Supplemental Data

Manuscript title:

REV-ERBα Regulates CYP7A1 through Repression of Liver Receptor Homolog-1

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Journal name:

Drug Metabolism and Disposition

Materials and Methods

Materials

β-MCA and T-β-MCA were purchased from Steraloids Inc. (Newport, RI). CA, DCA, CDCA, T-CDCA, T-DCA, T-CA, succinylacetone, and cholesterol were purchased from Sigma-Aldrich (St. Louis, MO). 7α-hydroxy-4-cholesten-3-one was purchase from Aladdin (Shanghai, China). Antibodies used in immunoblotting were as follows: anti-Rev-erbα (WH0009572M2, Sigma-Aldrich, MO, USA), anti-Cyp7a1/CYP7A1 antibody (MABD42, Millipore, Bedford, MA), anti-Cyp8b1 (ab191910, Abcam, Cambridge, MA), anti-Cyp27a1 (ab126785, Abcam, Cambridge, MA), anti-Pepck (ab40843, Abcam, Cambridge, MA), anti-Bmal1 (sc-365645, Santa Cruz Biotechnology, SantaCruz, CA), anti-E4bp4 (sc-9550, Santa Cruz Biotechnology, SantaCruz, CA), anti-Lrh-1/LRH-1 (AP21181c, Abgent, San Diego, CA, USA) anti-Gapdh/GAPDH (ab9484, Abcam, Cambridge, MA), and anti-β-actin (ab8226, Abcam, Cambridge, MA). For the ChIP studies, antibody against Rev-erbα (E1Y6D) was purchased from Cell Signaling Technology (Beverly, MA) and normal rabbit IgG from Cell Signaling Technology (Beverly, MA). Male C57BL/6 mice were obtained from Beijing HFK Bioscience Co., Ltd. (Beijing, China). Lrh-1 floxed mice (kindly gifted from Dr. Bruce Murphy, University of Montreal, Canada) were crossed with serum albumin-Cre mice (Model Animal Research Center of Nanjing University, China) and then further intercrossed to generate hepatocyte-specific Lrh-1 knockout (Alb-Cre;$Lrh1^{fl/fl}$) and wild-type ($Lrh1^{fl/fl}$) mice on a pure C57BL/6J background. Studies were performed in accordance with institutionally approved animal protocols.

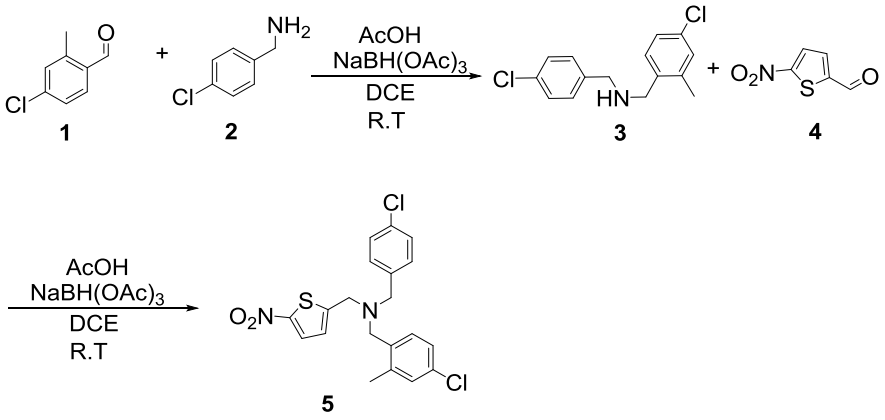
Plasmids

pGL4.35 [luc2P/9XGAL4UAS/Hygro] vector and pRL-TK vector were purchased from Promega (Madison, WI). GAL4-NR LBD construct, mBmal1(1 kb)-Luc, mCyp7a1(403)-Luc, mLrh-1(1 kb)-Luc, mLrh-1(700)-Luc, mLrh-1(500)-Luc, mLrh-1(250)-Luc, hLRH-1(1 kb)-Luc, hLRH-1(400)-Luc, hLRH-1(250)-Luc, pcDNA-mNr1d1, pcDNA-mNr1d2,

pcDNA-hNR1D1, pcDNA-hNR1D2, pcDNA-mlrh-1, and pcDNA-hLRH-1 were synthesized by Biowit Technologies (Shenzhen, China). The RevRE site mutated of Lrh-1/LRH-1 promoter construct were generated by polymerase chain reaction (PCR) amplification. The obtained fragments were cloned into the pGL4.11 plasmid (Promega, Madison, WI).

Synthesis and preparation of GSK2945

GSK2945 was synthesized as described previously (Trump et al., 2013). The reductive amination of 4-chloro-2-methylbenzaldehyde (1) with 4-chlorobenzylamine (2) and sodium triacetoxyborohydride gave secondary amine 2 (3). A second reductive amination with 5-nitro-2-thiophenecarboxaldehyde (4) gave tertiary amine 3 (5), namely, the final product GSK2945.



GSK2945

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, J = 4.2 Hz, 1H), 7.35 (m, 5H), 7.15 (m, 2H), 6.85 (m, 1H), 3.71 (brs, 2H), 3.59 (brs, 2H), 3.55 (d, J = 0.8 Hz, 2H), 2.27 (s, 3H). ^{13}C NMR (j-mod) (101 MHz, $CDCl_3$) δ 153.4, 151.0, 139.2, 136.4, 134.5, 133.5, 133.1, 130.8, 130.5, 130.3, 128.8, 128.7, 126.2, 124.6, 58.1, 55.7, 53.0, 19.4.

HRMS calcd for $C_{20}H_{19}N_2O_2Cl_2S$ $(M+H)^+$ requires m/z 421.054, found 421.045.

Pharmacokinetic studies

GSK2945 or SR8278 was formulated in 15% cremophor and administered to male C57BL/6 mice (22-24 g, *n* = 6 per each time point) at a dose of 10 mg/kg by

intraperitoneal injection. At each time point, 3 mice were rendered unconscious with isoflurane for blood and liver (0.08, 0.17, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10 and 12 h) sampling. The blood was collected by cardiac puncture. After washout of blood with ice-cold saline, the livers were rapidly removed, weighed, and stored at -80°C for further analysis.

The blood samples were centrifuged at 5,000 g (4°C) for 10 min and the plasma was transferred into Eppendorf (EP) tubes. A 100-µl aliquot of plasma sample was subjected to the deproteinization using 500-µl acetonitrile (containing SR9011 as the internal standard). After 3-min vortex, the sample mixture was centrifuged at 13,000 g (4°C) for 15 min. The resulting supernatant was collected and dried using Eppendorf Concentrator Plus (Hamburg, Germany). The dry residuals were re-dissolved in 200-µl of acetonitrile/water (50:50, v/v). After centrifugation (13,000 g, 15 min), a 5-µl aliquot of the supernatant was injected into the UPLC-QTOF/MS system (Waters, Milford, MA).

The livers were homogenized at a ratio of 1/2 (w/v) in saline solution. 0.2-ml of liver homogenate was mixed with 1-ml acetonitrile containing the internal standard SR9011. The mixture was vortexed for 3 min and subsequently subjected to 13,000 g centrifugation at 4°C for 15 min. The supernatant was collected and dried using Eppendorf Concentrator Plus (Hamburg, Germany). The dry residues were re-dissolved in 200-µl acetonitrile/water (50:50, v/v). After centrifugation (13,000 g, 15 min), the supernatant was subjected to UPLC-QTOF/MS analyses.

Drug quantification by UPLC-QTOF/MS

Concentrations of GSK2945 in plasma and liver samples were determined using a UPLC-QTOF/MS system consisting of Waters ACQUITY UPLC and Xevo G2 QTOF/MS (Liu et al., 2014). Chromatographic separation was performed on a BEH column (2.1 × 50 mm, 1.7 µm; Waters). A gradient elution was applied using formic acid (0.1%) in water (mobile phase A) versus acetonitrile (mobile phase B) at a flow rate of 0.45 ml/min. The gradient program consisted of 10% B at 0-0.5min, 10-95% B at 0.5-4.0 min, 95% B at 4.0-4.5 min, and 95-10 % B at 4.5-5.0 min. Data were collected at the positive scan mode using the electrospray ionization source (ESI). The capillary,

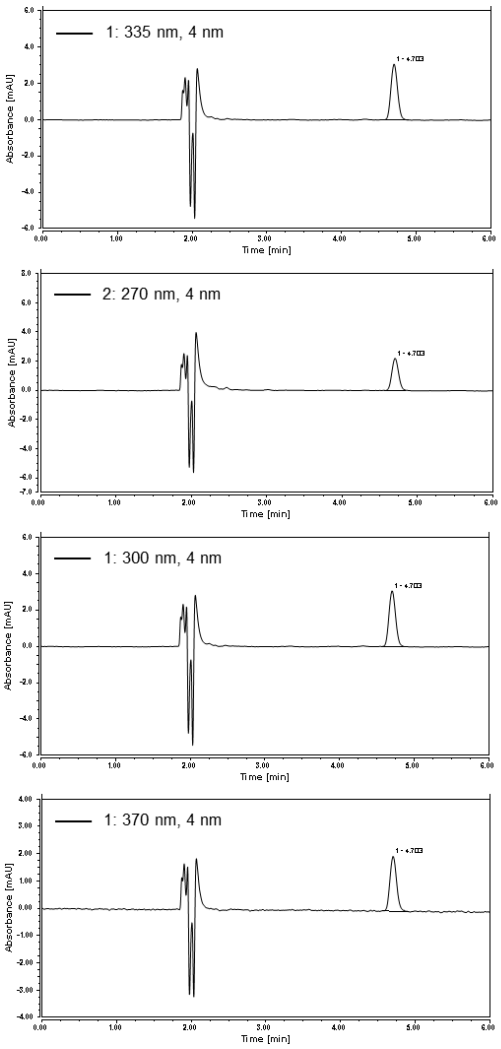
sampling cone, and extraction cone voltages were 3000, 20 and 4 V, respectively. The desolvation gas (nitrogen), and cone gas were set to 800 and 30 l/h, respectively. The desolvation and source temperature were 400°C, and 120°C, respectively. An external reference (lockspray) of 2 ng/ml leucine encephalin (*m/z* 556.2771) was infused at a rate of 2 μl/min to ensure the mass accuracy throughout an entire run.

References

Liu W, Liu HM, Sun H, Dong D, Ma ZG, Wang YF, and Wu BJ (2014) Metabolite elucidation of the Hsp90 inhibitor SNX-2112 using ultraperformance liquid chromatography/quadrupole time-of-flight mass spectrometry (UPLC-QTOF/MS). Xenobiotica 44:455-464.

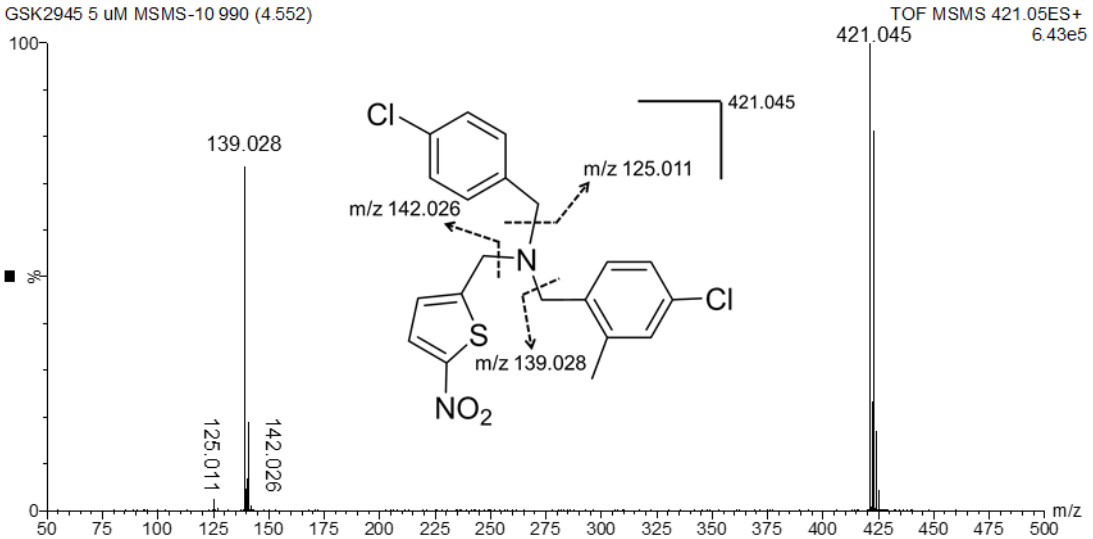
Trump RP, Bresciani S, Cooper AWJ, Tellam JP, Wojno J, Blaikley J, Orband-Miller LA, Kashatus JA, Boudjelal M, Dawson HC, Loudon A, Ray D, Grant D, Farrow SN, Willson TM, and Tomkinson NCO (2013) Optimized Chemical Probes for REV-ERB alpha. J Med Chem 56:4729-4737.

a

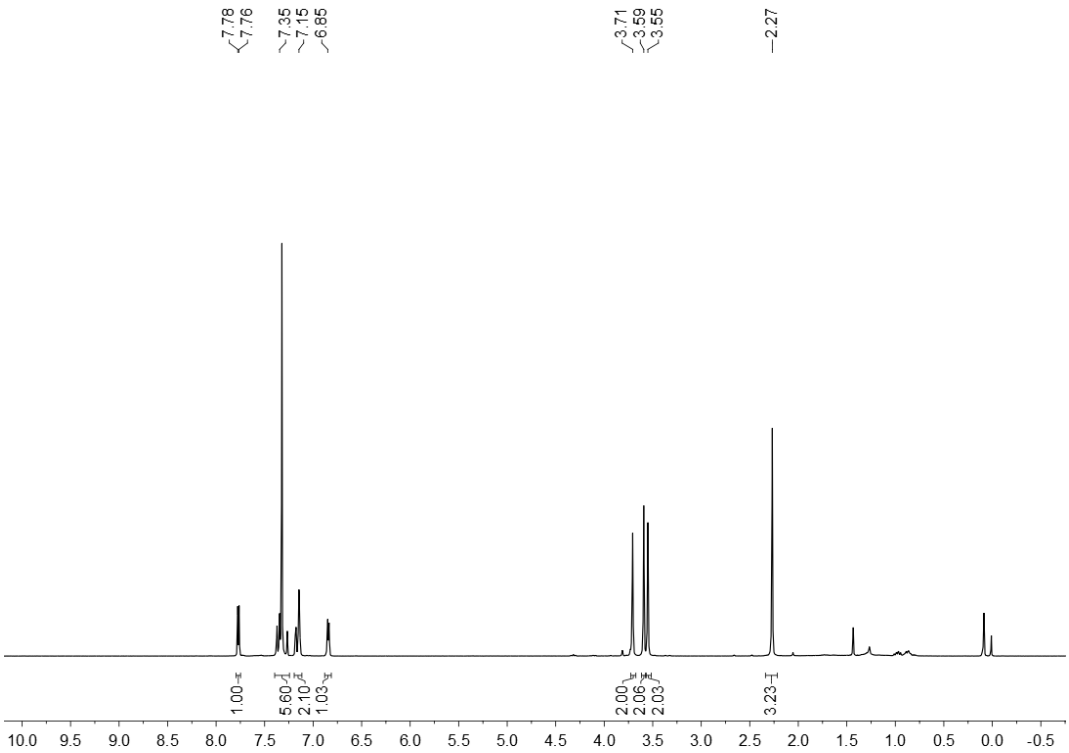


Mobile Phase A : B = 10 : 90
Gradient Time = 6 min
Flow Rate = 1 ml/min
Wavelength = 335 nm
PDA WL1:335 WL2:270 WL3:300 WL4:370
Solvent A = H_2O
Solvent B = ACN
Column: Accucore XL C18 250 × 4.6 mm

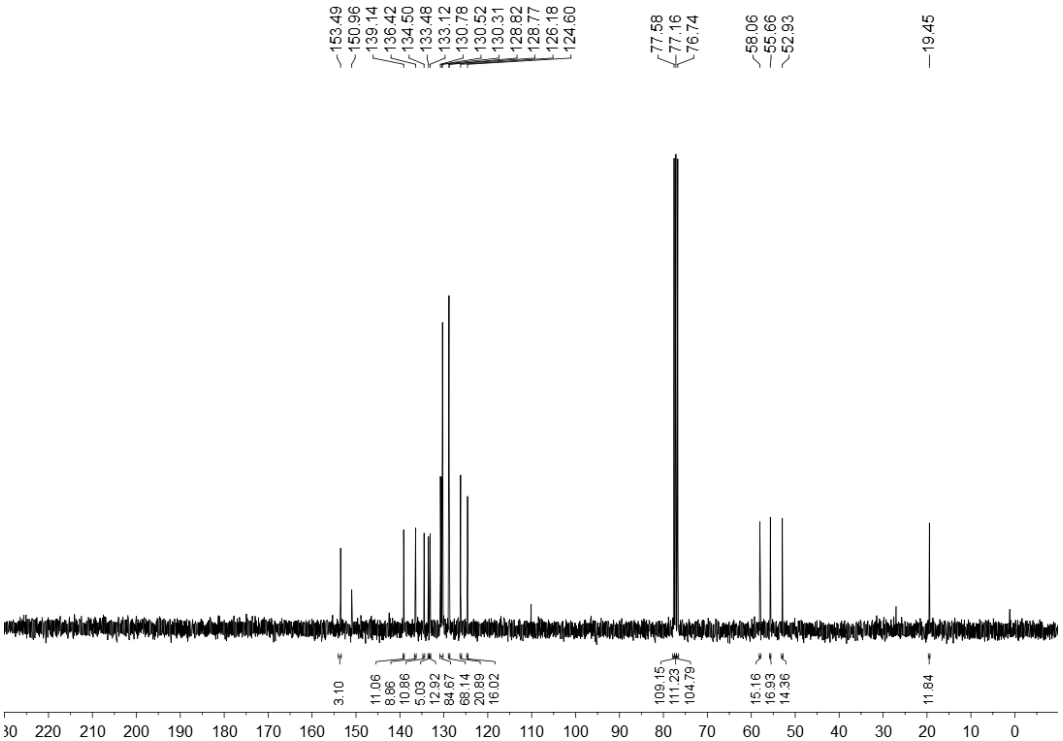
b



c

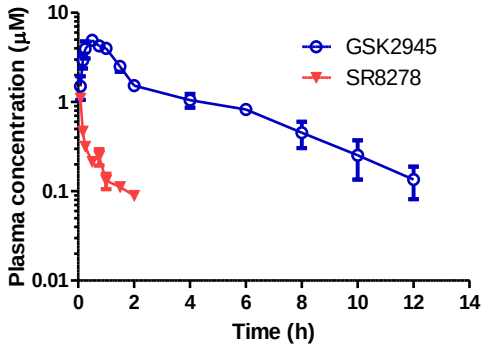


d



Supplementary Figure S1. Characterization of GSK2945. a, Sample purity of GSK2945 evidenced by HPLC. Four traces indicate data collected at distinct wavelengths (270, 300, 335 and 370 nm). Chemical identity of GSK2945 through b, MS/MS, c, ^{1}H-NMR and d, ^{13}C-NMR.

a



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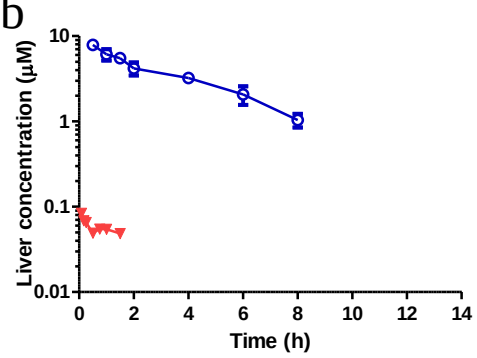
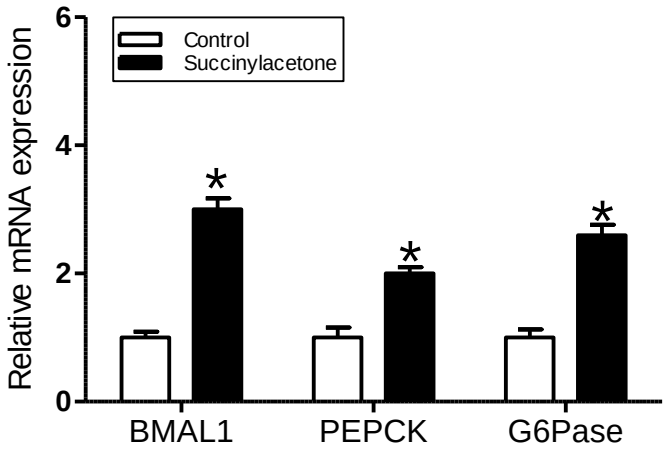


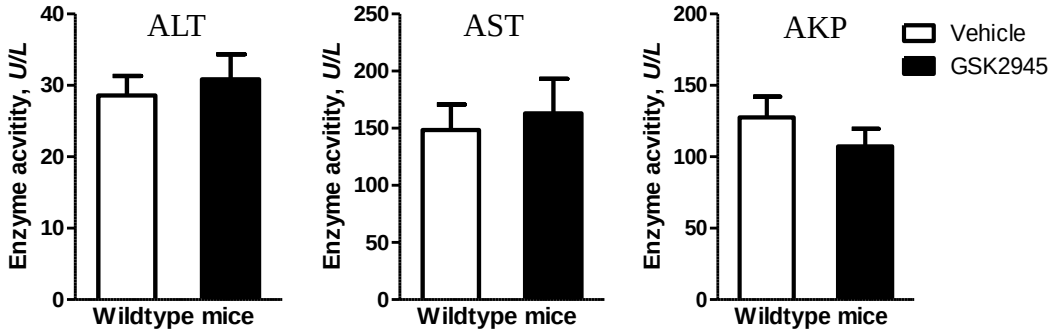
c

Parameter	Unit	Mean ± SD	
		GSK2945	SR8278
k_a	1/h	2.81 ±1.10	128 ± 51.4
k_{10}	1/h	0.68 ± 0.17	4.42 ± 1.21
k_{12}	1/h	1.12 ± 0.66	10.5 ± 3.08
k_{21}	1/h	0.86 ± 0.30	1.92 ± 0.36
$t_{1/2,\alpha}$	h	0.33 ± 0.17	0.05 ± 0.01
$t_{1/2,\beta}$	h	2.93 ± 0.92	1.36 ± 0.28
$t_{1/2,\ ka}$	h	0.28 ± 0.13	0.01± 0.01
V	L/kg	2.97 ± 0.74	4.18 ± 1.76
CL	L/h/kg	1.97 ± 0.38	16.1 ± 1.16
V_2	L/kg	3.56 ± 1.37	19.5 ± 2.50
CL_2	L/h/kg	3.00 ± 1.19	36.7 ± 9.38
T_{max}	h	0.56 ± 0.27	0.02 ± 0.01
C_{max}	μM	3.93 ± 0.88	1.69 ± 0.25
$AUC_{0\text{-}t}$	μM*h	11.6 ± 1.64	0.46 ± 0.04
$AUC_{0\text{-}inf}$	μM*h	12.3 ± 2.27	0.62 ± 0.05
MRT	h	3.86 ± 1.07	1.50 ± 0.26

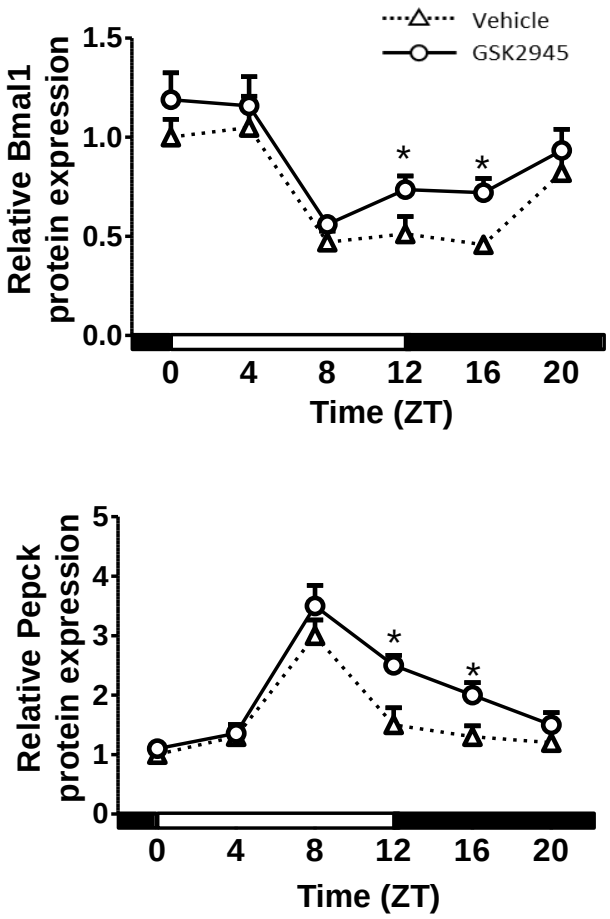
Supplementary Figure S2. Pharmacokinetic analyses of GSK2945 or SR8278 in mice (*n* = 6 per time point). a, plasma concentrations versus time profiles. b distribution of GSK26945 and SR8278 in mouse liver. c, pharmacokinetic parameters derived from fitting the plasma data with a two-compartment model.



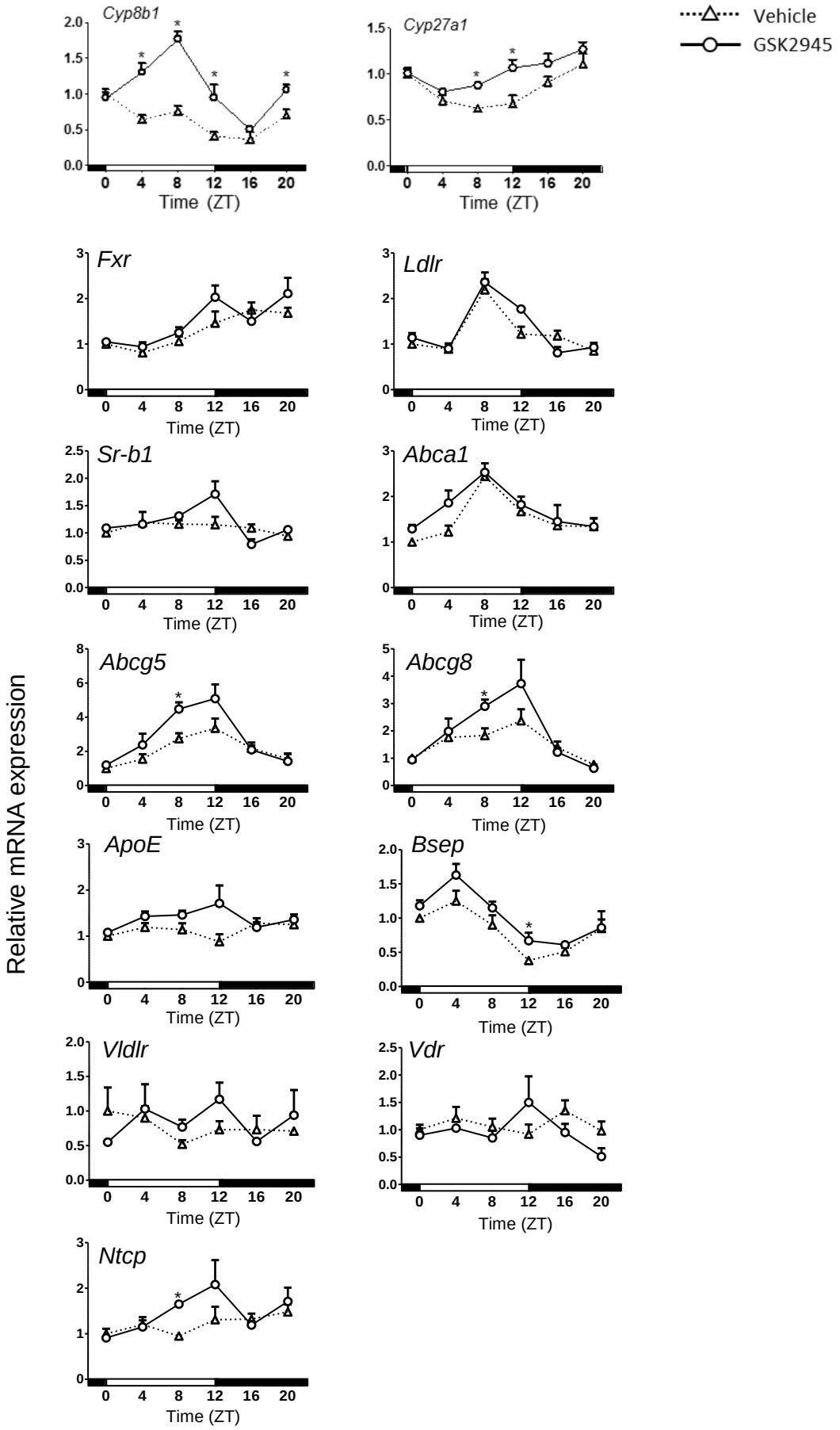
Supplementary Figure S3. Modulation of the expression of Rev-erbα target genes by succinylacetone (2 mM) in HepG2 cells. Gene expression was monitored by QPCR and normalized to GAPDH. *P < 0.05 versus vehicle control. Data are presented as mean ± SD (*n* = 3).



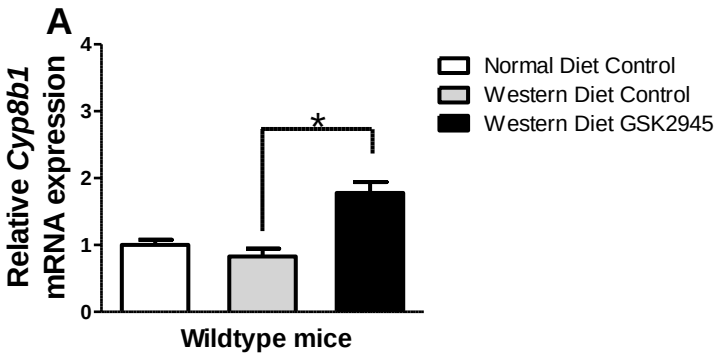
Supplementary Figure S4. Liver function tests of vehicle- versus GSK2945-treated mice. ALT, alanine transaminase; AST, aspartate aminotransferase; AKP, alkaline phosphatase. C57BL/6 mice were administered repeated doses of GSK2945 (10 mg/kg, i.p.) twice daily at ZT0 and ZT12 for 7 days. On day 8 (ZT12), groups of animals (n = 5) were sacrificed to collect blood. Plasma ALT, AST and AKP were determined by ALT kit, AST kit, and AKP kit (Jiancheng Bioengineering Institute, Nanjing, China), respectively.

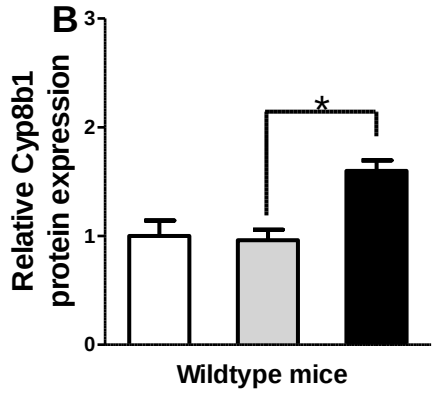


Supplementary Figure S5. GSK2945 increases hepatic protein expression of Bmal1 and Pepck in C57BL/6 mice. Mice were administered repeated doses of GSK2945 (10 mg/kg, i.p.) twice daily at ZT0 and ZT12 for seven days and groups of mice (*n* = 5) were sacrificed and protein expression was assessed by Western blotting. Data are presented as mean ± SEM (*n* = 5). *p < 0.05 versus vehicle control.

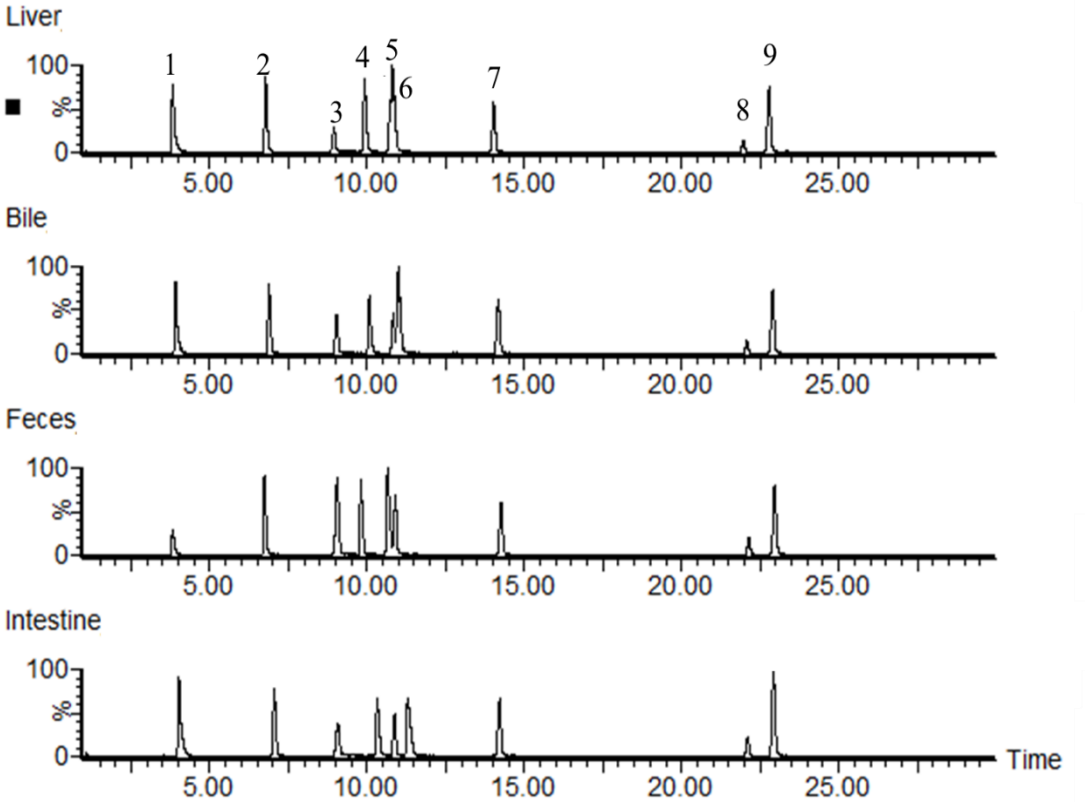

Vehicle
GSK2945
Relative mRNA expression
Cyp8b1
Cyp27a1
Fxr
Ldlr
Sr-b1
Abca1
Abcg5
Abcg8
ApoE
Bsep
Vldlr
Vdr
Ntcp
Time (ZT)
0 4 8 12 16 20

Supplementary Figure S6. Rev-erbα regulation of cholesterol-related genes in the liver. C57BL/6 mice were administered repeated doses of GSK2945 (10 mg/kg, i.p.) twice daily at ZT0 and ZT12 for 7 days and groups of mice (*n* = 5) were sacrificed and gene expression was assessed by qPCR. *P < 0.05 versus vehicle control. Data are presented as mean ± SEM (*n* = 5).

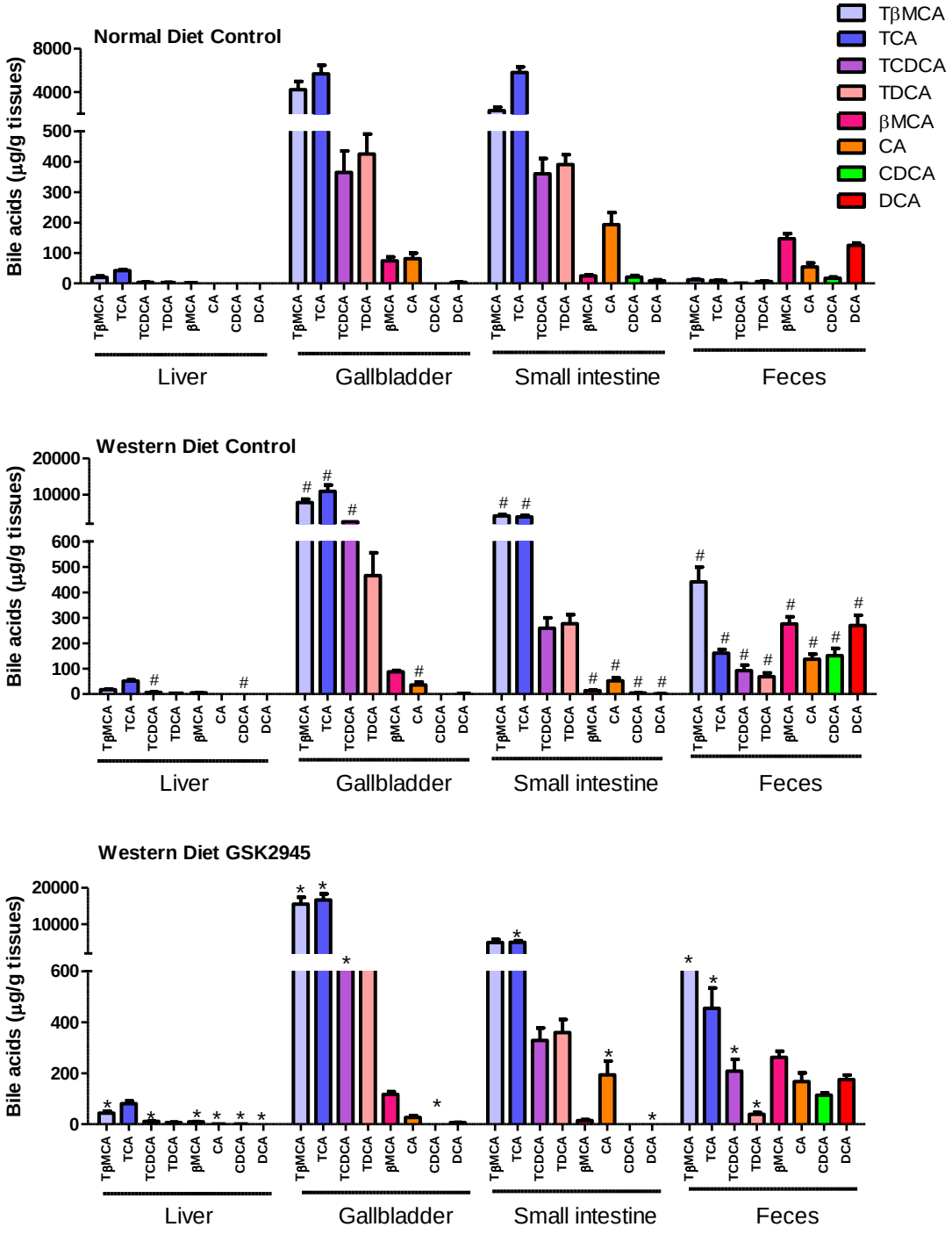




Supplementary Figure S7. GSK2945 treatment increases hepatic Cyp8b1 mRNA (**A**) and protein (**B**) expression in hypercholesterolemic mice. Mice were administered repeated doses of GSK2945 (10 mg/kg, i.p.) twice daily at ZT0 and ZT12 for 7 days.*p < 0.05 vehicle-treated versus GSK2945-treated hypercholesterolemic mice. Data are presented as mean ± SEM (*n* = 5)



Supplementary Figure S8. Representative chromatograms for quantification of 8 bile acids (BAs) in tissues and feces by UPLC-QTOF/MS. 1, T-β-MCA; 2, TCA; 3, dhCA(IS); 4, TCDCA; 5, TDCA; 6, β-MCA; 7, CA; 8, CDCA; 9, DCA. CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; β-MCA, β-muricholic acid; T, tauro-conjugated species; dhCA, dehydrocholic acid.



Supplementary Figure S9. Quantitative levels of various bile acids in liver, gallbladder, small intestine, and feces after vehicle- and GSK2945-treated mice by UPLC-QTOF/MS analyses. Data are presented as mean ± SEM (*n* = 5). # $p < 0.05$ Western diet versus normal diet; *$p < 0.05$ vehicle-treated versus GSK2945-treated hypercholesterolemic mice. CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; β-MCA, β-muricholic acid; T, tauro-conjugated species.

Supplementary Table S1. Mouse primer sequences for quantitative real-time PCR (qPCR)

Gene	Gene bank no.	Forward (5'-3' sequence)	Reverse (3'-5' sequence)
mAbca1	NM_013454.3	GTGGGCTCCTCCCTGTTTTT	TCTGAGAAACACTGTCCTCCTTTT
mAbcg5	NM_031884.2	GTCCTGCTGAGGCGAGTAAC	CGCCCTTTAGCGTGTTGTTC
mAbcg8	NM_026180.3	CTGTGGAATGGGACTGTACTTC	GTTGGACTGACCACTGTAGGT
mApoe	NM_001305844.1	CTGACAGGATGCCTAGCCG	CGCAGGTAATCCCAGAAGC
mBmal1	NM_007489.4	CTCCAGGAGGCAAGAAGATTC	ATAGTCCAGTGGAAGGAATG
mBsep	NM_021022.3	AAGCTACATCTGCCTTAGACACAGAA	CAATACAGGTCCGACCCTCTCT
mCyp27a1	NM_024264.5	GTGGACAACCTCCTTTGGGA	TTGCTCTCCTTGTGCGATGAA
mCyp7a1	NM_007824.2	AGCAACTAAACAACCTGCCAGTACTA	GTCCGGATATTCAAGGATGCA
mCyp8b1	NM_010012.3	GCCTTCAAGTATGATCGGTTCCT	GATCTTCTTGCCCGACTTGTAGA
mDbp	NM_016974.3	ACATCTAGGGACACACCCAGTC	AAGTCTCATGGCCTGGAATG
mDec2	NM_024469.2	CCCTCATTTGCAAGAGAGACAG	AGGTATCCTTGGTATCGTCTCG
mE4bp4	NM_017373.3	CTTTCAGGACTACCAGACATCCAA	GATGCAACTTCCGGCTACCA
mFxr	NM_001163700.1	GCACGCTGATCAGACAGCTA	CAGGAGGGTCTGTTGGTCTG
mHmgcr	NM_008255.2	ATGGCTGGGAGCATAGGCGG	CTGCATCCTGGCCACATGCG
mHnf4α	NM_008261.3	GACCATGGGCAATGACACGTCC	TTGCAGCCGTCACAGCTCGA
mInsig 2	NM_133748.2	CACGCCAGTGCTAAAGTAGAC	GGGTGACAACGGTTGCTAAG
mLdlr	NM_010700.3	CCGGAGTTGCAGCAGAAGA	TCAGGAATGCATCGGCTGAC
mLrh-1	NM_030676.3	GAACTGTCCAAAACCAAAAAAGG	CTTCCAGCTTCATCCCAAC
mLxra	NM_001177730.1	AGGAGTGTCGACTTCGCAAA	CTCTTCTTGCCGCTTCAGTTT
mNtcp	NM_011387.2	GAAGTCCAAAAGGCCACACTATGT	ACAGCCACAGAGAGGGAGAAAG
mPepck	NM_011044.2	ATGAAAGGCCGCACCATGTA	GCACAGATATGCCCATCCGA
mPparα	NM_013839.4	AGGCCGTTGCCACTGTTCAG	AGCCCTCTTCATCCCCAAGC
mRev-erbα	NM_145434.4	TTTTTCGCCGGAGCATCCAA	ATCTCGGCAAGCATCCGTTG
mRev-erbβ	NM_011584.4	GGAGTTCATGCTTGTGAAGGCTGT	CAGACACTTCTTAAAGCGGCACTG
mShp	NM_011850.3	CGATCCTCTTCAACCCAGATG	AGGGCTCCAAGACTTCACACA
mSr-b1	NM_016741.2	GGGAGCGTGGACCCTATGT	CGTTGTCATTGAAGGTGATGT
mSrebp2	NM_033218.1	CACAATATCATTGAAAAGCGCTACCGGTCC	TTTTTCTGATTGGCCAGCTTCAGCACCATG
mVdr	NM_009504.4	GCATCCAAAAGGTCATCGGC	AGCGCAACATGATCACCTCA
mVldlr	NM_001161420.1	CGAGAGTGTCAAAGGATCAATGT	CAAGAGAGGAAGGATGGCCC
mCyclophilin b	NM_011149.2	TCCACACCCTTTTCCGGTCC	CAAAAGGAAGACGACGGAGC

m：mouse

Supplementary Table S2. Human primer sequences for quantitative real-time PCR (qPCR)

Gene	Gene bank no.	Forward (5'-3' sequence)	Reverse (3'-5' sequence)
hCYP27A1	NM_000784.3	TCTGTGCCCTTTGGCTATGG	ACTTCTGGATCAGCCTTGCG
hCYP7A1	NM_000780.3	CGCAAGCAAACACCATTCCA	AGGATTGCCTTCCAAGCTGA
hCYP8B1	NM_004391.2	CTACCGCCTGCATCCTACAG	CGAGGACAGGCAGAACAGAG
hDBP	NM_001352.4	GGCGTCGGGTGTTTTGGTTT	CTCCTTCTACAAGGTGGGCG
hDEC2	NM_002591.3	TTAACCGCCTTAACCGAGCAA	AGTGGAACGCATCCAAGTCG
hE4BP4	NM_001289999.1	AGGGAAGCTGCAGAAGTCCTGAAA	AGTTGCTGGAGGATCGGTTGACTT
hFXR	NM_001206979.1	GGGTCTGCGGTTGAAGCTAT	GTCAGAATGCCCAGACGGAA
hHNF4α	NM_000457.4	TGCGACTCTCCAAAACCCTC	ATTGCCCATCGTCAACACCT
hINSIG2	NM_016133.3	AGTTGTGGGAGTGGAGGAGGAA	TCAAAGACTGACGCTTCAACGG
hLRH-1	NM_205860.2	CTTTGTCCCGTGTGTGGAGAT	GTCGGCCCTTACAGCTTCTA
hLXRα	NM_005693.3	AGACTTTGCCAAAGCAGGG	ATGAGCAAGGCAAACTCGG
hPPAR-α	NM_005036.4	GCAGCTGCAAGATCCAGAAAA	GTCCAAAACGAATCGCGTTG
hREV-ERB α	NM_005126.4	CCAACAACAACACAGGTGGCG	GGGGATGGTGGGAAGTAGGT
hSHP	NM_021969.2	CTCTTCCTGCTTGGGTTGGC	GCACATCGGGGTTGAAGAGG
hVDR	NM_001017536.1	CATCATTGCCATACTGCTGGAC	CTCCCTCCACCATCATTCACAC
hGAPDH	NM_002046.5	CATGAGAAGTATGACAACAGCCT	AGTCCTTCCACGATACCAAAGT

h: human

Supplementary Table S3. Oligonucleotide sequences for EMSA assays

Oligonucleotide	Forward (5'-3' sequence)
mBmal1-RevRE (+61/+91)	GATTGGTCGGAAAGTAGGTTAGTGGTGCGAC
mLrh1-RevRE(-186/-159)	GTCACTTTATAACAGGGTCCTCTTATCA
hBMAL1-RevRE(+44/+71)	GATTGGTCGGAAAGTAGGTTAGTGGTGC
hLRH1-RevRE(-99/-70)	TGTCACTTTATAACAGGGTCCTCTTATCAA

m: mouse; h: human

Supplementary Table S4. Primer sequences for ChIP assays

Gene	Forward (5'-3' sequence)	Reverse (3'-5' sequence)
mLrh-1	TGTGACCAGTTGAAGCCTATTGA	GCTCCAGTCCGTGTTCTCC
mBmal1	GGAAAGTAGGTTAGTGGTGCGAC	AAGTCCGGCGCGGGTAAACAGG
Non-specific region	GCAGAAGAACCCCTCCTTTTC	CATGACCCTGTGCCGGTT

m：mouse

Supplementary Table S5 Haematology test of vehicle- versus GSK2945-treated mice.

No significant differences were noted among any of the parameters examined consistent with no overt toxicity of GSK2945.

Parameters	Units	Vehicle	GSK2945
WBC	10^3/ul	3.35 ± 0.39	2.92 ± 0.41
RBC	10^6/ul	8.57 ± 0.15	8.86 ± 0.19
HGB	g/dl	14.7 ± 0.22	14.2 ± 0.21
HCT	%	40.2 ± 0.65	40.5 ± 0.60
MCV	fl	46.7 ± 0.20	46.7 ± 0.14
MCH	pg	17.3 ± 0.04	17.1 ± 0.08
MCHC	g/dl	35.6 ± 0.08	35.3 ± 0.14
PLT	10^3/ul	636 ± 57.6	681 ± 74.2
RDW	%	13.5 ± 0.07	13.5 ± 0.13
PCT	%	0.38 ± 0.02	0.40 ± 0.03
MPV	fl	5.98 ± 0.13	5.83 ± 0.14
PDW	%	16.0 ± 0.34	16.4 ± 0.40

C57BL/6 mice were administered repeated doses of GSK2945 (10 mg/kg, i.p.) twice daily at ZT0 and ZT12 for 7 days. On day 8 (ZT12), groups of animals (n = 5) were sacrificed to collect blood for assays. WBC, white blood cells; RBC, red blood cells; HGB, hemoglobin concentration; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelet count; RDW, red cell distribution width; PCT, plateletcrit; MPV, mean platelet volume; PDW, platelet distribution width. Data are presented as mean ± SEM (n = 5).