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GSK4112, a Small Molecule Chemical Probe for the Cell Biology of the Nuclear Heme Receptor Rev-erbα

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ABSTRACT The identification of nonporphyrin ligands for the orphan nuclear receptor Rev-erbα will enable studies of its role as a heme sensor and regulator of metabolic and circadian signaling. We describe the development of a biochemical assay measuring the interaction between Rev-erbα and a peptide from the nuclear receptor corepressor-1 (NCoR). The assay was utilized to identify a small molecule ligand for Rev-erbα, GSK4112 (**1**), that was competitive with heme. In cells, **1** profiled as a Rev-erbα agonist in cells to inhibit expression of the circadian target gene *bmal1*. In addition, **1** repressed the expression of gluconeogenic genes in liver cells and reduced glucose output in primary hepatocytes. Therefore, **1** is useful as a chemical tool to probe the function of Rev-erbα in transcriptional repression, regulation of circadian biology, and metabolic pathways. Additionally, **1** may serve as a starting point for design of Rev-erbα chemical probes with *in vivo* pharmacological activity.

Rev-erbα (NR1D1) and Rev-erbβ (NR1D2) are heme sensors and atypical members of the nuclear receptor superfamily. In response to fluctuations in heme levels in cells, the Rev-erbs repress the transcription of key genes involved in metabolic and circadian pathways (*1*, *2*). Expression of Rev-erbα is highest in liver, skeletal muscle, adipose tissue, and the brain (*3*). Both Rev-erbα and Rev-erbβ share many of the structural features characteristic of nuclear receptors but lack the critical C-terminal α-helix in their ligand binding domain (LBD) that is required for transcriptional activation (*4*). Consequently, the Rev-erbs function as constitutive repressors of transcription. Rev-erbα represses expression of its target genes by recruiting nuclear receptor corepressor-1 (NCoR) and the class I histone deacetylase 3 (HDAC3) to DNA response elements in the promoters of genes such as *g6pc*, *ppargc1a*, *apoc3*, and *bmal1* (*5*–*7*).

Many of the Rev-erbα target genes play important roles in regulation of metabolic processes. The catalytic subunit of glucose-6-phosphatase (G6 Pase), encoded by *g6pc*, mediates the final step in gluconeogenesis and plays a major role in the homeostatic regulation of blood glucose levels (*8*). PPARγ coactivator 1α (PGC1α) is a transcriptional coactivator of energy metabolism that regulates mitochondrial biogenesis, muscle fiber determination, and hepatic gluconeogenesis (*9*–*11*).

In addition, Rev-erbα regulates the expression of proteins that comprise the core circadian clock in mammals. Bmal1 is a basic helix–loop−helix transcription factor that dimerizes with CLOCK to form the positive

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limb of the circadian clock. This complex induces expression of the genes *cryptochrome* (*Cry)* and *period* (*Per)* that form the negative limb of the clock. The accumulation of PER and CRY proteins, in turn, leads to a repression of transcripts for *bmal1*, *clock*, and *reverba*, allowing the core loop and overall circadian cycle to propagate (*12*).

Rev-erbα knockout mice are viable but exhibit some metabolic phenotypes as well as disrupted circadian rhythmicity relative to wild type mice. Consistent with the role of Rev-erbα in the maintenance of normal hepatic physiology, Rev-erbα$^{-/-}$ mice have elevated apoC-III levels and increased serum triglycerides and VLDL levels (*6*). The oscillatory amplitude of *bmal1* expression is significantly attenuated in the knockout animals, and under constant darkness and constant light conditions the mice display shorter period length and greater period variability when compared with similarly entrained wild-type animals (*13*).

The characterization of the Rev-erbs as heme sensors was unprecendented among the other members of the nuclear receptor superfamily (*14*). The *Drosophila melanogaster* orthologue E75 was initially shown to be bound to heme by mass spectroscopy, UV–vis spectroscopy, and electron paramagnetic resonance (*15, 16*). Subsequent mass spectroscopy analysis of bacterially expressed Rev-erbα also showed heme bound to the protein. A shift in absorbance spectrum upon exposure to dithionite was consistent with reduction to the Fe(II) heme species that was functional in cells (*5*). A crystal structure of Rev-erbβ LBD in complex with Fe(III) heme and subsequent studies demonstrated that the receptor adopted multiple structural states, which were highly dependent on the iron oxidation state and coordination environment of the heme moiety (*17*).

The collective association of Rev-erbα activity with metabolic and circadian signaling prompted us to investigate the potential for pharmacological targeting of Rev-erbα as a means to influence the molecular clock and its crosstalk with metabolic processes such as hepatic gluconeogenesis. In addition, the identification of nonporphyrin ligands for Rev-erbα would aid the study of its molecular and biological function without the complicating factors associated with heme in biochemical and cellular assays.

We describe herein the development of a high-throughput screening assay for Rev-erbα ligands and the subsequent discovery of a series of tertiary amines, including GSK4112 (**1**). Our earlier report described the ability of **1** to phase-shift gene expression in synchronized cells that were engineered to stably express circadian reporter genes for *bmal1* and *per2* (*18*). We now describe the characterization of **1** as a functional Rev-erbα agonist in liver cells and its effect on repression of the hepatic gluconeogenesis pathway as evidence of its utility as a chemical probe for the cell biology of Rev-erbα.

RESULTS AND DISCUSSION

Several factors preclude the use of heme as a chemical probe for Rev-erbα, including its toxicity at higher concentrations and promiscuous interactions with other cellular targets such as p450 enzymes (*19, 20*). In an effort to identify small molecule nonporphyrin ligands of Rev-erbα, we developed a biochemical assay measuring the interaction between the LBD and a cofactor partner that could detect a ligand-induced conformational change in the receptor. Rev-erbα LBD was bacterially expressed, purified, and biotinylated. The isolated protein contained 1 equiv of Fe(III)-heme per receptor, presumably captured from the expression media (*5*). The protein was labeled with allophycocyanin and subjected to a fluorescence resonance energy transfer (FRET) peptide scan using biotinylated fragments from known NR cofactors conjugated with europium chelate (Supplementary Figure S1). Only peptides from the corepressors SMRT and NCoR showed a strong interaction with the receptor. A peptide representing the first interaction domain of NCoR (amino acids 2040 to 2065, NCoR ID1) was selected as the optimal partner for development of a FRET-based screening assay. Using this Rev-erbα LBD/NCoR ID1 FRET assay, the addition of hemin resulted in displacement of the peptide with an IC_{50} = 0.05 μM (*5*) (and data not shown). The observation that hemin, the Fe(III) form of heme, caused a decrease in the FRET signal suggested that the Rev-erbα—heme complex may contain Fe(II) under the reducing conditions of the assay and that the conformation of the receptor was sensitive to the oxidation state of the metal in the porphyrin ligand. Closely related observations have been made in Rev-erbβ, where the receptor structure was shown to be sensitive to the both the redox and coordination state of the iron (*17*).

Using the Rev-erbα biochemical assay we performed targeted screening of compound libraries synthesized around chemotypes known to bind to nuclear receptors

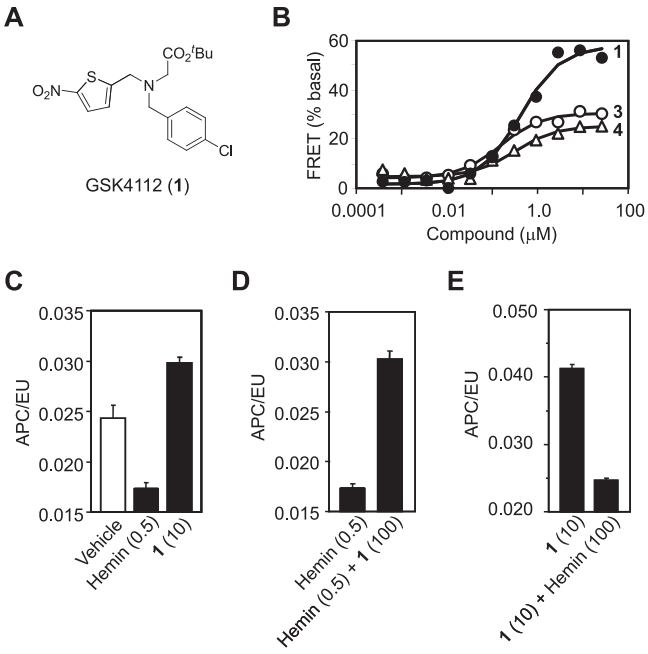
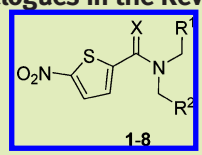


Figure 1. A) Chemical structure of GSK4112 (1). B) Representative curves for 1, 3, and 4 from the Rev-erbα/NCoR ID1 FRET assay. C–E) Competition of hemin and 1 for binding to Rev-erbα as measured in the FRET assay. Concentrations (μM) of hemin and 1 indicated in parentheses.

(*21*). One library, based on the tertiary amine chemotype found in the LXR agonist GW3965 (*10*), yielded several primary hits that increased the interaction between Rev-erbα and the corepressor peptide. Rescreening of the initial hits selected the nitrothiophene benzyl-substituted glycine ester GSK4112 (**1**) (Figure 1, panel A) as the most potent with EC_{50} = 0.4 μM (Figure 1, panel B).

To establish the structure–activity relationship for the binding of small molecules to Rev-erbα, we prepared a small array of tertiary amines related to **1** by one of two methods. Analogues of **1** could be synthesized by sequential reductive alkylation of *tert*-butyl glycine. Notably, the order of reaction was important for the chemistry to incorporate the nitrothiophene building block. 5-Nitrothiophenecarboxaldehyde could not be employed in the first reductive alkylation, as the resulting secondary amines were difficult to isolate. However, the desired products were successfully synthesized when 5-nitrothiophenecarboxaldehyde was utilized in the second of the reductive alkylation steps. In addition, use of 5-nitrothiophenecarboxylic acid in the second step resulted in the synthesis of the corresponding amide analogue **2**. As an alternative method of preparation, some of the tertiary amines were synthesized by a nucleophilic monoalkylation of a benzylic amine followed by a standard reductive alkylation. The resulting array was profiled in the Rev-erbα biochemical assay. EC_{50} values were obtained, and the maximal response for NCoR peptide recruitment relative to **1** was calculated (Table 1). The lack of activity of the tertiary

TABLE 1. Structure activity of 1 and its analogues in the Rev-erbα/NCoR ID1 FRET assay[a]



Compound	R^1	R^2	X	Rev-erbα EC_{50} (μM)	RE[b]
1	^{t}BuO$_2$C-	4-Cl-Ph-	none	0.4	1.0
2	^{t}BuO$_2$C-	4-Cl-Ph-	O	[c]	
3	Ph-	4-Cl-Ph-	none	0.2	0.4
4	Ph-	3,4-Cl$_2$-Ph-	none	0.3	0.4
5	MeO$_2$C-	4-Cl-Ph-	none	[c]	
6	Me$_2$N(O)C	4-Cl-Ph	none	>10	0.6

[a]All data ±15% with n = 3. [b]RE = relative efficacy where **1** = 1.0. [c]Inactive at 10 μM.

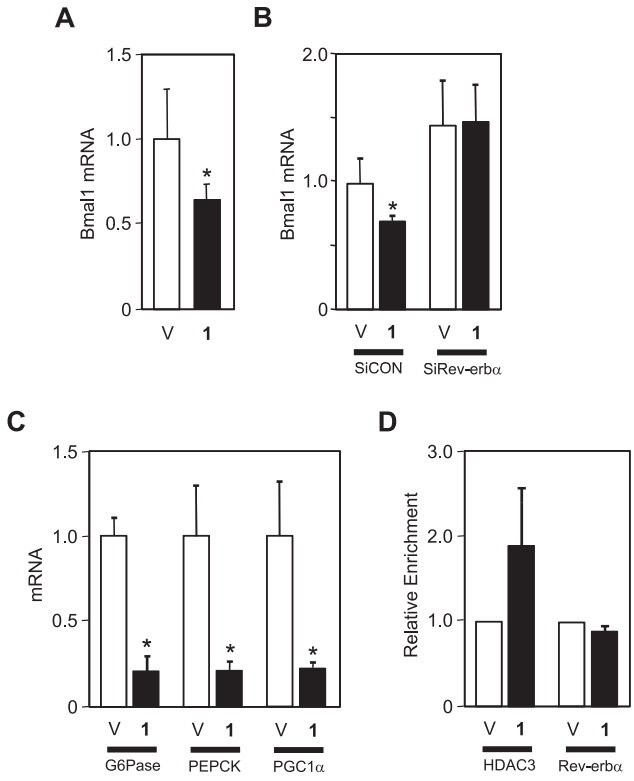


Figure 2. Repression of gene expression by 1 (10 μM) in HepG2 cells. The data were plotted as mean ± SD. *p-value <0.05 by a Student's t test. A) The value of Bmal1 mRNA was calculated using the standard curve method and normalized to the value of housekeeping gene control 18Srna. B) HepG2 cells were first transfected with pEntry-shβgal (Control shRNA) or pEntry-shRev (Rev-erbα shRNA), and 48 h after transfection, the mRNA levels of *bmal1* gene were measured and calculated. The experiment was repeated at least 3 times with similar results. C) The mRNA levels of G6 Pase, PEPCK, and PGC1α were calculated using the standard curve method and normalized to the value of housekeeping gene control 18Srna. D) Cells were harvested for ChIP analysis of HDAC3 and Rev-erbα recruitment to the *g6pase* promoter. The fold enrichment was calculated by setting the value of DMSO treatment as 1. The experiment was repeated twice with similar results

amide **2** suggested that Rev-erbα binding may be dependent on the conformational flexibility or basicity of the tertiary amine. Activity was maintained when the *tert*-butyl ester of **1** was replaced with a phenyl group in **3**, but notably, the maximal recruitment of NCoR was attenuated (Figure 1, panel B). Additional chlorine substitution, in **4**, led to a similar activity profile. The methyl ester analogue **5** was inactive, and the dimethylamide **6** had only partial activity at 10 μM, suggesting that the steric bulk of the *tert*-butyl ester was important for full Rev-erbα activation. In summary, the tertiary amine **1** and its analogues defined a series of nonporphyrin Rev-erbα ligands. Key features for activity were the presence of three lipophilic substituents on a basic amine. The ability to make small variations in the substituents while retaining activity demonstrated that **1** represents a tractable starting point for the development of new Rev-erbα ligands.

Addition of hemin has been shown to decrease NCoR peptide binding to Rev-erbα (*2, 5*). In contrast, the tertiary amine **1** increased interaction between Rev-erbα LBD and the NCoR ID1 peptide. Because hemin and **1** had opposite effects in our biochemical assay, we were intrigued to determine whether porphyrin and nonporphyrin Rev-erbα ligands would be competitive with each other for receptor binding. Using the Rev-erbα LBD/NCoR ID1 assay we studied the competition between hemin and **1**. In the presence of 0.5 μM hemin, recruitment of the NCoR1 ID1 peptide was reduced (Figure 1, panel C). In contrast, 10 μM **1** increased the interaction of the receptor with the peptide above the basal level, consistent with the data from the chemical array screening. Following saturation of the receptor with hemin, the tertiary amine **1** was able to reverse the suppression of the FRET signal (Figure 1, panel D). Likewise, a high concentration of hemin effectively antagonized the FRET signal generated by the Rev-erbα−**1** complex (Figure 1, panel E). The competitive actions of hemin and **1** in the FRET assay strongly suggested that their binding as ligands to Rev-erbα was mutually exclusive.

Although Fe(III)-hemin displaced the NCoR ID1 peptide from Rev-erbα *in vitro*, we previously found that Fe(II)-heme increased the assembly of the Rev-erbα/NCoR/HDAC3 complex (*22*) in liver cells and was able to repress the transcription of the *bmal1* gene (*5*). The apparent disconnect between the biochemical and cellular activity of hemin/heme may result from the difference in iron oxidation state or from the short NCoR ID1 peptide and Rev-erbα LBD inadequately reflecting the range of interactions possible with the full length proteins present in cells. For example, whereas Rev-erbα

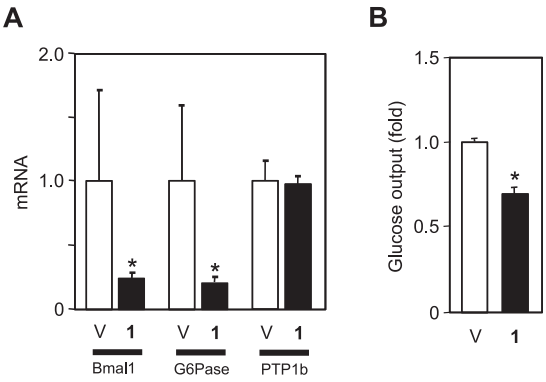


Figure 3. Repression of gluconeogenesis by 1 (10 μM) in primary mouse hepatocytes. The data were plotted as mean ± SD. *p-value <0.05 by a Student's t test. A) The gene expression analyses of the mRNA levels of Bmal1, G6 Pase, and PTP1b. The experiment was repeated at least 3 times and results were similar among experiments. B) Glucose levels following stimulation with dexamethasone and 8-CPT-cAMP.

will form a 1:1 complex with NCoR in solution, its function as a transcriptional repressor was only mediated through a dimeric Rev-DR2 DNA response element (*23*), leaving the stoichiometry of the active complex unknown. The availability of a nonporphyrin Rev-erbα ligand therefore has the potential to provide an important tool to study the Rev-erbα repression complex. To determine whether **1** exhibited functional activity as a Rev-erbα ligand in cells, we monitored the expression of *bmal1* mRNA in HepG2 cells. In the presence of 10 μM **1**, a statistically significant repression of *bmal1* expression was observed (Figure 2, panel A). This transcriptional repression was ablated in the presence of shRNA targeting Rev-erbα, thereby establishing the receptor specificity of the action (Figure 2, panel B).

To further characterize the utility of **1** as a tool to study Rev-erbα biology, we tested its effects on cellular pathways known from genetic studies to be controlled by the orphan receptor. The peripheral circadian clock plays a key role in the control of hepatic glucose output during the subjective night to mitigate the absence of food consumption (*24*). Rev-erbα has been shown to regulate Bmal1 expression in the liver and several critical genes required for hepatic gluconeogenesis (*12, 22*). We therefore investigated repression of gluconeogenesis in liver cells upon addition of **1**. The mRNA for *g6pc*, *pepck*, and *pgc1a* were all repressed by 10 μM **1** in HepG2 cells (Figure 2, panel C). These genes play pivotal roles in the pathway of hepatic gluconeogenesis: PEPCK is the rate-limiting enzyme, while G6 Pase catalyzes the final biosynthetic step. PGC1α is a Rev-erbα target gene that mediates feedback repression of heme biosynthesis (*25*) but is also a vital transcriptional regulator of hepatic gluconeogenesis (*11*). A ChIP experiment in the same cells showed that whereas no additional Rev-erbα was bound to the *g6pase* promoter, a doubling of HDAC3 recruitment was observed in the presence of **1** (Figure 2, panel D). These experiments demonstrate that **1** performed as a Rev-erbα agonist in cells to promote the formation of a transcriptional repressor complex with HDAC3 and repress target genes associated with the pathway of gluconeogenesis.

To confirm the functional consequence of Rev-erbα activation, primary murine hepatocytes were treated with **1**. Repression of *bmal1* and *g6pc* mRNA (Figure 3, panel A), but not the control gene *ptp1b* was observed. More importantly, **1** induced a 30% decrease in glucose output (Figure 3, panel B). Thus, **1** was able to function as a Rev-erbα agonist in liver cells to increase its activity as a transcriptional repressor and to effect a decrease in gluconeogenesis. Since we had previously demon-

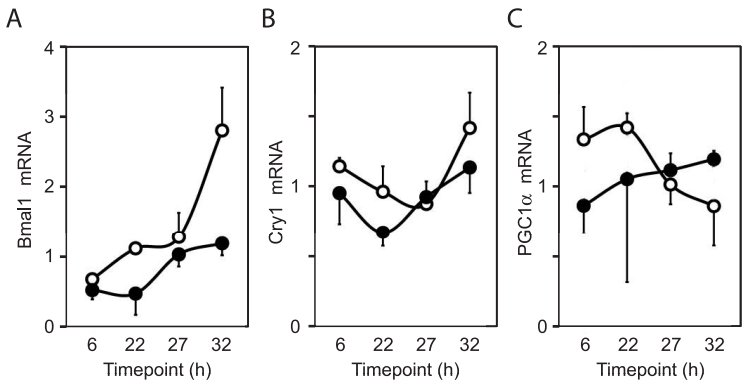


Figure 4. Circadian flux in gene expression regulated by 1 in primary mouse hepatocytes. Cells were synchronized by dexamethasone, and mRNA levels of Bmal1, Cry1, and PGC1α were measured at four time points. Open circles represent vehicle, and closed circles represent cells treated with 1 (10 μM). The data were plotted as mean ± SD.

strated that **1** had the ability to phase-shift gene expression in synchronized cells (*18*), we investigated whether its effects on the gluconeogenic pathway were also time-dependent. By sampling synchronized primary hepatocytes at four time points over a 32 h period, a rhythmicity was observed in the basal expression of the circadian clock genes *bmal1* and *cry1*, as well as the *ppargc1a* gene (Figure 4). Remarkably, in the presence of **1**, expression levels of these genes were both repressed and phase-shifted in their periodic expression pattern. The results demonstrated that **1** modulates the effect of Rev-erbα on oscillation of hepatic gene expression, perturbing the circadian clock and components of the gluconeogenic signaling pathway.

Further characterization of **1** verified its utility as a tool to elucidate Rev-erbα cell biology. Against a panel of cellular and biochemical nuclear receptor assays, including those for FXR, LXRα, LXRβ, PXR, and SF-1, **1** was inactive at a concentration of 10 μM. Moreover, **1** was inactive against a broader panel of pharmacologic targets, including protein kinases, GPCRs, ion channels, transporters, and enzymes. However, the utility of **1** as a chemical probe (*26*) was limited by its rapid clearance ($C_{int} > 1.0$ mL min^{-1} mg^{-1} protein) in rat liver microsomes and lack of oral bioavailability ($F = <1\%$ in mice, data not shown). As such, the compound is unlikely to be suitable for *in vivo* studies.

In conclusion, we have developed a biochemical assay to detect conformational changes in Rev-erbα employing a short NCoR peptide. The assay identified a tertiary amine nonporphyrin Rev-erbα ligand GSK4112 (**1**), which was characterized as a potent and selective small molecule Rev-erbα agonist. **1** will be a useful tool to study the molecular mechanism of Rev-erbα action as a transcriptional repressor, since it lacks the cell toxicity, target promiscuity, and redox chemistry of heme. Importantly, to facilitate open scientific research with this compound as a cell active Rev-erbα agonist, **1** has been made commercially available. (GSK4112 (**1**) is commercially available through Sigma-Aldrich (catalog no. G0673).) The additional investigations reported herein demonstrated that **1** mediated the repression of Rev-erbα target gene *bmal1* in liver cells and that it inhibited and shifted the phase of gluconeogenesis in primary mouse hepatocytes. Likewise, a recent report described the use of **1** to explore the role of Rev-erbα in adipogenesis (*27*). Future optimization of **1** will address its rapid clearance in liver microsomes to develop chemical probes for Rev-erbα pharmacology in animals.

METHODS

Rev-erbα LBD Expression and Purification. A modified polyhistidine tag (MKKGHHHHHHG) was fused in frame to residues 281–614 of Rev-erbα. Ten-liter fermentation batches were grown in LB media with 0.1 mg mL^{-1} ampicillin at 22 °C for 16 h until $OD_{600} = 14$. At this cell density, 0.25 mM IPTG was added, and induction proceeded for 4 h to a final $OD_{600} = 16$. Cells were harvested by centrifugation (20 min, 3500 × *g*, 4 °C), and concentrated cell slurries were stored in PBS at −80 °C. Approximately 200 g of cell paste was resuspended in 1 L of lysis buffer (50 mM Tris (pH 8.0), 150 mM NaCl). Cells were lysed by passing 3 times through a homogenizer (Rannie), and cell debris was removed by centrifugation (30 min, 20,000 × *g*, 4 °C). The cleared supernatant was filtered through coarse prefilters, and lysis buffer containing 500 mM imidazole was added to obtain a final imidazole concentration of 50 mM. This lysate was loaded onto a column (XK-26, 6 cm) packed with Ni^{2+}-charged Sepharose chelation resin (Pharmacia) and pre-equilibrated with buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 50 mM imidazole). After washing to baseline absorbance with the equilibration buffer, the column was developed with a linear gradient from 50 to 500 mM imidazole. Column fractions were pooled and dialyzed overnight against 2 L of buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM DTT and 0.5 mM EDTA). Following dialysis the protein sample was passed through prefilters and concentrated using Centri-prep 10K (Amicon) to 140 mL before diluting 7.5-fold with 50 mM Tris (pH 8.0), 5 mM DTT, and 0.5 mM EDTA. Following dilution the sample was loaded onto an equilibrated (50 mM Tris (pH 8.0), 20 mM NaCl, 5 mM DTT and 0.5 mM EDTA) column (XK-26, 3 cm, Poros HQ). After washing to baseline absorbance the column was developed with a linear gradient from 20 to 500 mM NaCl. The desired column fractions were pooled and concentrated to a volume of 25 mL before subjection to size exclusion (XK-26, 60 cm, Superdex 75). The final buffer was 50 mM Tris (pH 8.0), 150 mM NaCl, 5 mM DTT, and 0.5 mM EDTA.

Peptide Scan. The reagents for the FRET-based cell-free peptide scanning assays were prepared as described previously with minor modifications (*28*). Europium-labeled streptavidin (SA, 12 μL, 10 nM) was added to each well of a 384-well plate that contained 1 μL of 500 nM biotinylated test peptide and was allowed to equilibrate for 30 min at RT. Recombinant biotinylated Rev-erbα LBD labeled with 10 nM strepavidin-conjugated allophycocyanin (APC, 12 μL, 10 nM) was then added to the plate that contained the test peptide coupled europium-streptavidin complex (12 μL, 20 nM). The subsequent mixture was allowed to equilibrate for 1 h and was then imaged on a ViewLux 1430 ultraHTS microplate imager (Perkin-Elmer) in time-resolved mode. The final assay volume was 25 μL. Data are shown as the average and standard error of the means for four experiments and are shown as a ratio of the 665 to 620 nm with the value obtained in the absence of peptide subtracted.

FRET Assay. Proteins were set up in a 1:1 ratio of europium-labeled SA (Perkin-Elmer AD0063)-Rev-erbα LBD to APC-labeled SA (Perkin-Elmer CR130-150)-NCoR1 Complex. The NCoR peptide (ID1, 2040-2065) GQVPRTHRLITLADHICQIITQDFAR-NH_2 was

prepared by CPC Scientific. The buffer for this system was made at 50 mM Mops (pH 7.5), 50 mM NaF, and 1 mM EDTA in 1 L of deionized water. This buffer was then filtered with the Corning (431205) filter system with a 0.22 μm cellulose acetate filter. After filtering there was an addition of 0.1 mg mL^{-1} BSA (fatty acid free), and 50 μM Chaps. Before using the buffer in the assay 10 mM DTT was added to the appropriate amount of buffer. The proteins were incubated for 10 min, and then excess biotin was added to fill vacant SA sites. The protein mixture was added to prepared plates, which were then counted on the PerkinElmer Viewlux, and counts were then analyzed.

Compound Synthesis. Solvents and reagents were reagent grade and used without purification unless otherwise noted. All ^{1}H NMR spectra were recorded on a Varian 400 MHz spectrometer. Chemical shifts (δ) are reported downfield from tetramethylsilane (Me_4Si) in parts per million (ppm) of the applied field. Peak multiplicities are abbreviated: singlet, s; broad singlet, bs; doublet, d; triplet, t; quartet, q; multiplet, m. Coupling constants (*J*) are reported in hertz. LCMS analyses were conducted using a Waters Acquity UPLC system with UV detection performed from 210 to 350 nm with the MS detection performed on a Waters Acquity SQD spectrometer. Purities of compounds **1**–**5** were >98% as determined by LCMS.

***tert*-Butyl *N*-[(4-chlorophenyl)methyl]-*N*-[(5-nitro-2-thienyl) methyl]glycinate (1).** To a solution of 4-chlorobenzylamine (1.34 mL, 11.0 mmol) in THF (25 mL) was added K_2CO_3 (3.46 g, 25.0 mmol). *tert*-Butyl bromoacetate (1.48 mL, 10.0 mmol) was added dropwise over 3 min at RT, and the reaction mixture was stirred overnight at 40 °C. The resultant slurry was then diluted with EtOAc (80 mL) and washed with 2 × 50 mL of water and 50 mL of brine. The organic solution was dried with $MgSO_4$ and concentrated under reduced pressure. The crude material was purified by silica gel chromatography (Biotage 40 M column with 0 to 50% EtOAc in CH_2Cl_2 elution) to yield the intermediate *tert*-butyl *N*-[(4-chlorophenyl)methyl]glycinate as a clear, colorless oil: 2.08 g (81%); MS (ESI) *m*/*z* 256 $(M + H)^+$. To a stirring solution of *tert*-butyl *N*-[(4-chlorophenyl)methyl]glycinate (1.05 g, 4.11 mmol) in CH_2Cl_2 (20 mL) were added successively HOAc (0.5 mL), 5-nitro-2-thiophenecarboxaldehyde (0.774 g, 4.93 mmol), and sodium triacetoxyborohydride (1.740 g, 8.21 mmol). The reaction mixture was allowed to stir overnight, after which time it was diluted with 100 mL of EtOAc and washed with 2 × 50 mL of water and 50 mL of brine. After drying over $MgSO_4$ and concentration under reduced pressure, the resulting yellow oil was purified by silica gel chromatography (Biotage 40 M column, 0 to 50% CH_2Cl_2 in hexanes elution) to yield the product as a pale yellow oil: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.44 (s, 9H), 3.24 (s, 2H), 3.81 (s, 2H), 4.01 (s, 2H), 6.85 (d, J = 4.0 Hz, 1H), 7.29 (s, 4H), 7.75 (d, J = 4.0 Hz, 1H); ^{13}C (100 MHz, $CDCl_3$) δ 28.5, 53.2, 54.8, 56.8, 81.4, 126.0, 129.1, 131.1, 132.6, 137.8, 150.2, 155.9, 170.2; MS (ESI) *m*/*z* 397 $(M + H)^+$.

Cell Culture. HepG2 cells were incubated in DMEM without FBS for 16 h to deplete intracellular heme concentration and then switched to DMEM supplemented with either DMSO or **1** (10 μM) for 6 h. The cells were harvested for gene expression analysis by RT-QPCR or for ChIP analysis.

Mouse primary hepatocytes were freshly isolated from male C57BL6 mice age 12–16 wks. Cells were first seeded in DMEM with 10% FBS for 6 h to attach. Cells were then switched to serum-free DMEM for overnight incubation to deplete heme. Then, either DMSO or **1** (10 μM) was added and incubated with hepatocytes for another 16 h before harvest. For glucose output measurements, cells were washed three times with warm phosphate-buffered saline to remove glucose and cultured in the glucose-free medium containing gluconeogenic substrates (20 mM sodium lactate and 2 mM sodium pyruvate). Cells were stimulated with dexamethasone (1 nM) and 8-CPT-cAMP (500 μM) with or without **1** for 16 h. Glucose concentrations were determined with a glucose assay kit from Invitrogen and normalized to the cellular protein concentrations.

For observation of circadian biology, mouse primary hepatocytes were isolated and seeded as above. After 6 h of attachment, cells were switched to serum-free DMEM for 16 h and then synchronized with 100 nM of dexamethasone for 2 h (T2). Cells then were switched back to serum-free DMEM containing either DMSO or **1** (10 μM). Cells were then harvested at time T6, T21.5, T26.5, and T31.5 for RNA extraction and RT-QPCR analysis.

Chromatin Immunoprecipitation. Cells were cross-linked with 1% formaldehyde/PBS for 10 min at RT, then washed three times with PBS, and collected in ice-cold PBS with 1x protease inhibitor (Roche Applied Science, Indianapolis, IN). Cell pellets were resuspended and sonicated in modified RIPA buffer (50 mM Tris-HCl, pH 8.0, 125 mM NaCl, 0.1% SDS, 0.25% sodium deoxycholate, 1% Triton-X100) with protease inhibitor added. After incubating on ice for 10 min, the cell lysates were sonicated for 10 min using Bioruptor sonicator and then cleared by centrifugation at 14,000 × *g* for 10 min. Supernatants were collected and precleared with 2 μg of sheared salmon sperm DNA and protein A Sepharose (50 μL of a 50% slurry in 10 mM Tris-HCl (pH 8.1) and 1 mM EDTA) for 2 h at 4 °C. Immunoprecipitation with the following antibodies was performed at 4 °C overnight: anti-HDAC3 (Santa Cruz Biotechnology) and anti-Rev-erbα (Cell Signaling). Immunoprecipitated complexes were collected with protein-A sepharose beads followed by sequential washes in low salt, high salt, lithium, and TE buffer. Precipitates were eluted with elution buffer, and 5 M NaCl was added to reverse cross-links at 65 °C for 6 h. DNA fragments were purified with a PCR purification kit (Qiagen). A total of 2–3 μL of purified sample was used in 32–36 cycles of PCR using primers encompassing the RORE region of the human Bmal1 promoter. The sequences of the PCR primers included the following: (hG6pase) forward, 5′-*cctgggagacagagtgagacc*-3′ and reverse, 5′-*cctctggtttgggagatcaa*-3′; (h36B4) forward, 5′-*acgctgctgaacatgctcaa*-3′ and reverse, 5′-*gatgctgccattgtcgagca*-3′.

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Supporting Information Available: This material is available free of charge *via* the Internet at http://pubs.acs.org.

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