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Optimized Chemical Probes for REV-ERBα

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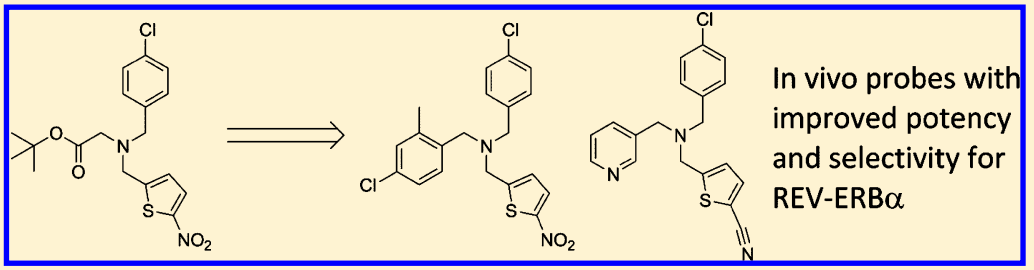
Supporting Information



ABSTRACT: REV-ERBα has emerged as an important target for regulation of circadian rhythm and its associated physiology. Herein, we report on the optimization of a series of REV-ERBα agonists based on GSK4112 (**1**) for potency, selectivity, and bioavailability.[1] Potent REV-ERBα agonists **4**, **10**, **16**, and **23** are detailed for their ability to suppress BMAL and IL-6 expression from human cells while also demonstrating excellent selectivity over LXRα. Amine **4** demonstrated in vivo bioavailability after either iv or oral dosing.

INTRODUCTION

The circadian clock aligns all the tissues in most organisms with the day and night cycle of our planet. Through a transcriptional mechanism, the clock controls many important biological pathways, such as metabolism, inflammation, and sleep−wake cycles.[2] REV-ERBα is a nuclear receptor that has been demonstrated, upon activation by heme, to form a complex with cofactors that represses the transcription of target genes.[3] REV-ERBα is at the heart of the circadian clock and is a mechanism by which the circadian clock gates inflammatory response and controls the metabolic state of the organism.[4] Investigation into the function of REV-ERBα will provide further insights into circadian biology and may identify the receptor as a therapeutic target for a variety of diseases.

Pharmacological investigations into the biological role of the REV-ERBα have used the suboptimal chemical probe GSK4112 (**1**) and its analogs SR9011 (**2**) and SR9009 (**3**) (Figure 1).[1,5] All three compounds are closely related by a common tertiary amine core and two of three identical substituents of the amine. Use of these compounds to interrogate REV-ERBα biology is complicated by high metabolic clearance rates that necessitate high dosing to achieve meaningful levels of exposure in vivo. In addition, the tertiary amine chemotype has known activity on the nuclear receptor LXRα, a potential liability for interpretation of results from cell-based and animal pharmacology.[6] Lack of selectivity for any chemical probe introduces challenges.[7] Specifically, LXRα has been shown to have effects in anti-inflammatory and metabolic pathways that overlap with those under investigation for the REV-ERBα. Herein we describe our optimization of the tertiary amine series of REV-ERBα agonists to address these liabilities that allow further exploration of the role of REV-ERBα in circadian control and inflammation.

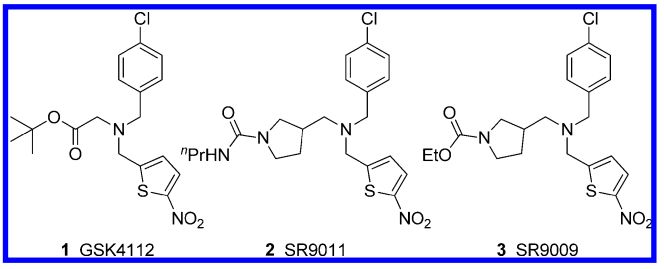


Figure 1. Reported REV-ERBα agonists **1**−**3**.

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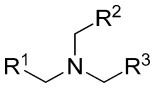
Table 1. REV-ERBα and LXRα Activity of Amine 1 Analogues



						LXRα		BMAL1	
compd	R^1	R^2	R^3	cLogP	REV-ERBα EC_{50} [a]	IC_{50} (μM)	fold selectivity	supp (%)[b]	phase delay[c]
1	$^tBuO_2C$-	4-Cl-C_6H_4-	5-nitrothiophen-2-yl	5.2	0.50	5.0	10	6	0
2	1-*N*-pentylcarboxamidepyrrolidin-3-yl	4-Cl-C_6H_4-	5-nitrothiophen-2-yl	5.3	>50	13		14	1.3
3	1-ethylcarboxylate-pyrrolidin-3-yl	4-Cl-C_6H_4-	5-nitrothiophen-2-yl	4.8	>50	6.3		20	1.3
4	4-Cl-2-Me-C_6H_3-	4-Cl-C_6H_4-	5-nitrothiophen-2-yl	6.8	0.050	63	1259	21	0
5	Ph-	4-Cl-C_6H_4-	5-nitrothiophen-2-yl	5.6	0.10	>100	>100	NT	NT
6	2-F-C_6H_4-	4-Cl-C_6H_4-	5-nitrothiophen-2-yl	5.7	0.063	20	320	38	0
7	isobutyl-	4-Cl-C_6H_4-	5-nitrothiophen-2-yl	5.9	0.16	16	100	21	0
8	3-pyridyl-	4-Cl-C_6H_4-	5-nitrothiophen-2-yl	4.1	0.16	13	79	NT	NT
9	3-pyridyl-	4-Me-C_6H_4-	5-nitrothiophen-2-yl	3.9	0.16	40	250	23	0
10	3-pyridyl-	4-F-C_6H_4-	5-nitrothiophen-2-yl	3.5	0.16	40	250	14	0
11	3-pyridyl-	2-Me-C_6H_4-	5-nitrothiophen-2-yl	3.8	0.20	32	160	37	0
12	3-pyridyl-	3-Me-C_6H_4-	5-nitrothiophen-2-yl	3.9	0.20	25	130	41	0.8
13	3-pyridyl-	4-OMe-C_6H_4-	5-nitrothiophen-2-yl	3.3	0.20	32	160	NT	NT
14	3-pyridyl-	3-OMe-C_6H_4-	5-nitrothiophen-2-yl	3.3	0.40	10	25	42	0.5
15	3-pyridyl-	3-CF_3-C_6H_4-	5-nitrothiophen-2-yl	4.3	0.40	16	40	NT	NT
16	3-pyridyl-	4-Cl-C_6H_4-	5-nitrilethiophen-2-yl	3.8	0.20	20	100	23	0
17	3-pyridyl-	4-Cl-C_6H_4-	4-Br-5-Me-thiophen-2-yl	5.7	0.25	32	130	16	0
18	3-pyridyl-	4-Cl-C_6H_4-	benzo[*b*]thiophen-2-yl	5.7	0.40	40	100	24	0
19	3-pyridyl-	4-Cl-C_6H_4-	3-(1*H*-pyrrol-1-yl)thiophen-2-yl	5.5	0.63	40	63	30	0
20	3-pyridyl-	4-Cl-C_6H_4-	thiazol-2-yl	3.0	>50	50	<1	NT	NT
21	3-pyridyl-	4-Cl-C_6H_4-	1*H*-imidazol-2-yl	2.5	>50	>100	NT	NT	NT
22	3-pyridyl-	4-F-C_6H_4-	5-nitrilethiophen-2-yl	3.2	0.20	NT	NT	NT	NT
23	3-pyridyl-	3,4-F_2-C_6H_3-	5-nitrilethiophen-2-yl	3.3	0.20	16	79	37	0
24	3-pyridyl-	4-OMe-C_6H_4-	5-nitrilethiophen-2-yl	3.0	0.63	>100	>160	NT	NT
25	3-pyridyl-	2,5-F_2-C_6H_3-	5-nitrilethiophen-2-yl	3.4	0.63	6.3	10	27	0
26	3-pyridyl-	2-F-C_6H_4-	5-nitrilethiophen-2-yl	3.3	>50	32	<1	6	0

[a] EC_{50} values are in μM. All active compounds demonstrated a 30−60% increase in NCOR peptide recruitment. [b] Suppression of BMAL1 expression after 40 h of 20 μM compound treatment. [c] Delay of peak of second cycle in hours of 20 μM treated cells relative to DMSO treated cells.

RESULTS AND DISCUSSION

To deliver a tool suitable for in vivo studies, we evaluated the literature compounds **1**−**3** to assess opportunities for improvement. First we investigated the ability of the compounds **1**−**3** to bind to both REV-ERBα and LXRα. The tertiary amine **1** induced recruitment of cofactor NCOR peptide fragment to purified REV-ERBα protein. This result indicated that the compound binds to and induces a change, potentially conformational, in the protein that alters the peptide recruitment profile (Table 1). Interestingly, neither compound **2** or **3** increased recruitment of the NCOR peptide fragment to REV-ERBα in vitro, a result that does not preclude the binding of compound **2** or **3** to the REV-ERBα but only that the potential binding of the compounds does not induce the same change in the protein to allow recruitment of our NCOR peptide fragment to our purified REV-ERBα. Amines **2** and **3** were reported to bind to the protein and have other activities associated with REV-ERBα activation, so we investigated the compounds' ability to activate the receptor by a different mechanism.[5] To determine if these two compounds were recruiting a different set of peptides to the REV-ERBα protein, we performed a scan of cofactor derived peptide fragments recruited to REV-ERBα in the presence of amine **1** (see Supporting Information Figure 1). Aside from NCOR and SMRT, the only other tested peptide fragments that showed significant interaction with the REV-ERBα was a fragment of PGC1-β. Full curve analysis of compounds **1**−**3** demonstrated the dose-dependent recruitment of PGC1-β peptide to REV-ERBα (Figure 2). This result demonstrated that amines **2** and **3** do bind to the REV-ERBα protein, as reported, to induce a

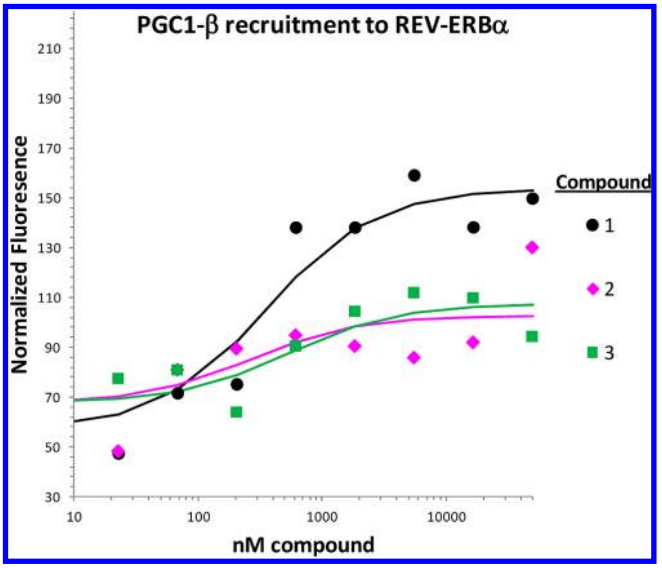


Figure 2. Effect of compounds **1**−**3** on PGC1-β peptide fragment recruitment to REV-ERBα.

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change in the protein that is different from that produced by amine **1**. These data raise the intriguing possibility that the different peptide recruitment profiles of the compounds may result in distinct biological activities. They also indicate that PGC1-β may have a yet uninvestigated role in REV-ERBα pharmacology. The exploration of these possibilities is currently under investigation and is beyond the scope of this paper.

Each of the compounds **1**−**3** was able to displace a radioligand from the LXRα binding site, confirming that the compounds bind to the LXRα at ∼10 μM (Table 1). We investigated if the observed binding of the compounds to both REV-ERBα and LXRα induced related effects in a living cell. To investigate REV-ERBα cellular efficacy, we measured the ability of agonists **1** and **2** to inhibit IL-6 production from human THP-1 cells following LPS stimulation (Figure 3a). To

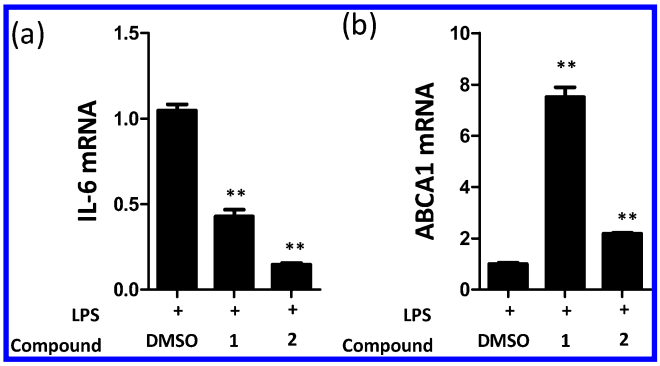


Figure 3. Effects of compounds **1** and **2** on cellular makers of (a) REV-ERBα and (b) LXRα activity. THP-1 cells were stimulated with LPS, 10 μg/mL, in the presence of DMSO, **1** (10 μM), or compound **2** (10 μM). After 6 h the cells were lysed and the mRNA was analyzed using RT-qPCR. Both compounds significantly repressed IL-6 mRNA expression but increased ABCA1 mRNA expression (**, $p < 0.01$, t test). A representative experiment of three experiments is shown.

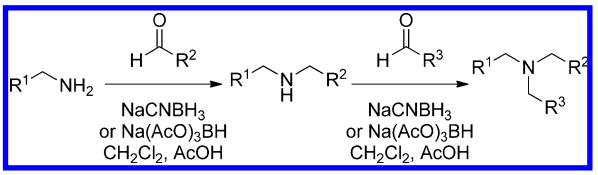
assess the cellular activity of the compounds in an LXRα-dependent system, we compared the effects of the compounds **1** and **2** on expression of ABCA1 in THP-1 cells (Figure 3b). Both compounds were able to significantly inhibit LPS-stimulated IL-6 mRNA expression and up-regulate ABCA1 mRNA levels, indicating significant cellular activity of each compound on both REV-ERBα and LXRα dependent phenotypes that correlated with the ability of the compounds to bind to target proteins.

The original report on compound **2** described a lack of activity in a chimeric LXRα-Gal4 reporter gene assay in which the binding domain of LXRα was fused to a yeast transcription factor. However, our results indicated that the compounds bound directly to purified LXRα protein and induced cellular affects through activation of the endogenous LXRα which contains multiple domains important for signal transduction that are absent in the chimeric LXRα-Gal4 construct. While we were able to replicate the published results with an artificial LXRα-Gal4 reporter assay (data not shown),[5] the assay may not be an appropriate system to measure the selectivity of the current series of REV-ERBα agonists because of the artificial nature of the LXRα employed. Another possibility is that, as noted above, compounds **2** and **3** induced a different profile of peptide recruitment to the REV-ERBα and may be acting as modulators of NR action, achieving selective activation of different NR responses. Importantly, compounds **1**−**3** bind to LXRα and up-regulate a marker of LXRα activation in primary cells. Since activation of LXRα has been reported to have diverse effects on multiple pharmacological pathways, including inflammation, cholesterol metabolism, and insulin resistance, we set a key goal for this project to improve the selectivity profile for the series.[6]

In addition to the observed off-target activity, compounds **1**−**3** have relatively high cLogP values of >5 that may contribute to off-target binding, particularly with nuclear receptors, and the high clearance observed in reported mouse DMPK studies.[8]

The goal of our synthetic efforts was therefore to provide an effective and readily accessible in vivo probe for REV-ERBα that could be used as an efficient tool to explore the biological profile of this fascinating receptor. To improve on the significant shortfalls of **1**−**3**, it was crucial to increase selectivity over LXRα to allow clear and precise interpretations of subsequent data and secondarily to reduce the lipophilicity of the compounds. Preparation of the target tertiary amines was through a series of reductive amination procedures (Scheme 1).

Scheme 1. Synthesis of Tertiary Amine Compounds



The overall process could be carried out under ambient reaction conditions or accelerated through microwave irradiation. It also proved possible to carry out the entire reaction sequence through a one-pot two-step protocol allowing rapid access to a range of tertiary amine scaffolds for evaluation. Pertinent data in this ligand development on a selection of the amines prepared are collected in Table 1.

Initial efforts focused on replacing the *tert*-butyl ester arm (R^1) of compound **1**. Alternative substitution at this position provided multiple options for improved REV-ERBα activity and LXRα selectivity but often at the cost of an increase in compound cLogP (Table 1); however, significant improvements in the overall compound profile proved to be possible. Replacement of the *tert*-butyl ester with both substituted benzyl (**4**−**6**) and alkyl (**7**) groups was not only well tolerated but led to stunning improvements in LXRα selectivity. Although many of these changes resulted in an increase in cLogP, it showed that LXRα activity could ultimately be removed from this tertiary amine series through simple structural modification. In order to address the lipophilicity issue, a variety of heterocyclic substituents were examined in this position, the vast majority of which were not tolerated (data not shown); however, introduction of a 3-pyridyl group (e.g., **8**) provided a modest increase in REV-ERBα activity (EC_{50} = 0.16), significant improvement in LXRα selectivity (79 fold), and importantly a meaningful lowering in cLogP (4.1).

Using compound **8** as the basis for continued exploration of scaffold modification, we examined variation of the 4-chlorobenzyl arm (R^2) of compound **1**. In contrast to substitution of R^1, both alkyl chains and heterocyclic substituents were not tolerated in this position with all changes resulting in compounds with no significant activity at REV-ERBα (data not shown). It was possible to replace this substituent with a variety of substituted benzyl groups (e.g., **9**−**15**) that provided multiple options that maintained REV-ERBα activity and concurrently improved LXRα selectivity and

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reduced cLogP. For example, introduction of a 4-fluorobenzyl unit (**10**) lowered the cLogP significantly (3.5) and improved selectivity over LXRα to >250-fold. Other changes at R^2 such as the introduction of a 4-methoxybenzyl substituent (**13**) provided a similar and substantial improvement in overall compound profile.

In parallel to these modifications, an array of compounds was prepared replacing the nitro- substituent on the thiophene ring (R^3). Interestingly, the majority of replacements for the nitrothiophene ring system with alternative polar five-membered ring heterocycles as exemplified by compounds **20** and **21** resulted in complete loss of activity in the primary NCOR peptide recruitment assay. It did prove to be possible to introduce different substituents to the thiophene ring and maintain REV-ERBα potency and improve LXRα selectivity as exemplified by compounds **17** and **18**; however, these changes often proved to be detrimental to the cLogP. One crucial exception to this trend was introduction of the 5-nitrile thiophene in compound **16**, which not only maintained REV-ERBα selectivity (EC_{50} = 0.2) but also resulted in a significant drop in cLogP (3.8) and a slight improvement in LXRα selectivity (100-fold).

Combination of the optimal substituents discovered for R^1, R^2, and R^3 resulted in the tertiary amines **22**–**26** which on evaluation revealed the key compound **23** which maintained potency in the NCOR peptide recruitment assay and also provided the desired LXRα selectivity profile and reduced lipophilicity.

Initially, to confirm the ability of the compounds to induce REV-ERBα-driven phenotypes in a cellular system, the ability to affect the circadian expression of BMAL in synchronized U2OS cells was examined. By stably expressing a BMAL promoter-driven luciferase reporter in U2OS cells, we were able to capture real time bioluminescent oscillations of synchronized cells.[9] A single end point assay of BMAL-luciferase suppression by REV-ERBα agonists (including compound **1**) has been described in the literature.[10] We confirmed that comparable data (a statistically significant 15% suppression) can be generated with compound **1** through our in-house U2OS reporter assay. Further, we showed that the natural ligand for REV-ERBα (heme) produced a >50% suppression in the same assay (data not shown). A representative data set from the assay for amine **4** is shown in Figure 4. Additional data from the assay are also shown in Table 1 and Supporting Information Figure 2. As can be seen, the increase in potency for recruitment of the NCOR peptide promoted by compound **4** correlates with the suppression and shift of the BMAL oscillation curve in a dose-dependent manner.

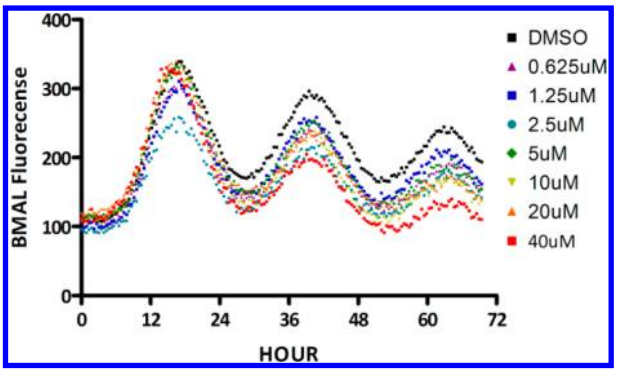

Figure 4. Oscillation of BMAL-Luc gene over time in synchronized U2OS cells with and without treatment with compound **4**.

During the evolution of this SAR knowledge, compounds **4** (GSK2945), **10** (GSK0999), **16** (GSK5072), and **23** (GSK2667) (Figure 5) were selected for further profiling and evaluation of their suitability to be adopted as in vivo probes for REV-ERBα.

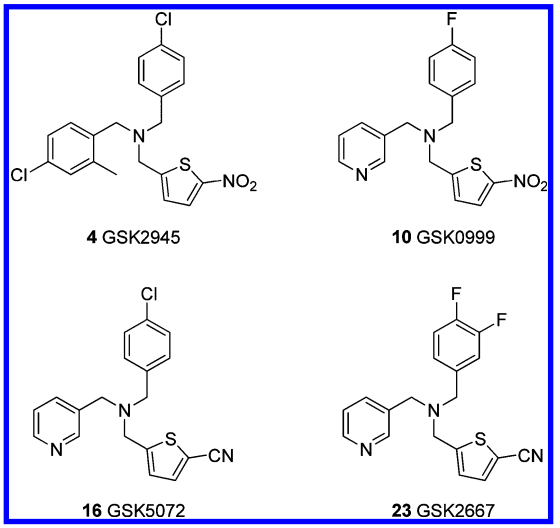

Figure 5. Key compounds selected during study for further evaluation as potential probes.

To assess the cellular efficacy of REV-ERBα activation and selectivity over a LXRα driven pathway, amines **4**, **10**, **16**, and **23** were profiled for their ability to inhibit LPS induction of IL-6 production and to up-regulate expression of ABCA1 in human THP-1 cells. All compounds demonstrated a significant reduction of IL-6 secretion following treatment with **4**, **10**, **16**, and **23** at 1 μM with no measurable effect on ABCA1 levels (Figure 6). Additionally, none of the compounds showed any toxicity in measurement of ATP levels in THP-1 cells (see Supporting Information Figure 3). These data indicated that each of these tertiary amines was able to potently activate REV-ERBα in cells, that suitable selectivity over LXRα had been achieved, and that no general toxicity was skewing the results.

The iv and oral DMPK profiles of compounds **3**, **4**, **10**, **16**, and **23** were evaluated in a 1 mg/kg cassette dose experiment in C57Bl/6 mice. Four of the compounds demonstrated essentially identical profiles with short half-lives, high clearance, and low oral bioavailability. Compound **4** (GSK2945) was differentiated from the group with a longer half-life of 2.0 h and an oral bioavailability of 23%, despite the higher cLogP of the compound (Table 2 and Supporting Information Figure 4). The profile of amine **4** is suitable to achieve plasma concentrations around the IC_{50} of the compound with oral doses around 20–30 mg/kg, a dosing regimen that is compatible with long-term in vivo studies. The remaining four compounds (**3**, **10**, **16**, **23**) would be suitable for acute time-of-day dosing by injection where short exposure of the compound at meaningful levels is desired. The specific advantage of **10**, **16**, and **23** over the previously reported tertiary amine **3** is the selectivity profile observed for REV-ERBα over LXRα.

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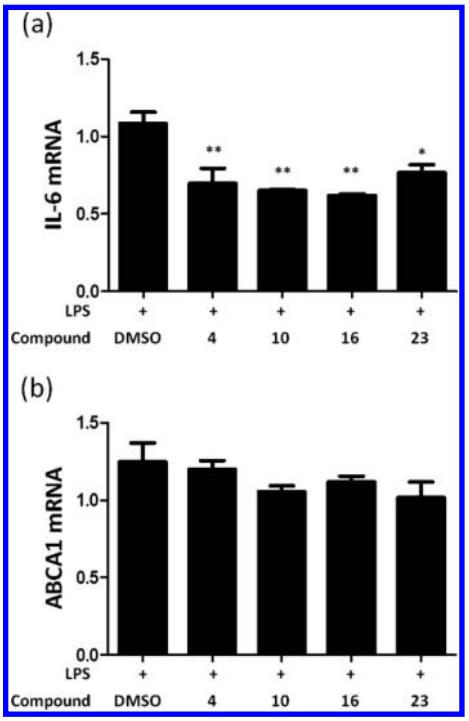

Figure 6. Effects of compounds **4**, **10**, **16**, and **23** on cellular makers of (a) REV-ERBα and (b) LXRα activity. THP-1 cells were stimulated with LPS, 10 μg/mL, in the presence of DMSO or compounds **4**, **10**, **16**, **23** (1 μM), in triplicate. After 6 h the cells were lysed and the RNA was analyzed using RT-qPCR. All compounds repressed IL-6 without affecting ABCA1 transcript expression (*, $p < 0.05$; **, $p < 0.01$; one-way ANOVA with post hoc Dunnett's).

CONCLUSION

Herein we report on the discovery of REV-ERBα agonists from the amine **1** series that demonstrated >1000-fold selectivity over LXRα and have improved DMPK properties. In addition, we have identified PGC1-β as a novel cofactor for REV-ERBα and have noted that reported ligands **1**–**3** recruit different cofactor peptides to REV-ERBα and may induce distinct pharmacology as a result. Tertiary amine **4** is a potent agonist with a profile suitable for chronic in vivo dosing via both oral and iv routes. Other compounds (**10**, **16**, and **23**) are identified with a shorter half-life in rodents that could be used for acute time-of-day dosing studies. These compounds represent valuable additions to the pharmacological toolbox to investigate the biology of REV-ERBα without the complication of activity on LXRα.

EXPERIMENTAL SECTION

General. All chemical reagents were purchased and used as received. ^{1}H NMR spectra were recorded on a Varian Unity 300, Varian Unity Plus-400, Brüker Avance 400, or a Brüker Avance 500. Chemical shifts are expressed in parts per million (ppm, δ units). Coupling constants are in units of hertz (Hz). Splitting patterns describe apparent multiplicities and are designated as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or brs (broad).

The LCMS system used to determine purity was a UPLC analysis conducted on a Waters Acquity system with a BEH C18, 2 mm × 50 mm, 1.7 μm column at 40 °C, 95% H_2O, 5% MeCN to 99% MeCN in 1.1 min, holding at 100% MeCN for 40 s. Water contained 0.2% v/v formic acid, and MeCN contained 0.15% v/v formic acid. The flow rate was 1 mL/min with 5 μL of solution injected. Mass spectra were recorded utilizing electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) switching between positive and negative modes with DAD scanning from 210 to 350 nm. All compounds tested were of ≥95% purity.

Normal phase chromatography was accomplished using either Isco or Biotage equipment using prepacked silica gel columns.

Reverse phase HPLC was accomplished using Agilent 110 series preparative HPLC systems using a C18 Phenomenex Luna, 75 mm × 30 mm, 5 μm column using the gradient described. The flow rate was 70 mL/min, and the product was collected based on UV detection at 220 or 254 nm.

General Procedures for Reductive Amination. General Method A (Used for Compounds 4, 10, 16, and 22–24). Aldehyde (1.0 equiv) was added to a solution of amine (1.0 equiv) in DCE (0.3 M) at room temperature. After the mixture was stirred for 5 min, acetic acid (1.0 equiv) and sodium triacetoxyborohydride (1.4 equiv) were added at room temperature. After the mixture was stirred for 16 h, the second aldehyde (1.0 equiv) was added followed by an additional portion of sodium triacetoxyborohydride (1.4 equiv). After being stirred for 24 h at room temperature, the mixture was quenched with saturated aqueous $NaHCO_3$ solution. The organic phase was dried over sodium sulfate, and the solvent removed under reduced pressure.

General Method B (Used for Compounds 5–9, 11–15, and 25–26). MP-sodium cyanoborohydride (2.0 equiv) was weighed into a 5 mL microwave reaction vessel. Amine (1.0 equiv) and aldehyde (1.1 equiv) were dissolved in a mixture of CH_2Cl_2, EtOH, and AcOH (2.5:2.5:1, 0.05 M) and then added to the reaction vessel. The mixture was heated to 120 °C for 10 min using microwave irradiation. The crude reaction was filtered, concentrated to an oil, dissolved in DMSO, and purified by reverse phase preparative HPLC (20–100% MeCN in water with 0.5% TFA) to give an oil. The oil was dissolved in CH_2Cl_2 and treated with 300 mg of MP-carbonate resin and filtered. The filtrate was treated with 2 mL of 1 N HCl in MeOH and concentrated to give the title compound.

General Method C (Used for Compounds 17–21). Aldehyde (0.3 mmol) was weighed into a 2 mL microwave vial. In a 20 mL scintillation vial, amine (0.3 mmol) was weighed, and to this 2 mL of a 3:3:1 CH_2Cl_2/EtOH/AcOH solution was added. The resulting solution was added to the mixture along with polymer supported sodium cyanoborohydride, 2 mM/g (4.77 mmol). The mixture was heated to 120 °C in the microwave for 10 min. The mixture was

Table 2. Pharmacokinetic Parameters

dose route (1 mg/kg)	PK parameter	3	4	10	16	23
iv	CL (L h^{-1} kg^{-1})	2.7	0.4	2.8	2.8	2.6
	V_{ss} (L/kg)	1.6	0.3	1.5	1.8	1.4
	terminal $t_{1/2}$ (h)	0.55	1.65	0.74	1.27	0.74
	AUC_{last} (h·ng/mL)	365	2,538	354	361	375
oral	T_{max} (h)	0.50	2.0	0.25	0.25	0.25
	C_{max} (ng/mL)	7.41	180	8.98	9.14	9.94
	AUC_{last} (h·ng/mL)	7.93	594	8.39	10.2	9.39
	F (%)	2.2	23.4	2.4	3.5	3.0

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filtered through a disposable filter funnel to remove the sodium cyanoborohydride and evaporated overnight under reduced pressure. The resulting solid was dissolved in DMSO and purified by reverse phase preparative HPLC (20−100% MeCN in water with 0.5% TFA).

***N*-(4-Chloro-2-methylbenzyl)-*N*-(4-chlorobenzyl)-1-(5- nitrothiophen-2-yl)methanamine (4).** By use of general method A, the title compound was synthesized using 4-chloro-2-methylbenzaldehyde, 5-nitrothiophene-2-carbaldehyde, and 4-chlorobenzylamine. The residue was purified over silica gel (10−30% EtOAc in petroleum ether) to give the title compound (0.42 g, 1.0 mmol, 50% yield) as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, J = 4.2 Hz, 1H), 7.37−7.29 (m, 5H), 7.18−7.11 (m, 2H), 6.86−6.81 (m, 1H), 3.70 (brs, 2H), 3.59 (brs, 2H), 3.55 (d, J = 0.8 Hz, 2H), 2.26 (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. LCMS: t_R = 1.60 min, 98%. MS m/z = 421.1 $(M + H)^+$. HRMS calcd for $C_{20}H_{19}N_2O_2Cl_2S$ $(M + H)^+$ requires m/z 421.0539, found 421.0536.

***N*-Benzyl-*N*-(4-chlorobenzyl)-1-(5-nitrothiophen-2-yl)-methanamine (5).** By use of general method B, the title compound was afforded as a white solid using amine **27** and benzaldehyde (27 mg, 59 μmol, 28%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (d, J = 4.1 Hz, 1H), 7.44−7.28 (m, 9H), 6.94−6.86 (m, 1H), 3.78 (brs, 2H), 3.73−3.62 (m, 4H). LCMS: t_R = 1.21 min, 100%. MS m/z = 373 $(M + H)^+$. HRMS calcd for $C_{19}H_{18}ClN_2O_2S$ $(M + H)^+$ requires 373.0778, found 373.0780.

***N*-(4-Chlorobenzyl)-*N*-(2-fluorobenzyl)-1-(5-nitrothiophen-2-yl)methanamine Hydrochloride Salt (6).** By use of general method B, the title compound was afforded as a white solid using amine **27** and 2-fluorobenzaldehyde (20 mg, 47.0 μmol, 22%). ^{1}H NMR (400 MHz, MeOD) δ 7.82 (d, J = 4.3 Hz, 1H), 7.52 (dd, J = 7.5, 1.2 Hz, 1H), 7.49−7.36 (m, 2H), 7.34−7.27 (m, 3H), 7.20−7.16 (m, 1H), 7.09−7.04 (m, 1H), 6.99 (d, J = 4.3 Hz, 1H), 3.79 (s, 2H), 3.72 (s, 2H), 3.64 (s, 2H). LCMS: t_R = 1.25 min, 100%. MS m/z = 391 $(M + H)^+$. HRMS calcd for $C_{19}H_{17}ClFN_2O_2S$ $(M + H)^+$ requires 391.0683, found 391.0684.

***N*-(4-Chlorobenzyl)-3-methyl-*N*-((5-nitrothiophen-2-yl)-methyl)butan-1-amine Hydrochloride Salt (7).** By use of general method B, the title compound was afforded as a white solid using amine **27** and 3-methylbutanal (13 mg, 36.0 μmol, 18%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.7 (brs, 1H), 8.12−8.10 (m, 1H), 7.79−7.36 (m, 5H), 4.71−4.51 (m, 1H), 4.48−4.25 (m, 2H), 3.12−3.02 (m, 1H), 2.80−2.30 (m, 2H), 1.77−1.38 (m, 3H), 0.80 (d, J = 6.5 Hz, 6H). LCMS: t_R = 1.13 min, 100%. MS m/z = 353 $(M + H)^+$. HRMS calcd for $C_{17}H_{22}ClN_2O_2S$ $(M + H)^+$ requires 353.1091, found 353.1091.

***N*-(4-Chlorobenzyl)-1-(5-nitrothiophen-2-yl)-*N*-(pyridin-3-ylmethyl)methanamine Hydrochloride Salt (8).** By use of general method B, the title compound was afforded as a white solid using amine **27** and 3-pyridylcarbaldehyde (27 mg, 59 μmol, 28%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.15 (brs, 1H), 8.71−8.70 (m, 1H), 8.62−8.60 (m, 1H), 8.19−8.16 (m, 1H), 7.73−7.63 (m, 2H), 7.28−7.19 (m, 4H), 6.84 (d, J = 4.3 Hz, 1H), 3.73 (s, 2H), 3.70 (s, 2H), 3.57 (s, 2H). LCMS: t_R = 0.81 min, 95%. MS m/z = 374 $(M + H)^+$. HRMS calcd for $C_{18}H_{17}ClN_3O_2S$ $(M + H)^+$ requires 374.0730, found 374.0731.

***N*-(4-Methylbenzyl)-1-(5-nitrothiophen-2-yl)-*N*-(pyridin-3-ylmethyl)methanamine Hydrochloride Salt (9).** By use of general method B, the title compound was afforded as a white solid using amine **28** and 4-methylbenzaldehyde (35 mg, 75 μmol, 32%). ^{1}H NMR (400 MHz, MeOD) δ 8.72−8.70 (m, 1H), 8.62 (d, J = 6.1 Hz, 1H), 8.44−8.42 (m, 1H), 7.85−7.83 (m, 2H), 7.25−7.24 (m, 2H), 7.13−7.11 (m, 2H), 7.02−7.01 (m, 1H), 3.88 (brs, 2H), 3.83 (brs, 2H), 3.66 (brs, 2H), 2.27 (s, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 154.7, 151.8, 149.8, 146.6, 145.6, 136.6, 134.7, 130.3, 130.0, 129.3, 129.1, 128.7, 125.8, 57.0, 54.1, 52.1, 20.7. LCMS: t_R = 0.82 min, 100%. MS m/z = 354 $(M + H)^+$. HRMS calcd for $C_{19}H_{20}N_3O_2S$ $(M + H)^+$ requires 354.1276, found 354.1278.

***N*-(4-Fluorobenzyl)-1-(5-nitrothiophen-2-yl)-*N*-(pyridin-3-ylmethyl)methanamine (10).** By use of general method A, the title compound was synthesized using 4-fluorobenzaldehyde and amine **28**. The residue was purified over silica gel (10−30% EtOAc in petroleum ether) and ISOLUTE SCX-2 SPE column (NH_3 solution, 2 N in MeOH) to give the title compound (0.29 g, 0.8 mmol, 40%) as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.61−8.55 (m, 1H), 8.52 (dd, J = 4.8, 1.7 Hz, 1H), 7.82−7.69 (m, 2H), 7.40−7.24 (m, 3H), 7.08−6.98 (m, 2H), 6.87−6.84 (m, 1H), 3.74 (brs, 2H), 3.64 (brs, 2H), 3.60 (brs, 2H). ^{13}C NMR (DEPTQ-135) (101 MHz, $CDCl_3$) δ 162.5 (d, J = 246.4 Hz), 153.4, 151.0, 150.2, 149.3, 136.4, 133.6, 133.5 (d, J = 3.3 Hz), 130.3 (d, J = 7.9 Hz), 128.8, 124.5, 123.7, 115.6 (d, J = 21.7 Hz), 57.5, 55.4, 52.9. ^{19}F NMR (377 MHz, $CDCl_3$) δ −114.6 to −114.7 (m, 1F); ^{19}F{H} NMR (377 MHz, $CDCl_3$) δ −114.6 (s, 1F). LCMS: t_R = 1.95 min, 100%. MS m/z = 358.1 $(M + H)^+$. HRMS calcd for $C_{18}H_{17}O_2N_3FS$ $(M + H)^+$ requires m/z 358.1020, found 358.1019.

***N*-(2-Methylbenzyl)-1-(5-nitrothiophen-2-yl)-*N*-(pyridin-3-ylmethyl)methanamine Hydrochloride Salt (11).** By use of general method B, the title compound was afforded as a white solid using 2-methylbenzaldehyde and amine **28** (31 mg, 66 μmol, 28%). ^{1}H NMR (400 MHz, MeOD) δ 8.62−8.60 (m, 1H), 8.58−8.56 (m, 1H), 8.35 (d, J = 8.2 Hz, 1H), 7.83−7.79 (m, 2H), 7.34−7.32 (m, 1H), 7.11−7.07 (m, 3H), 7.02 (d, J = 4.1 Hz, 1H), 3.92 (brs, 2H), 3.85 (brs, 2H), 3.73 (brs, 2H), 2.30 (s, 3H). LCMS: t_R = 0.79 min, 100%. MS m/z = 354 $(M + H)^+$. HRMS calcd for $C_{19}H_{20}N_3O_2S$ $(M + H)^+$ requires 354.1276, found 354.1278.

***N*-(3-Methylbenzyl)-1-(5-nitrothiophen-2-yl)-*N*-(pyridin-3-ylmethyl)methanamine Hydrochloride Salt (12).** By use of general method B, the title compound was afforded as a white solid using 3-methylbenzaldehyde and amine **28** (34 mg, 73 μmol, 31%). ^{1}H NMR (400 MHz, MeOD) δ 8.72−8.70 (m, 1H), 8.62−8.61 (m, 1H), 8.44 (d, J = 8.2 Hz, 1H), 7.86−7.83 (m, 2H), 7.40−7.30 (m, 1H), 7.19−7.17 (m, 2H), 7.06−7.04 (m, 1H), 7.02−7.01 (m, 1H), 3.89 (brs, 2H), 3.85 (brs, 2H), 3.67 (brs, 2H), 2.30 (s, 3H). LCMS: t_R = 0.81 min, 100%. MS m/z = 354 $(M + H)^+$. HRMS calcd for $C_{19}H_{20}N_3O_2S$ $(M + H)^+$ requires 354.1271, found 354.1271.

***N*-(4-Methoxybenzyl)-1-(5-nitrothiophen-2-yl)-*N*-(pyridin-3-ylmethyl)methanamine Hydrochloride Salt (13).** By use of general method B, the title compound was afforded as a white solid using 4-methoxybenzaldehyde and amine **28** (24 mg, 49 μmol, 21%). ^{1}H NMR (400 MHz, MeOD) δ 8.71−8.70 (m, 1H), 8.75−8.64 (m, 1H), 8.62−8.55 (m, 1H), 8.45−8.40 (m, 1H), 7.40−7.25 (m, 2H), 7.30−7.21 (m, 2H), 7.01 (d, J = 4.3 Hz, 1H), 6.40−6.36 (m, 1H), 3.88 (brs, 2H) 3.83 (brs, 2H), 3.74 (s, 3H), 3.65 (brs, 2H). LCMS: t_R = 0.75 min, 100%. MS m/z = 370 $(M + H)^+$. HRMS calcd for $C_{19}H_{20}N_3O_3S$ $(M + H)^+$ requires 370.1225, found 370.1229.

***N*-(3-Methoxybenzyl)-1-(5-nitrothiophen-2-yl)-*N*-(pyridin-3-ylmethyl)methanamine Hydrochloride Salt (14).** By use of general method B, the title compound was afforded as a white solid using 3-methoxybenzaldehyde and amine **28** (36 mg, 75 μmol, 32%). ^{1}H NMR (400 MHz, MeOD) δ 8.73−8.71 (m, 1H), 8.62−8.61 (m, 1H), 8.45−8.43 (m, 1H), 7.86−7.83 (m, 2H), 7.25−7.16 (m, 1H), 7.02 (d, J = 4.1 Hz, 1H), 6.98−6.92 (m, 2H), 6.81−6.77 (m, 1H), 3.90 (brs, 2H), 3.86 (brs, 2H), 3.77 (s, 3H), 3.68 (brs, 2H). LCMS: t_R = 0.75 min, 100%. MS m/z = 370 $(M + H)^+$. HRMS calcd for $C_{19}H_{20}N_3O_3S$ $(M + H)^+$ requires 370.1225, found 370.1220.

***N*-(3-Trifluromethylbenzyl)-1-(5-nitrothiophen-2-yl)-*N*-(pyridin-3-ylmethyl)methanamine Hydrochloride Salt (15).** By use of general method B, the title compound was afforded as a white solid using 3-trifluoromethylbenzaldehyde and amine **28** (21 mg, 40 μmol, 17%). ^{1}H NMR (400 MHz, MeOD) δ 8.71−8.68 (m, 1H), 8.62−8.58 (m, 1H), 8.35−8.32 (m, 1H), 8.23 (brs, 1H), 7.91−7.74 (m, 2H), 7.69−7.64 (m, 2H), 7.57−7.48 (m, 2H), 7.06 (d, J = 4.1 Hz, 1H), 3.90 (brs, 2H), 3.86 (brs, 2H), 3.80 (brs, 2H). LCMS: t_R = 0.84 min, 100%. MS m/z = 408 $(M + H)^+$. HRMS calcd for $C_{19}H_{17}F_3N_3O_2S$ $(M + H)^+$ requires 408.0994, found 408.0997.

5-(((4-Chlorobenzyl)(pyridin-3-ylmethyl)amino)methyl)-thiophene-2-carbonitrile (16). By use of general method A, the title compound was synthesized using 3-pyridinecarboxaldehyde, 5-formylthiophene-2-carbonitrile, and 4-chlorobenzylamine. The residue was purified over silica gel (30−70% EtOAc in petroleum ether) and ISOLUTE SCX-2 SPE column (NH_3 solution, 2 N in MeOH) to give the title compound (0.43 mg, 1.2 mmol, 61% yield) as a pale yellow

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oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.61−8.55 (m, 1H), 8.53 (dd, J = 4.8, 1.5 Hz, 1H), 7.75−7.69 (m, 1H), 7.46 (d, J = 3.8 Hz, 1H), 7.36−7.27 (m, 5H), 6.92−6.89 (m, 1H), 3.77 (brs, 2H), 3.62 (brs, 2H), 3.59 (brs, 2H). ^{13}C NMR (DEPTQ-135) (101 MHz, $CDCl_3$) δ 152.2, 150.2, 149.2, 137.6, 136.5, 136.3, 133.7, 133.5, 130.0, 128.9, 125.5, 123.7, 114.5, 109.0, 57.4, 55.3, 52.4. LCMS: t_R = 1.00 min, 98%. MS m/z = 354.1 $(M + H)^+$. HRMS calcd for $C_{19}H_{17}N_3ClS$ $(M + H)^+$ requires m/z 354.0826, found 354.0824.

1-(4-Bromo-5-methylthiophen-2-yl)-*N*-(4-chlorobenzyl)-*N*-(pyridin-3-ylmethyl)methanamine (17). By use of general method C, the title compound was afforded using 4-bromo-5-methylthiophene-2-carbaldehyde and [(4-chlorophenyl)methyl] (3-pyridinylmethyl)amine as a white solid (72 mg, 170 μmol, 54%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.68−8.64 (m, 1H), 8.61−8.57 (m, 1H), 8.16−8.11 (m, 1H), 7.68−7.62 (m, 1H), 7.40−7.34 (m, 4H), 6.92−6.89 (m, 1H), 3.71−3.63 (m, 4H), 3.57 (brs, 2H), 2.29 (s, 3H). LCMS: t_R = 0.98 min, 100%. MS m/z = 421 $(M + H)^+$. HRMS calcd for $C_{19}H_{19}BrClN_2S$ $(M + H)^+$ requires 421.0141, found 421.0139.

1-(Benzo[*b*]thiophen-2-yl)-*N*-(4-chlorobenzyl)-*N*-(pyridin-3-ylmethyl)methanamine (18). By use of general method C, the title compound was afforded using benzo[*b*]thiophene-2-carbaldehyde and [(4-chlorophenyl)methyl](3-pyridinylmethyl)amine as a white solid (24 mg, 62 μmol, 12%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.67−8.62 (m, 1H), 8.55−8.51 (m, 1H), 8.00−7.91 (m, 2H), 7.79−7.73 (m, 1H), 7.54−7.38 (m, 5H), 7.36−7.28 (m, 3H), 3.85 (brs, 2H), 3.68 (brs, 2H), 3.63 (brs, 2H). LCMS: t_R = 0.93 min, 100%. MS m/z = 379 $(M + H)^+$. HRMS calcd for $C_{22}H_{20}ClN_2S$ $(M + H)^+$ requires 379.1036, found 379.1038.

1-(3-(1*H*-Pyrrol-1-yl)thiophen-2-yl)-*N*-(4-chlorobenzyl)-*N*-(pyridin-3-ylmethyl)methanamine (19). By use of general method C, the title compound was afforded using 3-(1*H*-pyrrol-1-yl)-2-thiophenecarbaldehyde and [(4-chlorophenyl)methyl](3-pyridinylmethyl)amine as a white solid (44 mg, 110 μmol, 35%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.61−8.60 (m, 2H), 8.06−8.03 (m, 1H), 7.66−7.63 (m, 1H), 7.54 (d, J = 5.5 Hz, 1H), 7.36−7.31 (m, 4H), 7.06 (d, J = 5.5 Hz, 1H), 6.94−6.93 (m, 2H), 6.17 (t, J = 2.1 Hz, 2H), 3.71 (brs, 2H), 3.60 (brs, 2H), 3.54 (brs, 2H). LCMS: t_R = 0.90 min, 100%. MS m/z = 394 $(M + H)^+$. HRMS calcd for $C_{22}H_{21}ClN_3S$ $(M + H)^+$ requires 394.1147, found 394.1145.

***N*-(4-Chlorobenzyl)-1-(pyridin-3-yl)-*N*-(thiazol-2-ylmethyl)methanamine (20).** By use of general method C, the title compound was afforded using 1,3-thiazole-2-carbaldehyde and [(4-chlorophenyl)-methyl](3-pyridinylmethyl)amine as a white solid (53 mg, 160 μmol, 51%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.72−8.66 (m, 1H), 8.64−8.59 (m, 1H), 8.19−8.12 (m, 1H), 7.76−7.63 (m, 3H), 7.44−7.34 (m, 4H), 3.90 (brs, 2H), 3.75 (brs, 2H), 3.66 (brs, 2H). LCMS: t_R = 0.67 min, 95%. MS m/z = 330 $(M + H)^+$. HRMS calcd for $C_{17}H_{17}ClN_3S$ $(M + H)^+$ requires 330.0832, found 330.0836.

***N*-((1*H*-Imidazol-2-yl)methyl)-*N*-(4-chlorobenzyl)-1-(pyridin-3-yl)methanamine (21).** By use of general method C, the title compound was afforded using 1*H*-imidazole-2-carbaldehyde and [(4-chlorophenyl)methyl](3-pyridinylmethyl)amine as a white solid (47 mg, 150 μmol, 47%). ^{1}H NMR (400 MHz, MeOD) δ 8.77−8.76 (m, 1H), 8.64−8.62 (m, 1H), 8.41−8.38 (m, 1H), 7.82−7.79 (m, 1H), 7.37 (s, 2H), 7.31−7.25 (m, 4H), 4.01 (brs, 2H), 3.89 (brs, 2H), 3.70 (brs, 2H). LCMS: t_R = 0.43 min, 100%. MS m/z = 313 $(M + H)^+$. HRMS calcd for $C_{17}H_{18}ClN_4$ $(M + H)^+$ requires 313.1220, found 313.1220.

5-(((4-Fluorobenzyl)(pyridin-3-ylmethyl)amino)methyl)-thiophene-2-carbonitrile (22). By use of general method A, the title compound was synthesized using 3-pyridinecarboxaldehyde, 5-formylthiophene-2-carbonitrile, and 4-fluorobenzylamine. The residue was purified over silica gel (20−80% EtOAc in petroleum ether) and ISOLUTE SCX-2 SPE column (NH_3 solution, 2 N in MeOH) to give the title compound (0.17 g, 0.51 mmol, 51% yield) as a pale yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.63−8.57 (m, 1H), 8.57−8.51 (m, 1H), 7.80−7.75 (m, 1H), 7.47 (d, J = 3.7 Hz, 1H), 7.37−7.30 (m, 3H), 7.07−7.00 (m, 2H), 6.92−6.89 (m, 1H), 3.78 (brs, 2H), 3.63 (brs, 2H), 3.59 (brs, 2H). LCMS: t_R = 0.89 min, 100%. MS m/z = 338.1 $(M + H)^+$. HRMS $C_{19}H_{17}N_3FS$ $(M + H)^+$ requires m/z 338.1122, found 338.1128.

5-(((3,4-Difluorobenzyl)(pyridin-3-ylmethyl)amino)methyl)-thiophene-2-carbonitrile (23). By use of general method A, the title compound was synthesized using 3,4-difluorobenzaldehyde, 5-formylthiophene-2-carbonitrile, and 3-(aminomethyl)pyridine. The residue was purified over silica gel (30−70% EtOAc in petroleum ether) and ISOLUTE SCX-2 SPE column (NH_3 solution, 2 N in MeOH) to give the title compound (0.42 g, 1.2 mmol, 59% yield) as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.59−8.53 (m, 1H), 8.51 (dd, J = 4.8, 1.7 Hz, 1H), 7.74−7.68 (m, 1H), 7.45 (d, J = 3.8 Hz, 1H), 7.28 (ddd, J = 7.9, 4.8, 40.5 Hz, 1H), 7.24−7.15 (m, 1H), 7.15−7.04 (m, 2H), 6.93−6.88 (m, 1H), 3.77 (brs, 2H), 3.61 (brs, 2H), 3.56 (brs, 2H). ^{13}C NMR (DEPTQ-135) (101 MHz, $CDCl_3$) δ 151.8, 150.5 (dd, J = 248.9, 12.9 Hz), 150.1, 149.8 (dd, J = 248.0, 12.8 Hz), 149.2, 137.6, 136.3, 135.2 (dd, J = 5.2, 3.8 Hz), 133.4, 125.6, 124.5 (dd, J = 6.4, 3.6 Hz), 123.7, 117.3 (dd, J = 17.1, 7.5 Hz), 114.3, 109.0, 57.0, 55.3, 52.4, one carbon missing. ^{19}F NMR (377 MHz, $CDCl_3$) δ −139.3 to −139.1 (m, 1F), −137.2 (ddd, J = 21.8, 11.0, 6.8 Hz, 1F). ^{19}F{H} NMR (377 MHz, $CDCl_3$) δ −139.2 (d, J = 20.3 Hz, 1F), −137.2 (d, J = 20.3 Hz, 1F). LCMS: t_R = 0.92 min, 100%. MS m/z = 356.1 $(M + H)^+$. HRMS calcd for $C_{19}H_{16}N_3F_2S$ requires m/z 356.1028, found 356.1032.

5-(((4-Methoxybenzyl)(pyridin-3-ylmethyl)amino)methyl)-thiophene-2-carbonitrile (24). By use of general method A, the title compound was synthesized using 3-pyridinecarboxaldehyde, 5-formylthiophene-2-carbonitrile, and 4-methoxybenzylamine. The residue was purified over silica gel (20−70% EtOAc in petroleum ether) and ISOLUTE SCX-2 SPE column (NH_3 solution, 2 N in MeOH) to give the title compound (0.17 g, 0.5 mmol, 50% yield) as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.62−8.56 (m, 1H), 8.52 (dd, J = 4.8, 1.5 Hz, 1H), 7.78−7.72 (m, 1H), 7.46 (d, J = 3.8 Hz, 1H), 7.32−7.25 (m, 3H), 6.92−6.86 (m, 3H), 3.80 (s, 3H), 3.76 (brs, 2H), 3.62 (brs, 2H), 3.57 (brs, 2H). LCMS: t_R = 0.89 min, 100%. MS m/z = 350.1 $(M + H)^+$. HRMS calcd for $C_{20}H_{20}ONS$ requires m/z 350.1322, found 350.1327.

5-(((2,5-Difluorobenzyl)(pyridin-3-ylmethyl)amino)methyl)-thiophene-2-carbonitrile (25). By use of general method A, the title compound was synthesized using 2,5-difluorobenzaldehyde, 5-formylthiophene-2-carbonitrile, and *N*-methyl-1-(pyridin-3-yl)-methanamine as a white solid (23 mg, 60 μmol, 31%). ^{1}H NMR (400 MHz, MeOD) δ 8.92−8.91 (m, 1H), 8.79−8.77 (m, 1H), 8.73−8.71 (m, 1H), 8.08−8.04 (m, 1H), 7.61−7.59 (m, 1H), 7.31−7.30 (m, 1H), 7.29−7.27 (m, 1H), 7.25−7.00 (m, 2H), 4.17 (brs, 2H), 4.11 (brs 2H), 3.93 (brs, 2H). LCMS: t_R = 0.77 min, 100%. MS m/z = 356 $(M + H)^+$. HRMS calcd for $C_{19}H_{16}F_2N_3S$ $(M + H)^+$ requires 356.1033, found 356.1037.

5-(((2-Fluorobenzyl)(pyridin-3-ylmethyl)amino)methyl)-thiophene-2-carbonitrile (26). By use of general method A, the title compound was synthesized using 2-fluorobenzaldehyde, 5-formylthiophene-2-carbonitrile, and 3-aminomethylpyridine as a white solid (35 mg, 94 μmol, 50%). ^{1}H NMR (400 MHz, MeOD) δ 9.00−8.99 (m, 1H), 8.85−8.76 (m, 2H), 8.08−8.05 (m, 1H), 7.67−7.61 (m, 1H), 7.58−7.50 (m, 1H), 7.40−7.30 (m, 2H), 7.21−7.16 (m, 1H), 7.14−7.03 (m, 1H), 4.38 (brs, 2H), 4.31 (brs, 2H), 4.12 (brs, 2H). LCMS: t_R = 0.53 min, 100%. MS m/z = 338 $(M + H)^+$. HRMS calcd for $C_{19}H_{17}FN_3S$ $(M + H)^+$ requires 338.1129, found 338.1127.

***N*-(4-Chlorobenzyl)-1-(5-nitrothiophen-2-yl)methanamine (27).** 5-Nitrothiophene-2-carboxaldehyde (0.57 g, 3.6 mmol) was added to a solution of 4-chlorobenzylamine (0.44 mL, 3.6 mmol) in DCE (12.0 mL) at room temperature. After the mixture was stirred for 5 min, acetic acid (0.22 mL, 3.6 mmol) and sodium triacetoxyborohydride (1.10 g, 5.0 mmol) were added at room temperature. After being stirred for 16 h at room temperature, the mixture was quenched with saturated aqueous $NaHCO_3$ solution. The organic phase was dried over sodium sulfate, and the solvent was evaporated in vacuo. The residue was purified over silica gel (20−60% EtOAc in petroleum ether) to give the title compound as a pale yellow oil (0.55 g, 1.9 mmol, 54%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, J = 4.2 Hz, 1H), 7.32−7.26 (m, 4H), 6.86 (dt, J = 4.2, 1.0 Hz, 1H), 3.98 (d, J = 1.0 Hz,

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2H), 3.82 (brs, 2H). MS m/z = 283.0 (M + H)$^+$. HRMS calcd for $C_{12}H_{11}O_2N_2ClS$ requires m/z 283.0303, found 283.0306.

1-(5-Nitrothiophen-2-yl)-*N*-(pyridin-3-ylmethyl)-methanamine (28). 5-Nitrothiophene-2-carboxaldehyde (2.28 g, 14.4 mmol) was added to a solution of 3-aminomethylpyridine (1.46 mL, 14.4 mmol) in DCE (48.0 mL) at room temperature. After the mixture was stirred for 5 min, acetic acid (0.86 mL, 14.4 mmol) and sodium triacetoxyborohydride (4.40 g, 20.0 mmol) were added at room temperature. After being stirred for 16 h at room temperature, the mixture was quenched with saturated aqueous $NaHCO_3$ solution. The organic phase was dried over sodium sulfate, and the solvent was evaporated in vacuo. The residue was purified over silica gel (20−70% EtOAc in petroleum ether) to give the title compound as a brown gum (2.26 g, 9.0 mmol, 63% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.57−8.51 (m, 1H), 8.50 (dd, J = 4.8, 1.8 Hz, 1H), 7.76 (d, J = 4.2 Hz, 1H), 7.70 (dt, J = 7.8, 1.8 Hz, 1H), 7.26 (ddd, J = 7.8, 4.8, 0.5 Hz, 1H), 6.86 (ddd, J = 4.2, 1.0 Hz, 1H), 3.99 (d, J = 1.0 Hz, 2H), 3.86 (brs, 2H). MS m/z = 250.1 (M + H)$^+$. HRMS calcd for $C_{11}H_{11}O_2N_3S$ requires m/z 250.0645, found 250.0641.

5-(((Pyridin-3-ylmethyl)amino)methyl)thiophene-2-carbonitrile (29). 3-Aminomethylpyridine (1.51 g, 14.0 mmol) and 5-formyl-2-thiophenecarbonitrile (1.60 g, 11.7 mmol) were dissolved in anhydrous THF (40 mL) and acetic acid (10 mL) to give a clear solution. The mixture was stirred at room temperature for 30 min. Sodium triacetoxyborohydride (2.97 g, 14 mmol) was added, and the mixture was stirred at room temperature for 1 h. The mixture was concentrated to an oil which was purified over silica gel (2−10% MeOH in CH_2Cl_2) to give the title compound as an oil (1.34 g, 0.6 mmol, 50% yield) . ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.9 (brs, 1H), 9.17−9.11 (m, 1H), 8.98−8.85 (m, 1H), 8.80−8.72 (m, 1H), 8.11−8.02 (m, 1H), 7.95 (d, J = 4.2 Hz, 1H), 7.60 (d, J = 4.2 Hz, 1H), 4.54 (brs, 2H), 4.41 (brs, 2H). LCMS: t_R = 0.24 min, 100%. MS m/z = 230 (M + H)$^+$.

REV-ERBα NCOR FRET Assay. REV-ERBα was made at GSK. Proteins were chemically biotinylated using standard methods. Biotinylated NCOR1 was purchased from CPC Scientific. Streptavidin-labeled APC (CR130-150) and Eu-W1024 labeled streptavidin (AD0063) were purchased from Perkin-Elmer. Compounds were diluted in 100% neat DMSO at 10 mM. The compounds were dispensed into an intermediate plate (polypropylene Greiner PP V-bottom, 781280) to make serial dilutions in 100% neat DMSO. Approximately 100 nL of the serial dilution was added to the assay plate. The stock buffer used was a 0.5 M solution of MOPS made by adding 104 g of MOPS to 800 mL of H_2O in a graduated cylinder, using a calibrated pH meter, and adding increasing amounts of NaOH to give a final pH of 7.5. This solution was filtered using a Costar 0.2 μm filtering apparatus. The assay buffer used was prepared by adding 100 mL of 10× MOPS stock solution into a graduated cylinder to 800 mL. NaF (2.09 g), CHAPS (0.03 g), and BSA (0.1 g) were added to the flask. Water was added to give a final volume of 1 L. The assay buffer was filtered with a Costar 0.2 μm filtering apparatus. DTT was added to the assay buffer to a final concentration of 10 mM. To a polypropylene Costar conical centrifuge tube were added assay buffer and an appropriate amount of biotinylated-NCOR1 from the 100 μM stock solution to give a final concentration of 20 nM. To the above biotinylated NCOR1 solution, an appropriate amount of europium-labeled streptavidin was added to give a final concentration of 10 nM. The solution was incubated for 15 min at room temperature. In a separate polypropylene tube was added an appropriate amount of biotinylated REV-ERBα protein from the stock solution to give a final concentration of 20 nM. To the biotinylated-REV-ERBα solution was added an appropriate amount of APC-labeled streptavidin to give a final concentration of 10 nM. The resulting solution was incubated for 15 min at room temperature. A 20-fold excess of biotin from the 10 mM stock solution was added, and the resulting solution was incubated for 10 min at room temperature. The above solutions were gently mixed together to give a final solution containing 20 nM REV-ERBα 10 nM APC, and 20 nM NCOR1_10 nM SA_EU. The resulting mixture was incubated for 5 min, and a Thermo Combi Multidrop was used to add 10 μL of peptide/REV-ERBα solution to assay plates containing 100 nL of test compound. The plate was incubated for 1 h at room temperature and read on ViewLux in Lance mode for EU/APC. Raw data were analyzed using ABASE (IBDS) software. The data were normalized initially using the following equation: normalization =100 × [(basal HTRF − value)/(basal HTRF − minimal HTRF)]. The normalized data were then was fit to a four-parameter logistic equation.

Peptide Scan. By use of the same reagents as for the NCOR FRET assay, various biotinylated peptides were added to the Greiner assay plate to give a final concentration of 20 nM. An appropriate amount of Eu-W1024 was added to the plate to give a final concentration of 20 nM. The plate was incubated for 20 min at room temperature. At the same time, in a polypropylene tube was added an appropriate amount of biotinylated-REV-ERBα protein from the stock solution to give a final concentration of 20 nM. To the biotinylated-REV-ERBα solution, an appropriate amount of APC-labeled streptavidin was added to give a final concentration of 10 nM. The tube was inverted gently to mix and was incubated for 20 min at room temperature. Following the 20 min incubations, 20-fold excess biotin from the 10 mM stock solution was added, and the tube was inverted gently to mix. After a 15 min incubation at room temperature, a Thermo Combi Multidrop was used to add 5 μL of the REV-ERBα solution to the plates containing 100 nL of the various peptides. The plates were read on a ViewLux in Lance mode for EU/APC. The raw data were analyzed using an Excel template that averaged the four wells of each peptide and generated a standard deviation.

BMAL Reporter Gene Assay. Stable U2OS cell line expressing mouse BMAL-promoter luciferase was generated using a published procedure.[9] Freshly passaged cells were seeded at confluence in 384-well format. Robust circadian rhythms were induced by change of medium, and synchronization was evaluated by measuring the clock-driven luciferase reporter activity over 48 h. Synchronized cells were treated with compound, and real time bioluminescent oscillations were recorded for 72 h with measurements every 20 min using the temperature controlled luminometer Orion II. After 72 h of treatment, cells were washed and allowed to recover in full growth medium. The recovery oscillations are monitored and used as an internal toxicity counterscreen for off-target effects on cell transcription.

Cell Assays. THP-1 cells were obtained from ECACC. THP-1 cells were subcultured between 3×10^5 and 8×10^5 cells/mL in RPMI1640 (PAA) supplemented with 10% FBS (Invitrogen U.K.). Total RNA was prepared using the RNAeasy kit (Qiagen, U.K.) and treated with DNASe (Qiagen, U.K.). Reverse transcription was performed using a high capacity RNA to cDNA kit (Applied Biosystems, U.S.). The cDNA was analyzed by qPCR using Power Sybr Green master mix (Applied Biosystems, U.S.) and prevalidated Quantitect primers (Qiagen, U.K.). TATA box binding protein was the housekeeping gene. The cell viability assay was performed using the CellTiter-Glo kit (Promega, U.S.). The kit was used according to the manufacturer's instructions. Briefly, cells were incubated for 6 h with the chemical compounds. CellTiter-Glo was then added for 15 min before the luminescence was recorded.

DMPK Experiment. Plasma levels of a cassette of the five test compounds were evaluated in C57BL6 mice purchased from SLAC Laboratory Animal Co. (male mice, 22−25 g, n = 3 per time point, total of 9 animals per dosing regimen) administered by either iv tail vein injection (1 mg/kg) or po oral gavage administration (1 mg/kg). Compounds were dissolved in a 10% DMSO, 10% Solutol-HS-15, and 80% water solution. At 0, 0.083, 0.25, 0.5, 1, 2, 4, 8, and 24 h after dosing, blood was taken. Plasma was generated using standard centrifugation techniques and was frozen at −78 °C. Plasma was mixed with acetonitrile (1:15 (v/v)) and vortexed for 2 min and centrifuged at 14 000 rpm for 5 min. A 2 mL aliquot of the supernatant was analyzed for drug levels by liquid chromatography/tandem mass spectrometry.

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■ ASSOCIATED CONTENT

Supporting Information

Time course data, peptide recruitment scans, toxicity data, pharmacokinetics, and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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The manuscript was written with contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

REV-ERBα, NR1D1; LXRα, liver X receptor α (NR1H3); NCOR, nuclear receptor co-repressor 1; SMRT, nuclear receptor co-repressor 2; PGC1-β, peroxisome proliferator-activated receptor γ coactivator 1-β; IL-6, interleukin 6; THP-1, human acute monocytic leukemia cell line; LPS, lipopolysaccharide; ABCA1, ATP-binding cassette, subfamily A, member 1; GAL4, regulatory protein; BMAL, brain and muscle aryl hydrocarbon receptor nuclear translocator-like; U2OS, human osteosarcoma cell line; SAR, structure−activity relationship; AcOH, acetic acid

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