, gap junction, Type II diabetes mellitus, endocytosis, calcium signaling pathway, dilated cardiomyopathy, vascular smooth muscle contraction, MAPK signaling pathway, cholinergic synapse, Rap1 signaling pathway, dopaminergic synapse, Adherens junction, arrhythmogenic right ventricular cardiomyopathy, pathways in cancer, GnRH signaling pathway, oxytocin signaling pathway, transcriptional misregulation in cancer, estrogen signaling pathway, insulin secretion, retrograde endocannabinoid signaling, long-term depression, colorectal cancer, insulin signaling pathway, axon guidance, alcoholism, platelet activation, amphetamine addiction, herpes simplex infection, tight junction, thyroid hormone signaling pathway, acute myeloid leukemia, chronic myeloid leukemia, notch signaling pathway, and dorso-ventral axis formation.

EXPERIMENTAL

[0047] The present invention will be further illustrated by way of the following Examples. These Examples are non-limiting and do not restrict the scope of the invention. Unless stated otherwise, all percentages, parts, etc. presented in the Examples are by weight. The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention, and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Celsius, and pressure is at or near atmospheric. By "average" is meant the arithmetic mean. Standard abbreviations may be used, e.g., bp, base pair(s); kb, kilobase(s); pl, picoliter(s); s or sec, second(s); min, minute(s); h or hr, hour(s); aa, amino acid(s); kb, kilobase(s); bp, base pair(s); nt, nucleotide(s); i.m., intramuscular(ly); i.p., intraperitoneal (ly); s.c., subcutaneous(ly); and the like.

Example 1

[0048] Abbreviations

25HC	25-Hydroxycholesterol
25HC3S	25-Hydroxycholesterol 3-Sulfate
27HC	27-Hydroxycholesterol
27HC3S	27-Hydroxycholesterol 3-Sulfate
^{5m}C	5 -Methylcytosine
BAD	BCL2 associated agonist of cell death
CaV1	Calcium voltage-gated channel subunit alphal D, CACNA1D
CaV2	Calcium voltage-gated channel subunit alphal A, CACNA1A
CaV3	Calcium voltage-gated channel subunit alphal H, CACNA1H
CREB	cAMP response element-binding protein
DMEM	Eagle's minimal essential medium
DMGs	Differential methylated gene
DMRs	Differential methylated regions
DNMTs	DNA methyltransferases
DUSP	Dual-specificity phosphatase
FAS	Fatty acid synthase
GCN3	Giant congenital nevi
HDAC1	Histone deacetylase 1
HDAC10	Histone deacetylase 10
HDAC2	Histone deacetylase 2
HDAC3	Histone deacetylase 3
HDAC6	Histone deacetylase 6
HG	High glucose
HMGR	3-hydroxy-3-methylglutaryl-coenzyme A reductase
KDM6B-JMJD3	Lysine demethylase 6B
KEGG	Kyoto Encyclopedia of Genes and Genomes
LINE	Long interspersed nuclear element
LPS	Lipopolysaccharides
LXRs	Nuclear liver oxysterol receptors
MAPK	A mitogen-activated protein kinase
NAFLD	Non-alcoholic fatty liver diseases
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
p300	Histone acetyl transferase
Pcaf	KAT2B lysine acetyltransferase 2B
PGC-1α	Pparg coactivator 1 alpha
PPARγ	Peroxisome proliferator-activated receptor
PRDX6	Peroxiredoxin 6
RT-PCR	Reverse transcription-polymerase chain reaction
SREBP	Sterol regulatory element-binding protein
WGBS	Whole genome bisulfite sequencing
Xol	Cholesterol
Xol3S	Cholesterol 3-Sulfate

Materials and Methods

[0049] Materials

[0050] Cell culture reagents and supplies were purchased from GIBCO BRL (Grand Island, N.Y.); Huh-7 cells were obtained from American Type Culture Collection (Rockville, Md.). The reagents for real time RT-PCR were from AB Applied Biosystems (Warrington, UK). The chemicals used in this research were obtained from Sigma Chemical Co. (St. Louis, Mo.) or Bio-Rad Laboratories (Hercules, Calif.). All solvents were obtained from Fisher (Fair Lawn, N.J.) otherwise indicated.

[0051] Cell Culture

[0052] Huh-7 and HepG-2 cells were cultured in DMEM media supplemented with 10% heat-inactivated fetal bovine serum (FBS), high glucose (HG, 4.5 g/L) at 37° C. in a humidified atmosphere of 5% CO_2.

[0053] Extraction and Determination of DNA and mRNA Levels

[0054] After culturing Huh-7 cells in DMEM medium with HG for 72 hours followed by treating with 25 μM 25HC3S for 4 hours, genomic DNA from 5,000 cells were extracted using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Each sample, 2 μg, was sent to EpigenDx, Inc. (Hopkinton, Mass.) for analysis of global methylation bisulfite sequencing. The same samples, 6 μg, were sent to Novogene Co., Ltd (Tianjin, China) for analysis of whole genome bisulfite sequencing (WGBS). Total RNA was isolated using the Promega SV total RNA isolation system (Madison, Wis.) with DNase treatment. Each sample, 2 μg, was used for the first-strand cDNA synthesis as recom-

mended by the manufacturer (Invitrogen, Carlsbad, Calif.). Real-time RT-PCR was performed using SYBR Green as the indicator on ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, Calif.). Amplifications of j-actin or GAPDH were used as internal controls. Relative messenger RNA (mRNA) expression was quantified with the comparative cycle threshold (Ct) method using the primer set shown in Table 1.1 and was expressed as $2^{-\Delta\Delta ct}$.

TABLE 1.1

Primer Sequence for Real-time Polymerase Chain Reaction

Gene name	Forward Sequence	Reverse Sequence
DUSP8	TCAGCTCCGTCAACATCTGC (SEQ ID NO: 1)	CGCGTGCTCTGGTCATAGA (SEQ ID NO: 15)
DUSP7	ATATCCTCAATGTCACACCCAA (SEQ ID NO: 2)	ATCTTCTGCATCAGATAGGCC (SEQ ID NO: 16)
MAPK1	ATGGTGTGCTCTGCTTATGATA (SEQ ID NO: 3)	TCTTTCATTTGCTCGATGGTT G (SEQ ID NO: 17)
CREB5	GCAACAAGTCATCCCAGCATAA T (SEQ ID NO: 4)	AAGAATCGGATTCAGGTCTGT T (SEQ ID NO: 18)
PRDX6	TCAATAGACAGTGTTGAGGACC (SEQ ID NO: 5)	CCCGATTCCTATCATCGATGA T (SEQ ID NO: 19)
BAD	ATGTTCCAGATCCCAGAGTTTG (SEQ ID NO: 6)	ATGATGGCTGCTGCTGGTT (SEQ ID NO: 20)
CaV1	AACAACAAACCAGAAGTCAACC (SEQ ID NO: 7)	CTAAGAATGAAGAAAGCGCTC C (SEQ ID NO: 21)
CaV2	CGCTTCGGAGACGAGATGC (SEQ ID NO: 8)	TGCGCCATTGACTGCTTGT (SEQ ID NO: 22)
CaV3	CATGCTGGTAATCATGATCAAC (SEQ ID NO: 9)	CGAAAATGAAGGCGTCAAAGG (SEQ ID NO: 23)
PGC1A	CACCAGCCAACACTCAGCTA (SEQ ID NO: 10)	ACGTCTTTGTGGCTTTTGCT (SEQ ID NO: 24)
HMGR	GTCATTCCAGCCAAGGTTGT (SEQ ID NO: 11)	GGGACCACTTGCTTCCATTA (SEQ ID NO: 25)
FAS	TGTGGACATGGTCACGGAC (SEQ ID NO: 12)	GGCATCAAACCTAGACAGGTC (SEQ ID NO: 26)
β-ACTIN	CATGTACGTTGCTATCCAGGC (SEQ ID NO: 13)	CTCCTTAATGTCACGCACGAT (SEQ ID NO: 27)
GAPDH	CAATGACCCCTTCATTGACC (SEQ ID NO: 14)	TTGATTTTGGAGGGATCTCG (SEQ ID NO: 28)

[0055] Chemical Synthesis and Characterization of Sterol Sulfates, 25HC3S, Xol3S, 27HC3S

[0056] 5-Cholesten-3β, 25-diol 3-sulfate (25-Hydroxycholesterol 3-Sulfate, 25HC3S); 5-cholesten-3β-ol, 3-sulfate (Cholesterol 3-Sulfate, Xol3S); 5-cholesten-3β, 27-diol 3-sulfate (27-Hydroxycholesterol 3-Sulfate, 27HC3S) were synthesized as previously described with mild modification. Briefly, a mixture of 25-hydroxycholesterol, cholesterol, or 27-hydroxycholesterol (6.5 mg, 0.016 mmol) and triethylamine-sulfur trioxide (3.5 mg, 0.019 mmol) was dissolved in dry pyridine (300 μl) and was stirred at room temperature for 2 hours. The solvents were evaporated at 40° C. under nitrogen gas stream, and the syrup was added into 2 ml of 50% acetonitrile (loading buffer). The products were applied to a 6 cc Oasis cartridge (Waters), which had been primed by methanol (15 ml) and water (15 ml). The cartridge was successively washed with the loading buffer (15 ml), water (15 ml), methanol (15 ml), 50% methanol (15 ml), 5% ammonia hydroxide in 10% methanol (15 ml), and 5% ammonia hydroxide in 50% methanol (15 ml). The retained sulphated sterol was eluted with 5% ammonia hydroxide in 80% methanol (10 ml), respectively. After dilution with 10 times volume of acetonitrile, the solvents were evaporated to dryness under nitrogen gas stream, and the sterol sulphates were obtained in white powder form.

[0057] Enzyme Kinetic Study of 5-Cholesten-3, 25-Diol 3-Sulfate

[0058] For the DNMT1 activity assay, the substrate solution, 0.001 mg/ml Poly(dI-dC):Poly(dI-dC) in 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM EDTA, 5 mM DTT, 1 mM PMSF, 5% glycerol, 0.01% Brij35, 1% DMSO was used. For the DNMT3a/3b activity assay, 0.0075 mg/ml Lambda DNA in 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM EDTA, 5 mM DTT, 1 mM PMSF, 5% glycerol, 1% DMSO, was used. The indicated DNMT1, DNMT3a, or DNMT3b was added to the appropriate substrate solution and gently mixed. Amounts of cholesterol (Xol), 25HC, 27HC, Xol3S, 25HC3S, or 27HC3S ranging from 5.08E-09 to 0.0001 M in DMSO were added to the reaction mixture by using Acoustic Technology (Echo 550, LabCyte Inc. Sunnyvale, Calif.). The mixtures were first incubated for 15 min, then ^{3}H-SAM was added to the reaction mixture to initiate the reaction, and the mixture was incubated for 60 min at 30° C. Following incubation, the reaction mixture was finally transferred to filter-paper for detection of radioactivity counts.

[0059] Analysis of Global Methylation, Long Interspersed Nucleotide Element 1(LINE-1) Assay

[0060] For global DNA methylation analysis, 500 ng of extracted genomic DNA was bisulfite-treated using the EZ DNA Methylation Kit™ (Zymo Research, Inc., CA). PCR reaction and product purification were performed as per the manufacturer's protocol (GE Healthcare Life Sciences). The PCR products, 10 μl, were sequenced by Pyrosequencing on the PSQ96 HS System following the manufacturer's instructions (Pyrosequencing, Qiagen). The methylation status of each CpG site was determined individually as an artificial C/T SNP using QCpG software (Pyrosequencing, Qiagen). The methylation level at each CpG site was calculated as the percentage of the methylated alleles divided by the sum of all methylated and unmethylated alleles. The mean methylation level was calculated using methylation levels of all measured CpG sites within the targeted region of each gene. Each experiment included non-CpG cytosines as internal controls to detect incomplete bisulfite conversion of the input DNA. In addition, a series of unmethylated and methylated DNA were included as controls in each PCR assay. Furthermore, PCR bias testing was performed by mixing unmethylated control DNA with in vitro methylated DNA at different ratios (0%, 5%, 10%, 25%, 50%, 75%, and 100%), followed by bisulfite modification, PCR, and Pyrosequencing analysis.

[0061] Analysis of Whole Human Genome Bisulfite Sequencing (WGBS)

[0062] Each sample, 5.2 μg of genomic DNA spiked with 26 ng lambda DNA, was fragmented by sonication to 200-300 bp with Covaris S220, followed by end repair and adenylation. Cytosine-methylated barcodes were ligated to sonicated DNA per the manufacturer's instructions. These

DNA fragments were treated twice with bisulfite using EZ DNA Methylation-Gold™ Kit™ (Zymo Research) before the resulting single-strand DNA fragments were PCR amplified using KAPA HiFi Hot Start Uracil and Ready Mix (2×). Library concentration was quantified by Qubit® 2.0 Flurometer (Life Technologies, CA, USA) and quantitative PCR, and the insert size was assayed on an Agilent Bioanalyzer 2100 system.

[0063] The library preparations were sequenced on an Illumina Hiseq 2500/4000 or Novaseq™ platform and 125 bp/150 bp paired-end reads were generated. Image analysis and base calling were performed with Illumina CASAVA pipeline. Trimmomatic (Trimmomatic-0.36) software was used for quality control. Bismark software (version 0.16.3; Krueger F, 2011) was used to perform alignments of bisulfite-treated reads to a reference genome (–X 700-dovetail). DSS software (23) was used to identify differentially methylated regions (DMRs). KOBAS software was used to test the statistical enrichment of DMR related genes in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

[0064] Transcriptional Profiling and Data Analysis

[0065] Total RNA was extracted and purified from HepG-2 cells using SV total RNA isolation system (Promega, Madison, Wis.). cDNAs were prepared and analyzed using GeneChip® Human Genome U133 Plus 2.0 Array, Affymetrix (Santa Clara, USA) as previously described with technical support from Shanghai Biotechnology Corporation. Direct target genes in the present study were selected based on more than 2-fold of reduction together with array detect signal more than 5 in both samples. Genes showing fold changes greater than 2 and array-detected signals greater than 7 in at least one sample were selected as differently expressed genes. DAVID software (https://david.ncifcrf.gov/conversion.jsp) was used to analyze GO enrichment of differently expressed genes.

Results

[0066] 25-hydroxycholesterol-3-sulfate (25HC3S) Specifically Inactivate DNMT Activities

[0067] In order to study the effects of sterol sulfates on the epigenetic regulating targets, 25HS3S, Xol3S, and 27HC3S (FIG. **1**A) were synthesized and purified to more than 95% purity using triethylamine sulphate complex methods as shown in FIG. **1**B. Results show that 25HC3S significantly inhibits only DNMT-1, 3a, and 3b activities with IC_{50}=4.04, 3.03, and 9.05×10^{-6} M, respectively (FIG. **1**C, Left), while its precursor 25HC activates DNMT-1 activity by 8-fold with $EC_{50}=3.5\times10^{-6}$ M (FIG. **1**D, Left). As controls, Xol as well as Xol3S did not significantly affect enzymatic activities although Xol3S slightly inhibits DNMT3a with $IC_{50}=8.2\times10^{-5}$ M, which is most likely not physiologically significant (FIG. **1**C, Middle). Compared with 25HC3S, 27HC3S did inhibit DNMTs with similar $IC_{50}=3.58\times10^{-6}$ M for DNMT1, 8.88×10^{-6} M for DNMT3a, and 2.68×10^{-6} M for DNMT3b as shown in FIG. **1**C. Right. In contrast, its precursor 27HC, was much less potent in activation of DNMT-1 with $EC_{50}=3.3\times10^{-5}$ M and had no effect on other enzymes (FIG. **1**D, Right). In contrast to the 3 DNMTs, the 9 other epigenetic enzymes are not affected by these oxysterols or sterol sulfates (data not shown). As a positive control, S-adenosyl homocysteine (SAH) inhibited DNMT1 activity by 95% at 1 μM (data not shown), as previously reported. The results demonstrated that both 25HC3S and 27HC3S are potent inhibitors of DNMTs. However, only 25HC3S has been discovered in vivo in human hepatocyte nuclei: first found in concentration of 20 ug/g (~40 uM) following overexpression of mitochondrial cholesterol delivery protein, StarD1. The kinetic study shows that the IC50s are between 1-10 uM.

[0068] 25-hydroxycholesterol-3-sulfate (25HC3S) Decreases ^{5m}CpG Levels in Global Promoter Regions

[0069] Previous studies have shown that global DNA methylation and the methylation of specific genes are involved in adipogenesis, lipid metabolism, and inflammation in visceral adipose tissues, which, in turn, are related to the specific etiology of metabolic syndrome. To study the effects of 25HC3S on methylation status of ^{5m}CpG in global promoter regions, LINE-1 analysis was first performed to estimate global demethylation. Methylation usually occurs in repetitive elements, such as LINE elements. There are ~500,000 LINE elements and 750 million copies in total human genome. Human LINE-1 is a retro-transposable region (promoter region) and has only 700,000 copies, which correlates to ~17% of the human genome. The specific sequence includes four CpG dinucleotides (Pos 1, 2, 3, and 4), which serve as methylation/demethylation targets in LINE-1. As shown in FIG. **2**A, culturing Huh-7 cells in high glucose media (HG), Pos 3 and Pos 4 had higher methylation, while all 4 Pos increased methylation after culturing cells in ethanol control. Reduction of methylation (demethylation) at Pos1 (–5%), Pos3 (–10%), and Pos 4 (–5.6%) occurred after incubating cells with 25HC3S for 4 hours. The results indicate that 25HC3S significantly reduce ^{5m}CpG methylation in promoter regions induced by HG or ethanol.

[0070] Profiles of Whole Genome-Wide DNA Methylation in 25HC3S-Treated Human Hepatocytes

[0071] To understand the possible cellular functions of ^{5m}CpG demethylation in 25HC3S-treated Huh-7 cells, the cells were harvested for the construction of bisulfite-treated genomic DNA libraries. In 5 total WGBS, there were 366 million (Vehicle) and 370 million (25HC3S-treated) raw reads generated from the two libraries by paired-end sequencing, respectively. Among clean reads, 360 million, from vehicle library, 77% (277 million) were uniquely mapped to the reference genome of "human reference genome (hg38)", while among 365 million clean reads from the 25HC3S-treated library, 78% (286 million) were uniquely mapped to the reference genome, exhibiting an 10 average read depth of 22 and 20, respectively. In these two libraries, more than 80% of cytosine residues were covered by at least ten reads in "human reference genome (hg38)".

[0072] The depth and density of the sequencing were enough for a high-quality genome-wide methylation analysis. Meanwhile, the efficiencies of bisulfite conversion, represented by the lambda DNA to the libraries, were over 99%, providing reliable and accurate results for the WGBS (Table 1.2).

TABLE 1.2

Summary of The Whole Genome Bisulfite Sequencing Data

Sample name	Raw reads	Clean reads	Clean ratio (%)	Uniquely mapped reads	Uniquely mapped rate (%)	Sites_covgMean	Sites_num Covg10	BS conversion rate(%)
Vehicle	366,129,468	360,798,889	90.30	276,588,428	76.66	19.68	80.78	99.727
25HC3S	370,635,495	365,154,331	90.15	285,623,717	78.22	21.09	83.17	99.795

[0073] CpG methylation and demethylation are well documented to related with gene expression. A total of about 7,136 differential methylated regions (DMRs) under CG context were identified as hypomethylated regions located in 1,106 genes (differential methylated genes, DMGs). In 97% (1,074) of the DMGs, the hypomethylated regions were identified in their promoters (FIG. **2**B). The hypomethylated genes were highly enriched in 75 KEGG pathways ($p<0.05$) (Table 1.3). The top 20 pathways (from the most significance, $p<10^{-9}$) were shown in FIGS. **2**E and **2**F. Among these pathways, MAPK-ERK and calcium-cAMP signaling are believed as the master pathways regulating cell survival, anti-oxidants, anti-apoptosis, energy metabolism, and lipid homeostasis. The pathways identified from whole genome are shown in FIG. **2**E, and those identified from promoter regions are shown in FIG. **2**F. Both sets of pathways, from whole genome or from promoter regions, are very similar. All pathways identified from promoter regions were hypomethylated without any hypermethylated CpGs in their promoter regions, indicating up-regulated gene expressions.

[0074] DNA methylation levels in whole genome and differential methylated regions (DMRs) are shown in FIG. **2**B. To present the global DNA methylation profiles of the two libraries, the uneven methylation levels throughout the chromosomes under CG, CHG (H represents adenosine or thymidine residues), and CHH contexts are shown in FIG. **2**B. A total of 6,923 differentially methylated genes (DMGs) were screened out among the two libraries. Moreover, 1,510 were 20 identified under CG context, 420 under CHG context, and 3,359 under CHH context. 83 were identified under CG and CHG contexts, 481 under CG and CHH contexts, 793 under CHG and CHH contexts, while only 277 were identified under CG, CHG, and CHH contexts. Furthermore 2,853 were identified as promoter regions, and 1,413 were identified under CG context, 186 under CHG context, and 787 under CHH context. For these DMGs, 59 were identified under CG and CHG contexts, 46 under CG and CHH, 260 under CHG and CHH contexts, and only 103 were identified under CG, CHG, and CHH contexts. While in these DMGs, 80.93% (5,603) were identified as hypomethylated, and 37.55% (2,104) were identified as hypomethylated in promoter 5 regions (FIG. **2**C).

TABLE 1.3

Significant Enrichment KEGG Pathways of Hypomethylated DMGs in Promoter Region under CG Context

Pathway name	P-value	Pathway name	P-value
cAMP signaling pathway	3.69E−07	Cocaine addiction	0.014096
cGMP-PKG signaling pathway	2.65E−05	Viral carcinogenesis	0.01429
Circadian entrainment	5.68E−05	Pancreatic cancer	0.015201

TABLE 1.3-continued

Significant Enrichment KEGG Pathways of Hypomethylated DMGs in Promoter Region under CG Context

Pathway name	P-value	Pathway name	P-value
Glutamatergic synapse	8.40E−05	Inflammatory mediator regulation of TRP channels	0.015802
Adrenergic signaling in cardiomyocytes	0.000108	Hippo signaling pathway	0.017014
Gap junction	0.000227	Melanogenesis	0.017221
Type II diabetes mellitus	0.000505	Neurotrophin signaling pathway	0.018561
Endocytosis	0.000656	Salmonella infection	0.018615
Calcium signaling pathway	0.000656	Chagas disease (American trypanosomiasis)	0.01954
Dilated cardiomyopathy	0.000656	Thyroid hormone synthesis	0.021071
Vascular smooth muscle contraction	0.000752	HTLV-I infection	0.021071
MAPK signaling pathway	0.000877	Prostate cancer	0.021071
Cholinergic synapse	0.001123	GABAergic synapse	0.021665
Rap1 signaling pathway	0.001123	Salivary secretion	0.021665
Dopaminergic synapse	0.001194	Cell adhesion molecules (CAMs)	0.022648
Adherens junction	0.001194	Amoebiasis	0.023054
Arrhythmogenic right ventricular cardiomyopathy	0.001203	Viral myocarditis	0.024448
Pathways in cancer	0.001203	Type I diabetes mellitus	0.024448
GnRH signaling pathway	0.001679	Focal adhesion	0.024448
Oxytocin signaling pathway	0.002196	Ras signaling pathway	0.024601
Transcriptional misregulation in cancer	0.002515	Fructose and mannose metabolism	0.025877
Estrogen signaling pathway	0.002932	One carbon pool by folate	0.027147
Insulin secretion	0.003045	Serotonergic synapse	0.027147
Retrograde endocannabinoid signaling	0.003437	Endocrine and other factor-regulated calcium reabsorption	0.029495
Long-term depression	0.003463	Hypertrophic cardiomyopathy (HCM)	0.034694
Colorectal cancer	0.004132	Long-term potentiation	0.036088
Insulin signaling pathway	0.004549	Ovarian steroidogenesis	0.036088
Axon guidance	0.005282	Wnt signaling pathway	0.036088
Alcoholism	0.005377	Endometrial cancer	0.038405
Platelet activation	0.006325	AMPK signaling pathway	0.040972
Amphetamine addiction	0.006325	Fc epsilon RI signaling pathway	0.041787
Herpes simplex infection	0.006736	Allograft rejection	0.045211

TABLE 1.3-continued

Significant Enrichment KEGG Pathways of Hypomethylated DMGs in Promoter Region under CG Context

Pathway name	P-value	Pathway name	P-value
Tight junction	0.007081	Bile secretion	0.045703
Thyroid hormone signaling pathway	0.007681	Prolactin signaling pathway	0.045703
Acute myeloid leukemia	0.007681	Chemokine signaling pathway	0.046416
Chronic myeloid leukemia	0.008581	Neuroactive ligand-receptor interaction	0.046416
Notch signaling pathway	0.012054	Fc gamma R-mediated phagocytosis	0.047019
Dorso-ventral axis formation	0.013526		

[0075] Previous report has shown that high glucose incubation (HG), an in vitro model for study of NAFLD, induces lipid accumulation via increasing DNA promoter methylation signaling. It was noted that the hypermethylated ^{5m}CpG in the promoter regions induced by HG were demethylated by 25HC3S. 25HC3S demethylated ^{5m}CpG in promoter regions of 23 genes in MAPK signaling pathway (Table 1.4), 19 genes in Calcium pathway (Table 1.5), and 28 genes in cAMP pathway (Table 1.6). No hypermethylated DMR was found in the genes involved in the signaling pathways. The chromosome and sequence location of the hypermethylated ^{5m}CpG by HG and the hypomethylated CpG by 25HC3S in promoter regions are compared in the tables. It is observed that these genes are also involved in many other KEGG pathways including insulin, Type II Diabetes Mellitus, and cGMP-PKG signaling pathways. The results indicate that the global regulatory mechanisms of 25HC3S are through demethylation of ^{5m}CpG in promoter regions of the key genes, such as the DUSP and Calcium channel families, involved in MAPK-ERK and calcium-cAMP master signaling pathways.

[0076] DNA methylation levels generally show a varied distribution across different functional regions of the genome. The methylation levels in the CGI (CG island), CGI-shore (up to 2 k bp away from the CGI), promoter (upstream 2 k bp sequence from transcription starting site), 5' untranslated region (UTR5), exon, intron, 3' untranslated region (UTR3), and repeat were 10 significant different between vehicle and 25HC3S treated groups. It is interesting that 25HC3S treatment resulted in significantly higher hypomethylation levels than vehicle (FIG. **2**D). In a total of 34,508 DMRs identified, 3,676 (1,549 hypermethylated and 2,127 hypomethylated) were distributed in CGI, 2206 (627 hypermethylated and 1,579 hypomethylated) in CGI-shore, 3,263 (1,213 hypermethylated and 2,050 hypomethylated) in exon, 9,850 (2,340 hypermethylated and 15 7,510 hypomethylated) in intron, 3,696 (1,187 hypermethylated and 2,509 hypomethylated) in promoter, 8,956 (1,882 hypermethylated and 7,774 hypomethylated) in repeat region, 61 (16 hypermethylated and 45 hypomethylated) in TES elements, 452 (179 hypermethylated and 273 hypomethylated) in TSS elements, 403 (123 hypermethylated and 280 hypomethylated) in UTR3 regions, and 1245 (432 hypermethylated and 813 hypomethylated) in UTR5 regions. In almost all 20 DMRs, CpGs are significantly more hypomethylated than hypermethylated. It has been reported that CG methylation in promoter regions plays a key role in silencing gene expression.

[0077] In a total of 6,923 DMGs, the genes under CG context were highly enriched in 120 KEGG pathways (69 hypomethylated and 51 hypermethylated). The genes under CHG context were enriched in 48 pathways (33 hypomethylated and 15 hypermethylated), while those under CHH context, enriched in 136 pathways (101 hypomethylated and 35 hypermethylated). DMGs in promoter regions were highly enriched in 114 (31 hypermethylated and 83 hypomethylated) pathways, of which 75 (0 hypermethylated and 75 hypomethylated) under CG context, 13 (13 hypermethylated and 0 hypomethylated) under CHG context, and 26 (18 hypermethylated and 8 hypomethylated) under CHH context (Table 1.3).

TABLE 1.4

Demethylation of ^{5m}CpG in Promoter Regions of MAPK Signaling Genes (P = 0.00087)

	DMR location in promoter region			DMR (Methylation %)	
Gene name	Chromosome	Start	End	HGLG	25HC3S-Vehicle
MAPK8	Chr10	48306510	48306561		−29.24
DUSP8	Chr11	1572973	1573032		−30.51
		1573583	1573855	+41.72	
MAX	Chr14	65102273	65102358		−31.75
DUSP7	Chr3	52056416	52056485		−40.47
		52056538	52056778	+31.75	
NTF3	Chr12	5432796	5432882		−33.48
CACNA1D	Chr3	53494583	53494705		−20.46
		53493469	53494019	+49.03	
CACNA1H	Chr16	1194928	1195015		−26.48
		1194670	1195215	+41.26	
CACNA1A	Chr19	13226635	13226711		−39.91
		13238959	13239237	+9.51	
MAPK1	Chr22	21867450	21867512		−7.66
		21867333	21867621	+20.15	
HRAS	Chr11	535071	535127		−18.85
		536242	537214	+42.8	
PDGFB	Chr22	39243967	39244084		−30.51
		39242292	39242477	+29.29	
CACNA1C-AS1	Chr12	2691335	2691438		−20.97

TABLE 1.4-continued

Demethylation of ^{5m}CpG in Promoter Regions of MAPK Signaling Genes (P = 0.00087)

	DMR location in promoter region			DMR (Methylation %)	
Gene name	Chromosome	Start	End	HGLG	25HC3S-Vehicle
CACNB2	Chr10	18140401	18140547		−21.91
		18340356	18341053	+47.13	
MAP4K1	Chr19	38596260	38596495		−34.3
RAPGEF2	Chr4	1.59E+08	1.59E+08		−36.05
CTB-186G2.1	Chr19	38596260	38596495		−34.3
RELA	Chr11	65661914	65662035		−35.1
GADD45B	Chr19	2474806	2474873		−28.6
AL671762.1	Chr6	31828019	31828149		−27.17
CACNG8	Chr19	53961377	53961479		−31.18
MAP4K4	Chr2	1.02E+08	1.02E+08		−13.63
		1.02E+08	1.02E+08	+17.93	
TGFB3	Chr14	75982395	75982557		−23.74

[0078] Table 1.4—After culturing Huh-7 cells in DMEM medium with HG for 72 hours followed by treating with ethanol (vehicle) and 25 μM 25HC3S for 4 hours, genomic DNA from 5,000 cells were extracted using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Each sample (6 μg) was used for analysis of the whole genome bisulfite sequencing (WGBS). The KEGG analysis shows that the demethylated genes are involved in MAPK signaling pathway (p=0.00087). Of the 257 total genes in the MAPK signaling pathway, 23 were demethylated by the 25HC3S treatment. Of these 23 genes, 10 were found to be methylated by a HG environment (shown in bold). The first column represents the gene name, the second column (DMR location in promoter region) shows the location of differential methylation region in the chromosome, the third column (DMR (Methylation %)) shows the methylation rates by high glucose (HG) and demethylation rates induced by 25HC3S.

TABLE 1.5

Demethylation of ^{5m}CpG in Promoter Regions of Calcium Signaling Genes (P = 0.00066)

	DMR location in promoter region			DMR (Methylation %)	
Gene name	Chromosome	Start	End	HGLG	25HC3S-Vehicle
ADCY4	Chr14	24334570	24334719		−18.63
		24334404	24334719	+32.6	
DRD5P2	Chr1	1.43E+08	1.43E+08		−34.73
EDNRB	Chr13	77919496	77919743		−34.09
ADRB1	Chr10	1.14E+08	1.14E+08		−33.1
GRIN2A	Chr16	10183302	10183414		−19.63
		10084383	10084724	+8.62	
GNA11	Chr19	3094383	3094442		−32.66
		3092574	3092876	+6.13	
SPHK1	Chr17	76383180	76383291		−24.74
CACNA1C-AS1	Chr12	2691335	2691438		−20.97
HRH1	Chr3	11154292	11154354		−25.48
ITPKB	Chr1	2.27E+08	2.27E+08		−25.12
		2.27E+08	2.27E+08	+23.84	
PLCD1	Chr3	38029863	38030016		−24.31
		38029901	38030042	+26.02	
GNAS	Chr20	58888598	58888893		−25.68
		58888560	58888756	+23.2	
AC005264.2	Chr19	3156392	3156457		−15.76
CACNA1D	Chr3	53494583	53494705		−20.46
		53493469	53494019	+49.03	
GNAQ	Chr9	78032198	78032440		−11.92
GRIN2C	Chr17	74861739	74861853		−37.6
		74854901	74855100	+36.66	
SLC8A3	Chr14	70188747	70189089		−28.51
		70046033	70046320	+65.66	
CACNA1H	Chr16	1194928	1195015		−26.48
		1194670	1195215	+41.26	

[0079] Table 1.5—Cells preparation and DNA methylation as described in Table 1.1. The KEGG analysis shows that the demethylated genes are involved in calcium signaling pathway (P=0.00066). Of the 180 total genes in the Calcium signaling pathway, 19 were demethylated by the

25HC3S treatment. Of these 19 genes, 10 were found to be methylated by a HG environment (shown in bold). The first column represents the gene name, the second column (DMR location in promoter region) shows the location of differential methylation region in the chromosome, the third column (DMR (Methylation %)) shows the methylation rates by high glucose (HG) and demethylation rates induced by 25HC3S.

TABLE 1.6

Demethylation of ^{5m}CpG in Promoter Regions of cAMP Signaling Genes (P = 3.69E−07)

	DMR location in promoter region			DMR (Methylation %)	
Gene name	Chromosome	Start	End	HG-LG	25HC3S-Vehicle
MAPK8	Chr10	48306510	48306561		−29.24
PTCH1	Chr9	95508446	95508561		−12.09
ADCY5	Chr3	1.23E+08	1.23E+08		−30.44
		1.23E+08	1.23E+08	+15.8	
GLI3	Chr7	42228171	42228223		−19.62
		42184839	42185064	+8.27	
PPP1CB	Chr2	28751716	28751798		−10.93
		28793684	28794075	+7.5	
GNAS	Chr20	58888598	58888893		−25.68
		58888560	58888756	+23.2	
CACNA1D	Chr3	53494583	53494705		−20.46
		53493469	53494019	+49.03	
GRIN2A	Chr16	10183302	10183414		−19.63
MAPK1	Chr22	21867450	21867512		−7.66
		21867333	21867621	+20.15	
GABBR2	Chr9	98708549	98708610		−21.84
CREB5	Chr7	28489448	28489601		−29.31
		28776194	28776325	+27.34	
GRIN3B	Chr19	1000331	1000405		−34.67
CACNA1C-AS1	Chr12	2691335	2691438		−20.97
VAV2	Chr9	1.34E+08	1.34E+08		−33.11
		1.34E+08	1.34E+08	+44.31	
ABCC4	Chr13	95301634	95301736		−33.06
PDE4D	Chr5	59893498	59893718		−27.84
		59215742	59215941	+39.66	
ROCK2	Chr2	11344628	11344733		−23.14
ADCY1	Chr7	45574500	45574591		−14.76
		45574683	45574877	+25.79	
ADCY4	Chr14	24334570	24334719		−18.63
		24334404	24334719	+32.6	
DRD5P2	Chr1	1.43E+08	1.43E+08		−34.73
ADRB1	Chr10	1.14E+08	1.14E+08		−33.1
GIPR	Chr19	45668710	45668767		−10.04
		45669075	45669744	+43.62	
RELA	Chr11	65661914	65662035		−35.1
AFDN	Chr6	1.68E+08	1.68E+08		−30.68
BAD	Chr11	64286088	64286179		−42.33
ATP1A3	Chr19	41999134	41999316		−15.75
GRIN2C	Chr17	74861739	74861853		−37.64
		74854901	74855100	+36.66	

[0080] Table 1.6—Cells preparation and DNA methylation as described in Table 1.1. The KEGG analysis shows that the demethylated genes are significantly involved in cAMP signaling pathway (P=3.69E-07). Of the 200 total genes in the cAMP signaling pathway, 28 were demethylated by the 25HC3S treatment. Of these 28 genes, 13 were found to be methylated by a HG environment (shown in bold). The first column represents the gene name, the second column (DMR location in promoter region) shows the location of differential methylation region in the chromosome, the third column (DMR (Methylation %)) shows the methylation rates by high glucose (HG) and demethylation rates induced by 25HC3S.

[0081] Relationship Between ^{5m}CpG Demethylation in Promoter Regions and Gene Expression: 25HC3S Decreases HG-Induced ^{5m}CpG Levels in Promoter Regions

[0082] To explore the relationship of promoter ^{5m}CpG demethylation and gene expression from the results of KEGG pathway analysis, the expression of key genes (DUSP7,8 and MAPK1) and their target genes CREB5, PRDX6, and BAD in the MAPK pathway, as well as the key genes CACNAlD (CaV1), CACNA1A (CaV2), and CACNAlH (CaV3) (encoding for calcium voltage-gated channel subunits) and their targeting genes (PGC1A, HMGR, and FAS) in the calcium-AMK pathway were determined by RT-PCR analysis. The DUSP-MAPK signaling pathway is the major pathway involved in cell survival/death and anti-oxidization, and the calcium signaling pathway controls lipid and energy metabolism. As expected,

25HC3S increased expression of DUSP8 by 5-fold and its targeting gene, CREB5, by up to 20-fold, which is the key element involved in cell survival and death (FIGS. **3**A and **3**B). Meanwhile, 25HC3S treatment significantly increased expression of key genes involved in the calcium signaling pathway, and its down-stream element, PGC1A, by 12-fold, while it decreased expression of HMGR and FAS genes by ~90%, which encode the key enzymes controlling energy metabolism in mitochondria, cholesterol biosynthesis, and fatty acid biosynthesis, as shown in FIGS. **3**C and **3**D.

[0083] Transcriptional Array Analysis in Hepatocytes

[0084] To examine the effect of 25HC3S on whole gene expression in human hepatocytes, human Genome U133Aplus2.0 Genechip® array analysis of 38,500 full length genes and EST (expressed sequence tags) clusters showed that treatment with 25HC3S in HpG-2 cells significantly modulated many clusters of gene expressions. The major clusters affected are genes involved in cholesterol and triglyceride metabolism, cell survival, and inflammation. Genes associated with cholesterol and triglyceride biosynthesis were significantly down-regulated, while genes associated with cell survival, proliferation, and anti-oxidization were significantly upregulated as shown in FIG. **4**. Altogether, 25HC3S modulated the transcription of 1,276 genes (>1.6 fold) in a time-dependent manner. Genetic analysis of different GO processes, a collection of genes associated with a specific biological functional process, revealed that at 8 hours, the majority of up-regulated pathways are involved in cell survival (FIGS. **4**A and B); in contrast, majority of down-regulated genes are involved in lipid metabolism (FIGS. **4**C and D). The up-regulated genes related with anti-apoptosis (increased by 3 to 12-fold at 8 hours) are listed in FIG. **4**E; and the down-regulated genes related with lipid metabolism (decreased by 50% to 95%) are listed in FIG. **4**F. The detailed individual up-regulated genes are listed in Table 1.7; the down-regulated genes are listed in Table 1.8. Many studies have shown that epigenetic modification could globally regulate gene expression involved in vital cellular functions, including metabolism, inflammation, and cell death/proliferation. Our data demonstrates that 25HC3S epigenetically regulates gene expressions via DNA ^{5m}CpG demethylation in promoter regions.

TABLE 1.7

Up Regulated Gene List of Huh-7 Cells Treated by 25HC3S for 8 hours.

Gene Symbol	Fold Change	Gene Name or Function
IL8	11.91	Interleukin 8
ANKRD1	8.67	Ankyrin Repeat Domain 1
FSTL3	8.24	Follistatin like 3
CYR61	8.08	Cysteine rich angiogenic inducer 61
EDN1	8.03	Endothelin 1
C11orf96	6.30	Description: chromosome 11 open reading frame 96
BIRC3	5.81	Baculoviral IAP repeat containing 3
IL11	5.53	Interleukin 11
HBEGF	4.43	Heparin binding EGF like growth factor
CYP24A1	4.33	Cytochrome P450 family 24 subfamily A member 1
SERPINE1	4.20	Serpin family E member 1
DDIT3	4.10	DNA damage inducible transcript 3
ATF3	4.08	Activating transcription factor 3
HSPA6	3.92	Heat shock protein family A (Hsp70) member 6
TNS1	3.89	Tensin 1
DUSP1	3.88	Dual specificity phosphatase 1
KLF5	3.88	Kruppel like factor 5
THBS1	3.82	Thrombospondin 1
SLC2A14	3.73	Solute carrier family 2 member 14
PMAIP1	3.65	Phorbol-12-myristate-13-acetate-induced protein 1
CXCL2	3.63	Chemokine (C-X-C motif) ligand 2
KRTAP3-1	3.49	Keratin associated protein 3-1
SKIL	3.36	SKI like proto-oncogene
AKAP12	3.30	A-kinase anchoring protein 12
TCIM	3.29	Transcriptional and immune response regulator
ICAM1	3.22	Intercellular adhesion molecule 1
GABBR1	3.20	Gamma-aminobutyric acid type B receptor subunit 1
UBASH3B	3.15	Ubiquitin associated and SH3 domain containing B
SOCS2	3.15	Suppressor of cytokine signaling 2
CREB5	3.12	cAMP responsive element binding protein 5

TABLE 1.8

Down Regulated Gene List of Huh-7 Cells Treated by 25HC3S for 8 hours.

Gene Symbol	Percentage Change (%)	Gene name or function
SC4MOL	−81.12	Methylsterol monooxygenase 1
SLCO4C1	−72.30	Solute carrier organic anion transporter family member 4C1
HMGCR	−71.32	3-hydroxy-3-methylglutaryl-CoA reductase
PNPLA3	−70.45	Patatin like phospholipase domain containing 3
ANKRD43	−69.19	Sosondowah ankyrin repeat domain family member A
HMGCS1	−67.71	3-hydroxy-3-methylglutaryl-CoA synthase 1
TUBB8	−67.29	Tubulin beta 8 class VIII
IDI1	−66.48	Isopentenyl-diphosphate delta isomerase 1
TNFSF10	−65.42	TNF superfamily member 10
NCMAP	−65.26	Non-compact myelin associated protein
RHOBTB1	−64.85	Rho related BTB domain containing 1
EHHADH	−64.46	Enoyl-coa hydratase and 3-hydroxyacyl coa dehydrogenase
SQLE	−64.42	Squalene epoxidase
PCSK9	−62.51	Proprotein convertase subtilisin/kexin type 9
KANK4	−61.28	KN motif and ankyrin repeat domains 4
SPTLC3	−60.32	Serine palmitoyltransferase long chain base subunit 3
PAQR8	−60.05	Progestin and adipoq receptor family member 8
RALGPS1	−59.86	Ral GEF with PH domain and SH3 binding motif 1
MAP2K6	−59.78	Mitogen-activated protein kinase kinase 6

TABLE 1.8-continued

Down Regulated Gene List of Huh-7 Cells Treated by 25HC3S for 8 hours.

Gene Symbol	Percentage Change (%)	Gene name or function
ZNF385B	−58.25	Zinc finger protein 385B
PLPPR1	−57.91	Phospholipid phosphatase related 1
SEC16B	−57.72	SEC 16 homolog B, endoplasmic reticulum export factor
ID3	−57.51	Inhibitor of DNA binding 3, HLH protein
VAV3	−57.09	Vav guanine nucleotide exchange factor 3
KLLN	−56.31	Killin, p53 regulated DNA replication inhibitor
SCN1A	−56.24	Sodium voltage-gated channel alpha subunit 1
PLA2G12B	−56.10	Phospholipase A2 group XIIB
FRMD3	−55.75	FERM domain containing 3
ID4	−55.58	Inhibitor of DNA binding 4, HLH protein
SLCO2B1	−55.27	Solute carrier organic anion transporter family member 2B1
KLB	−54.22	Klotho beta
FABP1	−54.20	Fatty acid binding protein 1
SORBS2	−53.92	Sorbin and SH3 domain containing 2
POU2AF1	−53.59	POU class 2 homeobox associating factor 1
METTL7A	−53.26	Methyltransferase like 7A
RAB11FIP4	−53.16	RAB11 family interacting protein 4
MAT1A	−53.04	Methionine adenosyltransferase 1A
CELSR2	−53.00	Cadherin EGF LAG seven-pass G-type receptor 2
AGTR1	−52.98	Angiotensin II receptor type 1
ELOVL6	−52.72	ELOVL fatty acid elongase 6
MVK	−52.63	Mevalonate kinase
CYB5B	−52.60	Cytochrome b5 type B
CYP51A1	−52.40	Cytochrome P450 family 51 subfamily A member 1
FDFT1	−52.07	Farnesyl-diphosphate farnesyltransferase 1
PRLR	−51.88	Prolactin receptor
ALAD	−51.76	Aminolevulinate dehydratase
PAQR9	−51.51	Progestin and adipoq receptor family member 9
FMC1	−51.27	Formation of mitochondrial complex V assembly factor 1 homolog
P2RY8	−50.91	P2Y receptor family member 8
TAB3	−50.37	TGF-beta activated kinase 1 (MAP3K7) binding protein 3
ADH6	−50.18	Alcohol dehydrogenase 6 (class V)
NAP1L5	−50.17	Nucleosome assembly protein 1 like 5
TMEM170B	−50.02	Transmembrane protein 170B

[0085] The Calcium, AMPK, and PPAR signaling pathways are ones involved in regulation of energy, lipids, and carbohydrate metabolisms. The Ca^{2+}/calmodulin-dependent protein kinase (CaMKK) and AMPK signaling pathway increases expression and decreases acetylation of PGC-1a, which regulates mitochondrial biogenesis and lipid metabolism. The data shown in Example 1 from analysis of Whole Genome-Wide DNA Methylation (genomic level) and transcriptional Array of Human Genome U133Aplus2.0 Genechip® (mRNA level) showed that 25HC3S treatment significantly demethylated ^{5m}CpG in the promoter regions of key genes including calcium channels, as well as genes of CaMKK and AMPK, increased their expression, and modulated downstream elements. These results provided evidence that 25HC3S globally regulated metabolic pathways mainly via the Calcium-AMPK signaling pathway as shown in FIG. **5**. 25HC and 25HC3S are potent modulators in regulating DNA methylation. 25HC methylates CpG, and 25HC3S demethylates ^{5m}CpG, while also down- and up-regulating expression of the key genes. PGC-1a is a key regulator of mitochondrial biogenesis, oxidative phosphorylation, and mitochondrial antioxidant defense, and it is also responsible for maintaining metabolic homeostasis. PGC-1α expression is up-regulated by the CREB protein and the AMPK signaling pathway. The present finding shows that 25HC3S up-regulates expression of CREB and AMPK via demethylating ^{5m}CpG in their promoter regions, and subsequently increases intracellular PGC-1a levels (FIG. **3**), which provides a detailed mechanism for how 25HC3S functions as proposed in FIG. **5**. 25HC3S suppresses DNMTs activities and demethylates ^{5m}CpG in the key promoter regions. The demethylation up-regulates gene expression and increases MAPK-CREB signalings, which blocks cell apoptosis, induces cell proliferation. The demethylation also up-regulates calcium-AMPK signaling, resulting in inhibition of SREBP-1 activity by which inhibits fatty acid and triglyceride biosynthesis, and inhibition of HMGCR expression, decreases in cholesterol biosynthesis, and increases in the levels of malnonyl-CoA as shown FIG. **5**.

Conclusion

[0086] The oxysterol sulfate, 25-hydroxycholesterol-3-sulfate (25HC3S) has been shown in this example to play an important role in lipid metabolism, inflammatory response, and cell survival. Example 1 provides a study of the molecular mechanism by which 25HC3S functions as an endogenous epigenetic regulator. The kinetic study of epigenetic enzymes demonstrated that 25HC3S specifically inhibited DNA methyltransferases, DNMT1, DNMT3a, and DNMT3b with IC50=4.04, 3.03, and 9.05×10^{-6} M, respectively. In human hepatocytes, high glucose induces lipid

accumulation by increasing promoter CpG methylation of key genes involved in development of non-alcoholic fatty liver diseases (NAFLD). Using this model, 25HC3S converted the 5mCpG to CpG in the promoter regions of 1074 genes involved in 79 KEGG pathways. Expression of the demethylated genes, which are involved in the master signaling pathways, including MAPK-ERK, calcium-AMPK, and type II diabetes mellitus pathways, increased. Messenger RNA array analysis showed that the up-regulated genes encoding for key elements in keeping cell survival and the down-regulated genes encoding for key enzymes in decreasing lipid biosynthesis. The results shown in Example 1 indicate that the expression of these elements and enzymes are regulated by the demethylated signaling pathways, and 25HC3S DNA demethylation of 5mCpG in promoter regions is a potent regulatory mechanism.

Example 2

Objective

[0087] The objectives of this study were to determine the plasma pharmacokinetics of [4-^{14}C]-25HC3S-derived radioactivity in male Sprague Dawley rats, determine the routes of elimination and excretion mass balance of [4-^{14}C]-25HC3S-derived radioactivity in male Sprague Dawley rats, determine the tissue distribution and tissue pharmacokinetics of [4-^{14}C]-25HC3S-derived radioactivity using quantitative whole body autoradiography methods in male Sprague Dawley and Long Evans rats following a single intravenous (bolus) dose, and to provide plasma, urine, and fecal homogenate samples for metabolite profiling of [4-^{14}C]-25HC3S-derived radioactivity.

Study Design

[0088] Nine male Sprague Dawley rats (Group 1) were designated for the pharmacokinetic phase, 3 male Sprague Dawley rats (Group 2) for the excretion mass balance phase, and 7 male Sprague Dawley rats (Group 3) and 9 male Long Evans rats (Group 4) for the tissue distribution phase. All animals received a single intravenous dose of [^{14}C]-25HC3S at 10 mg/kg and a target radioactivity of 225 pCi/kg. Blood samples were collected from all Group 1 animals at approximately 0.083, 0.25, 0.5, 1, 2, 4, 8, 12, 24, 48, and 72 hours post-dose. Urine and feces were collected from all Group 2 animals periodically through 168 hours post-dose. At approximately 0.083, 0.5, 1, 4, 8, 24, and 168 hours post-dose for Group 3 and at approximately 0.083, 0.5, 1, 4, 8, 24, 168, 336, and 504 hours post-dose for Group 4, 1 animal/group/time point was anesthetized with isoflurane and a blood sample collected. Following blood collection, animals were euthanized by CO_2 inhalation and carcasses frozen in a dry ice/hexane bath for processing by quantitative whole body autoradiography. Whole blood, plasma, urine, feces, cage rinse, and cage wash were analyzed for total radioactivity by liquid scintillation counting.

Results and Key Findings

[0089] After a single intravenous (bolus) dose of [4-^{14}C]-25HC3S administered to rats at 10 mg/kg, the mean plasma C_0 was 25,900 ng-equiv./g, and AUC_{last} was 27,900 h*ng-equiv./g. The terminal elimination phase $T_{1/2}$ was 26.6 hours.

[0090] Based on the excretion data, approximately 100.2% of the dose administered was recovered over 168 hours in urine, feces, and cage rinse from rats following a single intravenous (bolus) dose of [4-^{14}C]-25HC3S at 10 mg/kg. The majority of the recovered radioactivity was in feces (83.0%), indicating that biliary excretion is the primary route of excretion in rats.

[0091] After a single intravenous (bolus) dose of [4-^{14}C]-25HC3S to male Sprague Dawley rats in Group 3 at 10 mg/kg, [4-^{14}C]-25HC3S and/or its metabolites were broadly distributed and detected by quantitative whole body autoradiography in all tissues except the eye (lens). Plasma concentrations were similar to those determined in the pharmacokinetics phase. The whole blood C_{max} was 8530 ng-equiv/g, and AUC_{last} was 25,200 h*ng-equiv./g. There was a negligible difference in plasma and whole blood exposure, as measured by the plasma:whole blood AUC_{last} ratio of 0.79, indicating that the 25HC3S partitioned equally into plasma and blood cells. The $T_{1/2}$ was 44.3 hours in plasma and 52.2 hours in whole blood; differences in plasma $T_{1/2}$ between the PK phase and the QWBA phase are due to the difference in blood collection time points.

[0092] The C_max and AUC_{last} for [4-^{14}C]-25HC3S-derived radioactivity were highest in the liver: up to 87,900 ng-equiv./g and 364,000 h-ng/g, respectively. Kidney (all sections), small intestine (wall), lung, and adrenal gland concentrations ranged from 43,200 ng-equiv./g to 13,600 ng-equiv./g, higher than the maximum plasma concentration of 12,400 ng-equiv./g. Thymus, bone (femur), uveal tract, fat, testes, and brain concentrations were lowest relative to the other tissues: <5000 ng-equiv./g (around 1500 ng-equiv./g). Remaining tissues had concentrations between 5000 and 10,800 ng-equiv./g. The T_{max} was most often 0.083 to 0.5 hours post-dose. Concentrations were below quantitation limit in all tissues except adrenal gland, harderian gland, liver, and small intestine by 168 hours post-dose. As calculated using AUC_{last}, the tissue:plasma ratios were high for liver and small intestine (wall) at 11.4 and 7.44, respectively. High liver and small intestine concentrations are consistent with extensive biliary (fecal) excretion following an intravenous dose. All other tissue:plasma ratios demonstrated limited affinity for remaining tissue types.

[0093] Administration of a single intravenous dose of [4-^{14}C]-25HC3S to male Long Evans rats at 10 mg/kg revealed no substantial difference in plasma or whole blood concentrations over the first 168 hours post-dose versus Sprague Dawley rats; plasma and whole blood concentrations were below quantitation limit in plasma and whole blood by 336 hours post-dose in pigmented animals. There appeared to be no difference in binding to pigmented or non-pigmented skin or the uveal tract; for all tissues, the concentrations were below quantitation limit by 168 hours post-dose.

[0094] Plasma, urine, and feces from rats were analyzed for determination of 25HC3S related radiolabeled materials. Samples were profiled using high performance liquid chromatography with radiodetection and metabolic characterization was performed using mass spectrometry and tandem mass spectrometry analysis.

[0095] Plasma pools were made from Group 1 rats at the 0.083, 0.25, 0.5, and 1-hour collection time points. From these Group 1 sample pools and from a Group 3 0.083-hour plasma sample, the largest component present in the 0.083- and 0.25-hour collections was attributed to the parent

25HC3S representing about 58% to 92% of the radioactivity. Three metabolites present at >10% of the radioactivity in the 0.5- and 1-hour collections were M14 (up to 15% relative observed intensity), M24 (up to 13% relative observed intensity), and M28 (up to 83% relative observed intensity). Among the time points with suitable radioactivity for metabolite profiling and characterization (up to 1 hour post-dose), approximately 54% of the exposure (AUC) to 25HC3S related radioactivity was attributable to 25HC3S, approximately 34% to M28, and the remainder to the minor metabolites.

[0096] Urine pools were prepared for Group 2 at 0 to 6 and 6 to 12 hours post-dose. The largest component present was attributed to the parent 25HC3S representing about 78% to 93% of the radioactivity. A total of 4 metabolites were identified, although no metabolites were present at >1.2% of dose or >10% relative observed intensity. Four metabolites present at <10% relative observed intensity in at least 1 sample were M7 (<5% relative observed intensity), M16 (<3% relative observed intensity), M19 (<6% relative observed intensity), and M25 (<5% relative observed intensity).

[0097] Feces pools were prepared for Group 2 at 0 to 12, 12 to 24, and 24 to 48 hours post-dose.

[0098] A total of fourteen metabolites were identified. Four metabolites present at >5% of dose were M1 (21% of dose and 23% to 30% relative observed intensity), M2 (7% of dose and 4% to 12% relative observed intensity), M3 (15% of dose and 13% to 23% relative observed intensity), and M4 (8% of dose and 6% to 12% relative observed intensity). Parent 25HC3S was present at 2% of dose (1% to 5% relative observed intensity).

[0099] The primary metabolic pathways involved oxidation of 25HC3S resulting in the conversion of the sulfate group to a hydroxyl group followed by further oxidation to form bile acid structures related to deoxycholic acid and cholic acid or their isomers. In addition, glutathione conjugation of deoxycholic acid (or an isomer of deoxycholic acid) was suggested by the presence of a metabolite having the corresponding molecular weight for that structure. Neither desmosterol sulfate nor 25-hydroxycholesterol was detected in any of the plasma, urine, or feces samples.

Example 3

[0100] After a single oral (gavage) dose of [^{14}C]-25HC3S administered to rats at 75 mg/kg, plasma C_{max} was 3800 ng equiv./g, and AUC_{last} was 96,400 h-ng equiv./g. The terminal elimination phase $T_{1/2}$ was 27.3 hours.

[0101] Based on the excretion data, approximately 94.5% of the dose administered was recovered in urine, feces, and cage rinse from rats following a single oral (gavage) dose of [^{14}C]-25HC3S at 75 mg/kg. The majority of the recovered radioactivity was in feces (94.2%), indicating that biliary excretion is the primary route of excretion for absorbed 25HC3S in rats.

[0102] After a single oral (gavage) dose of [^{14}C]-25HC3S to male Sprague Dawley rats at 75 mg/kg, [^{14}C]-25HC3S and/or its metabolites were broadly distributed and detected by quantitative whole body autoradiography in all tissues except the eye (lens). No [^{14}C]-25HC3S-derived radioactivity was detected in the eye (lens). Plasma concentrations were similar to those determined in the pharmacokinetics phase, and were above the lower limit of quantitation. The whole blood C_max was 2850 ng equiv/g, and AUC_{last} was 127,000 h-ng equiv./g. There was a negligible difference in plasma and whole blood exposure, as measured by the plasma:whole blood AUC_{last} ratio of 1.12, indicating that the 25HC3S partitioned approximately equally into plasma and blood cells.

[0103] For the tissues analyzed by quantitative whole-body autoradiography, the C_{max} for [^{14}C]-25HC3S-derived radioactivity, where measurable, was highest in the small intestine (wall) followed by the stomach (wall): 424,000 ng equiv./g and 204,000 ng equiv./g, respectively. Pancreas and liver concentrations ranged from 23,500 ng equiv./g to 28,100 ng equiv./g. Uveal tract and brain concentrations were lowest relative to the other tissues and were approximately 1000 ng equiv./g. Skin, thymus, prostate, and pituitary tissue concentrations were <3000 ng equiv./g. Remaining tissues had concentrations between 3600 ng-equiv./g and 10,700 ng equiv./g. The T_{max} was 6 hours post-dose or less. By 168 hours post-dose, tissue concentrations were near or below the quantitation limit in all tissues except adrenal gland and liver. As calculated using AUC_{last}, the tissue: plasma ratios were highest for the small intestine (wall, 15.4) followed by the liver and adrenal gland at 6.96 and 6.64, respectively. High liver and small intestine concentrations are consistent with oral administration and biliary (fecal) excretion. All other tissue:plasma ratios demonstrated limited affinity for remaining tissue types.

[0104] Radiolabeled components in plasma and feces extracts were profiled and identified using radio-high performance liquid chromatography (HPLC) and high performance liquid chromatography/mass spectrometry (HPLC/MS) methods.

[0105] There were no urine samples that contained sufficient radioactivity to require metabolite profiling and identification.

[0106] Plasma pools were prepared for Group 1 (75 mg/kg, [^{14}C]-25HC3S) samples collected at 2, 4, and 6 hours post-dose. In the 2 hour post-dose plasma, the primary radiolabeled component was parent 25HC3S which was present at 63% relative observed intensity (ROI) and a concentration of 2090 ng-equiv./g. One metabolite M29 was identified as 25-hydroxycholesterol with 37% ROI and a concentration of 1233 ng-equiv./g. The plasma collections at 4 and 6 hours post-dose did not contain sufficient concentrations for radioprofiling.

[0107] Feces pools were prepared for Group 2 (75 mg/kg, [^{14}C]-25HC3S) samples collected from 0 to 24, 24 to 48, 48 to 72, 72 to 96, 96 to 120, 120 to 144, and 144 to 168 hours post-dose. A total of eleven metabolites were identified. None of the metabolites were present at >5% of dose. Metabolites present at 2-5% of dose were M1 (4.5% of total dose and 1%-69% ROI), M3 (4.6% of total dose and 1%-44% ROI), M4 (2.0% of total dose and 0%-10% ROI), M8 (3.1% of total dose and 1%-46% ROI), M29 (1.9% of total dose and 0%-2% ROI), and M30 (3.3% of total dose and 0%-5% ROI). The primary radiolabeled component was parent 25HC3S which was present at 71.1% of total dose (0%-88% ROI).

[0108] Radiolabeled desmosterol sulfate was not found in any of the plasma or feces samples.

[0109] The primary metabolic pathways involved oxidation of 25HC3S, resulting in the conversion of the sulfate group to a hydroxyl group followed by further oxidation to form bile acid structures related to deoxycholic acid and cholic acid or their isomers and 25-hydroxycholesterol.

[0110] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

[0111] Accordingly, the preceding merely illustrates the principles of the invention. It will be appreciated that those skilled in the art will be able to devise various arrangements which, although not explicitly described or shown herein, embody the principles of the invention and are included within its spirit and scope. Furthermore, all examples and conditional language recited herein are principally intended to aid the reader in understanding the principles of the invention and the concepts contributed by the inventors to furthering the art, and are to be construed as being without limitation to such specifically recited examples and conditions. Moreover, all statements herein reciting principles, aspects, and embodiments of the invention as well as specific examples thereof, are intended to encompass both structural and functional equivalents thereof. Additionally, it is intended that such equivalents include both currently known equivalents and equivalents developed in the future, i.e., any elements developed that perform the same function, regardless of structure. Moreover, nothing disclosed herein is intended to be dedicated to the public regardless of whether such disclosure is explicitly recited in the claims.

[0112] The scope of the present invention, therefore, is not intended to be limited to the exemplary embodiments shown and described herein. Rather, the scope and spirit of present invention is embodied by the appended claims. In the claims, 35 U.S.C. § 112(f) or 35 U.S.C. § 112(6) is expressly defined as being invoked for a feature in the claim only when the exact phrase "means for" or the exact phrase "step for" is recited at the beginning of such feature in the claim; if such exact phrase is not used in a feature in the claim, then 35 U.S.C. § 112(f) or 35 U.S.C. § 112(6) is not invoked.

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1. A method of treating at least one of cancer related to Epstein-Barr virus and non-cancerous transformation related to Epstein-Barr virus in a subject in need thereof, comprising:

administering to the subject an effective amount of at least one compound selected from 25-hydroxycholesterol-3-sulfate (25HC3S), 25-hydroxycholesterol-disulfate (25HCDS), 27-hydroxycholesterol-3-sulfate (27HC3S), 27-hydroxycholesterol-disulfate (27HCDS), 24-hydroxycholesterol-3-sulfate (24HC3S), 24-hydroxycholesterol-disulfate (24HCDS), and 24,25-epoxycholesterol-3-sulfate, or salt thereof.

2. The method of claim 1, wherein the at least one of cancer related to Epstein-Barr virus and non-cancerous transformation related to Epstein-Barr virus is at least one cancer related to Epstein-Barr virus.

3. The method of claim 2, wherein the at least one cancer comprises at least one of Hodgkin's lymphoma, soft tissue sarcoma, leiomyosarcoma, nasopharyngeal carcinoma, Burkitt's lymphoma, T-cell lymphoma, gastric carcinoma, invasive breast cancer, and hierarchically organized carcinoma.

4. The method of claim 3, wherein the hierarchically organized carcinoma comprises at least one of pancreatic ductal adenocarcinoma, urothelial cancer, colorectal cancer, head and neck cancer, non-small cell lung cancer, esophagus cancer, breast cancer, thyroid cancer, oral cancer, cervical cancer, ovarian cancer, and liver cancer.

5. The method of any one of claims 1 to 4, wherein the method comprises administering to the subject an effective amount of 25-hydroxycholesterol-3-sulfate (25HC3S) or salt thereof.

6. The method of any one of claims 1 to 5, wherein the at least one compound is administered in an amount selected from the group consisting of:

(a) an amount ranging from 0.001 mg/kg/day to 100 mg/kg/day;

(b) an amount ranging from 0.1 mg/kg to 100 mg/kg, based on body mass of the subject; and

(c) an amount ranging from 1 mg/kg to 10 mg/kg, based on body mass of the subject.

7. The method of any one of claims 1 to 6, wherein the administering is performed from once to 3 times per day.

8. The method of any one of claims 1 to 7, wherein the administering comprises at least one of oral administration, enteric administration, sublingual administration, transdermal administration, intravenous administration, peritoneal administration, parenteral administration, administration by injection, subcutaneous injection, and intramuscular injection.

9. The method of any one of claims 1 to 8, wherein the administering comprises administering a pharmaceutical composition comprising the at least one compound and a physiologically acceptable excipient, diluent, or carrier.

10. The method of claim 9, wherein the pharmaceutical composition is formulated in unit dosage form.

11. The method of any one of claims 9 or 10, wherein the pharmaceutical composition is in solid form.

12. The method of any one of claims 9 to 11, wherein the pharmaceutical composition: (a) is in the form of a powder, a tablet, a capsule, or a lozenge; and/or (b) comprises the at least one compound in freeze-dried form together with a bulking agent.

13. The method of claim 9 or 10, wherein the pharmaceutical composition comprises a carrier that is a liquid.

14. The method of claim 13, wherein the at least one compound is solubilized in the liquid or dispersed in the liquid.

15. The method of claim 13 or 14, wherein: (a) the liquid is aqueous; or (b) the liquid is sterile water for injections or phosphate-buffered saline.

16. The method of any one of claims 9 and 13 to 15, wherein the pharmaceutical composition is in a sealed vial, ampoule, syringe, or bag.

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