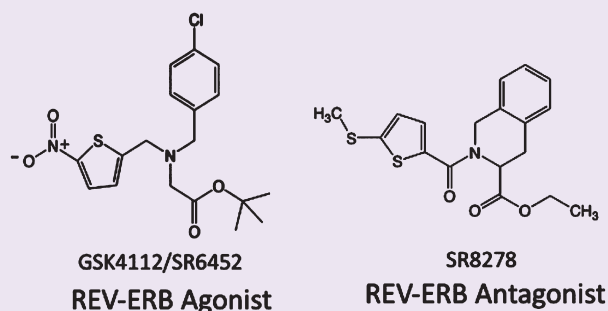


# Identification of SR8278, a Synthetic Antagonist of the Nuclear Heme Receptor REV-ERB

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**ABSTRACT:** REV-ERB $\alpha$  is a member of the nuclear receptor superfamily that functions as a receptor for the porphyrin heme. REV-ERB $\alpha$  suppresses transcription of its target genes in a heme-dependent manner. Recently, the first nonporphyrin synthetic ligand for REV-ERB $\alpha$ , GSK4112, was designed, and it mimics the action of heme acting as agonist. Here, we report the identification of the first REV-ERB antagonist, SR8278. SR8278 is structurally similar to the agonist but blocks the ability of the GSK4112 to enhance REV-ERB $\alpha$ -dependent repression in a cotransfection assay. Additionally, whereas GSK4112 suppresses the expression of REV-ERB $\alpha$  target genes involved in gluconeogenesis, SR8278 stimulates the expression of these genes. Thus, SR8278 represents a unique chemical tool for probing REV-ERB function and may serve as a point for initiation of further optimization to develop REV-ERB antagonists with the ability to explore circadian and metabolic functions.



REV-ERB $\alpha$  and REV-ERB $\beta$  are unusual members of the nuclear receptor (NR) superfamily that displays ligand-dependent transcriptional repression activity. We and others identified the porphyrin heme as the physiological ligand for these receptors.<sup>1,2</sup> Heme binding is required for these receptors to recruit corepressors such as NCoR to actively repress transcription of its target genes. Although considerably more is known about the function of REV-ERB $\alpha$  than REV-ERB $\beta$ , it is assumed that they play similar physiological roles based on their conserved patterns of expression and recognition of identical DNA binding sites. REV-ERB $\alpha$  plays a critical role in regulation of the circadian rhythm where it directly modulates the expression of *Bmal1* expression.<sup>3,4</sup> Additionally, REV-ERB $\alpha$  has been shown to regulate metabolic processes including lipid and glucose metabolism.<sup>2,5–7</sup>

Soon after characterization of REV-ERB as a ligand-dependent NR, the first synthetic ligand was identified.<sup>8</sup> This ligand acted as an agonist, mimicking the action of heme, in cell-based assays and also was able to reset the circadian rhythm in a phasic manner.<sup>8</sup> Our lab examined this compound, GSK4112/SR6452 (Figure 1A), and found that it induced adipogenesis in a manner similar to treatment with heme or overexpression of REV-ERB $\alpha$  in 3T3-L1 cells.<sup>9</sup>

Upon further analysis of compounds within the GSK4112 tertiary amine scaffold, we identified one compound, SR8278 (Figure 1A and B), that displayed REV-ERB $\alpha$  antagonist activity in contrast to the agonist activity displayed by GSK4112. The activity of SR8278 was first assessed in a cotransfection assay in HEK293 cells where chimeric receptors composed of the DNA-binding domain of GAL4 fused to either REV-ERB $\alpha$  or REV-ERB $\beta$  were transfected in the cells along with a luciferase reporter containing GAL4 binding sites in the promoter. We

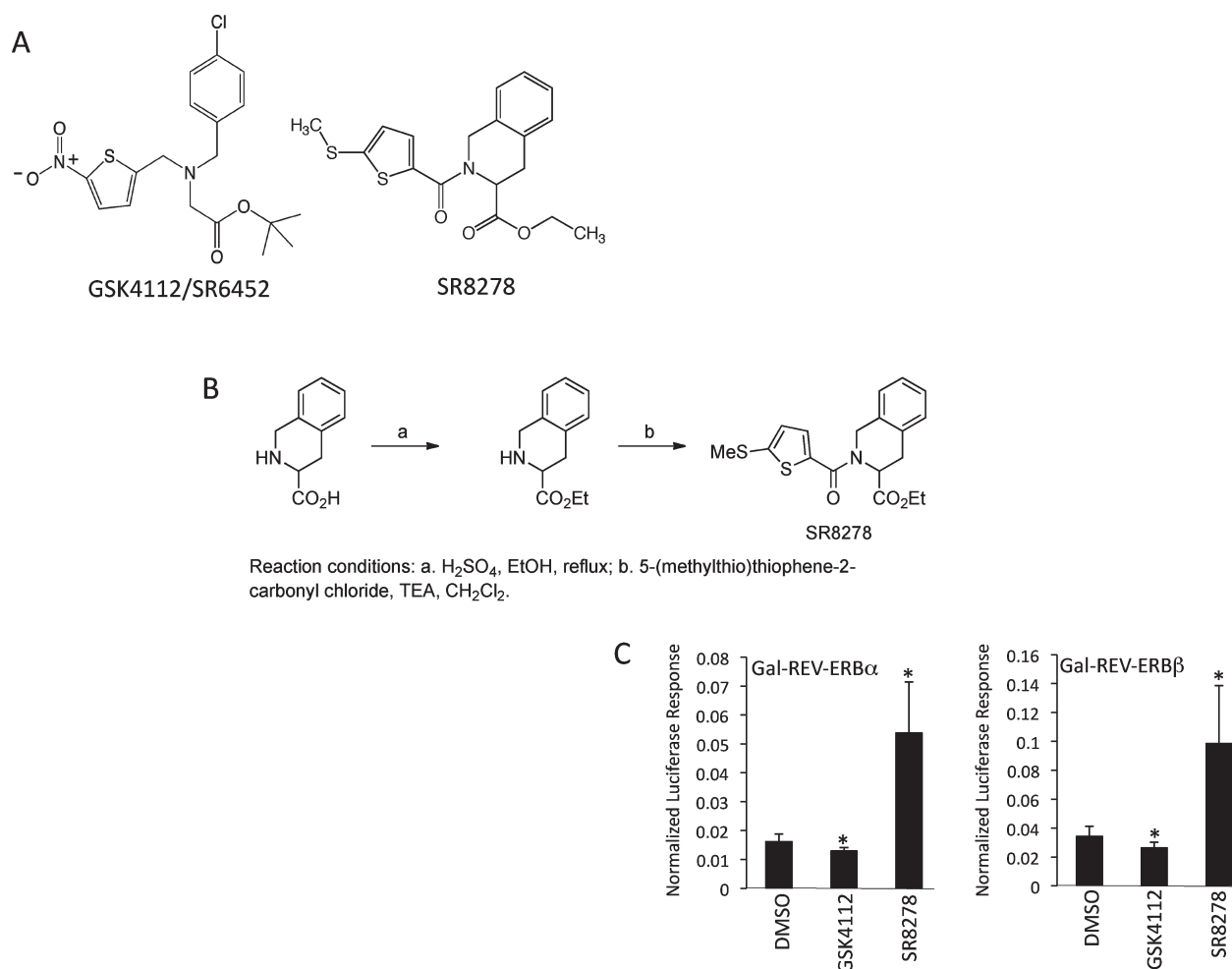
previously used this assay system to demonstrate that the repressive effects of REV-ERB $\alpha$  were heme-dependent since elimination of the heme binding activity of the receptor eliminated the ability of the receptor to repress transcription of the reporter gene.<sup>10</sup> We took advantage of this cell-based system where heme, a REV-ERB agonist, is always present and mediating repressor activity of the receptor to assess the activity of the antagonist, SR8278. As shown in Figure 1C, addition of GSK4112 to either the REV-ERB $\alpha$  or REV-ERB $\beta$  assay results in enhanced repression. Addition of SR8278 shows the opposite effect, enhancing the expression of the reporter gene presumably due to blocking the action of the endogenous agonist heme. We performed a similar assay where we transfected the cells with full-length REV-ERB $\alpha$  and a luciferase reporter driven by the three different promoters all derived from REV-ERB $\beta$  target genes. The *Bmal1*, *glucose 6-phosphatase (G6Pase)*, and *phosphoenolpyruvate carboxykinase (PEPCK)* promoters fused to luciferase were used. As shown in Figure 2, we treated the cells with either GSK4112 or SR8278 and observed a pattern very similar to that observed when we performed the GAL4 chimeric receptor assay. Generally, GSK4112 suppressed reporter gene expression and SR8278 enhanced expression.

We utilized the *Bmal1* reporter to examine the activity of SR8278 more closely. GSK4112 dose-dependently suppressed transcription driven by the *Bmal1* promoter ( $IC_{50} = 2.3 \mu M$ ) (Figure 3A). It should be noted that activity displayed by GSK4112 is in addition to the heme-dependent activity that is associated with REV-ERB $\alpha$  as is the case with all the cell-based

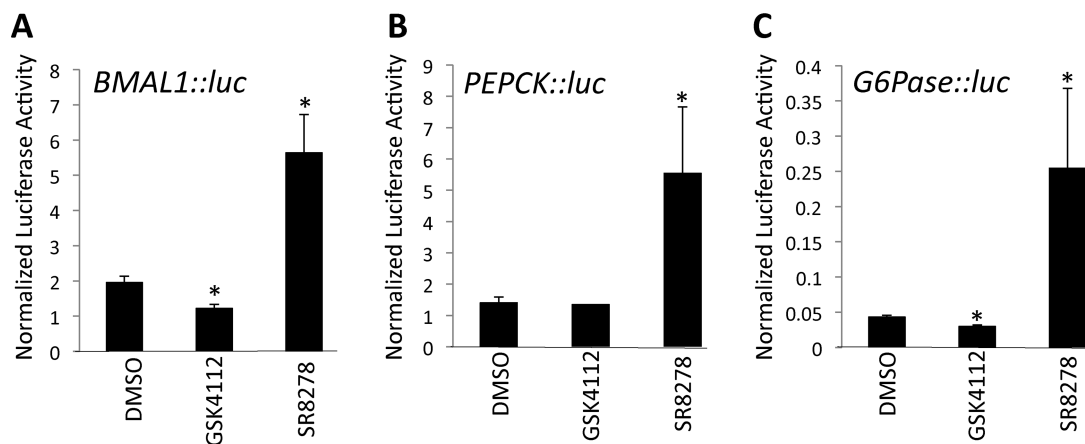
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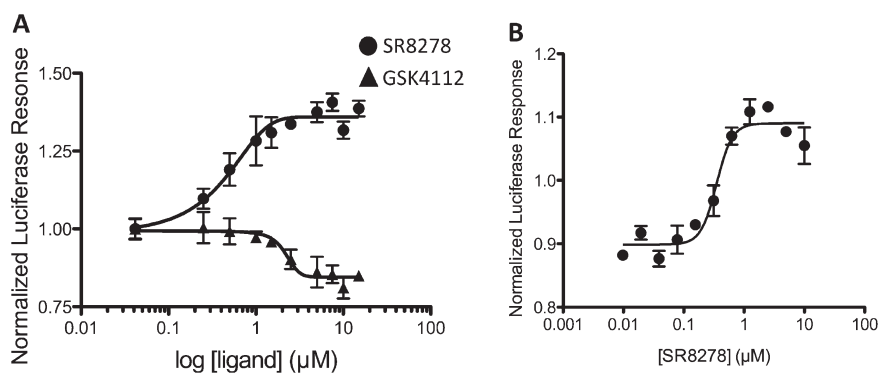
**Figure 1.** Identification of a REV-ERB $\alpha$  antagonist. (A) Structure of GSK4112 compared to SR8278. (B) Schematic illustrating the synthesis of SR8278. (C) GAL4-REV-ERB cotransfection assay illustrating the activity of GSK4112 and SR8278; 10  $\mu\text{M}$  drug was used. An internal Renilla luciferase control is included to normalize the data for all cotransfection assays. The action of REV-ERB is to suppress transcription so ligands that induce this effect are called agonists and ligands that block this effect are termed antagonists. Since heme is the natural agonist and is ubiquitous, these data are consistent with the antagonists blocking the endogenous activity of heme. REV-ERB that is incapable of binding heme is inactive and unable to suppress transcription. \* denotes  $p < 0.05$  via Student's  $t$  test.



**Figure 2.** Comparison of the cell-based activity of a REV-ERB agonist and antagonists. HEK293 cells were transfected with REV-ERB $\alpha$  and a luciferase reporter under the control of the promoter of REV-ERB target gene *Bmal1* (A), *PEPCK* (B), or *G6Pase* (C). Cells were then treated with 10  $\mu\text{M}$  compound for 24 h followed by the luciferase assay. \* denotes  $p < 0.05$  via Student's  $t$  test.

assays. It is clear that a mutant REV-ERB $\alpha$  that is devoid of heme binding activity is transcriptionally inactive and unable to modulate

transcription due to inability to recruit NCoR.<sup>1,2</sup> Addition of the antagonist, SR8278, results in inhibition of the REV-ERB $\alpha$

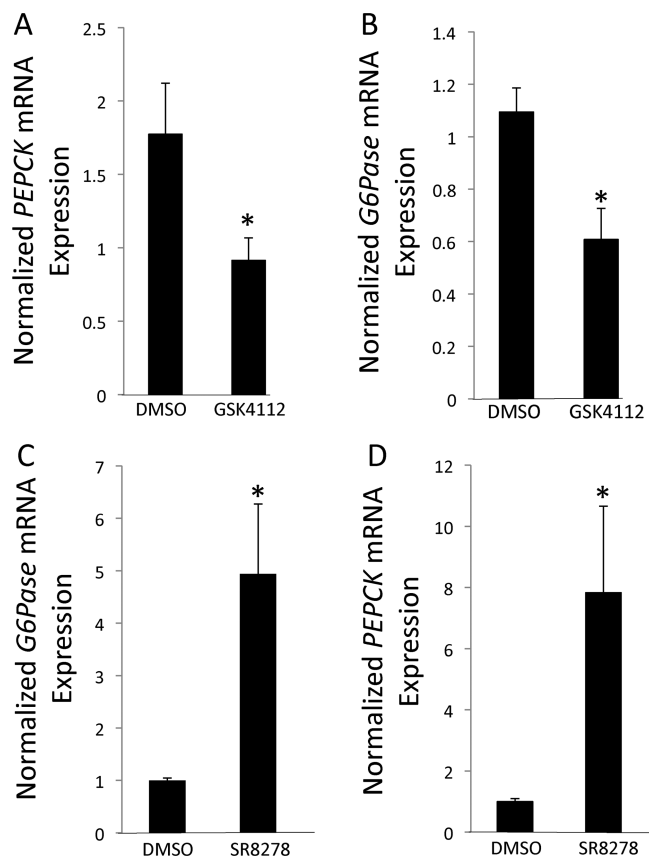


**Figure 3.** SR8278 functions as a REV-ERB antagonist. (A) Comparison of the cell-based activity of a REV-ERB agonist and antagonist. BMAL1-luciferase/REV-ERB $\alpha$  cotransfection assay was performed as in Figure 2, and cells were then treated with various doses of compounds for 24 h followed by the luciferase assay. (B) BMAL1-luciferase/REV-ERB $\alpha$  cotransfection assay was performed as in Figure 2 in the presence of the agonist, SR6452 (5  $\mu\text{M}$ ), and increasing amounts of the antagonist SR8278 were added. SR6452 decreased transcription (agonist activity), and increasing amounts of SR8278 blocked this activity dose-dependently ( $\text{IC}_{50}$  = 0.35  $\mu\text{M}$ ). This assay is a cell-based assay, and there are physiological levels of heme present. This likely results in the ability of SR8278 to bring the activity above baseline — the compound is antagonizing heme activity.

transcriptional repression activity in a dose-dependent manner ( $\text{EC}_{50}$  = 0.47  $\mu\text{M}$ ) (Figure 2). SR8278 displays potency nearly 5 times greater than that of GSK4112 in this assay. We also assessed the ability of SR8278 to block the action of GSK4112 in the cotransfection assay. Cells were treated with 5  $\mu\text{M}$  GSK4112, resulting in suppression of transcription as shown in Figure 3B. Increasing doses of SR8278 eliminated the activity of GSK4112, and as illustrated in Figure 3, activity increased above baseline due to the effect of SR8278 also blocking the action of heme. The potency of SR8278 in this assay was 0.35  $\mu\text{M}$ .

In order to further characterize the activity of SR8278, we assessed the ability of this compound to modulate the expression of target gene expression in cells that express REV-ERB $\alpha$  endogenously. REV-ERB $\alpha$  regulates glucose production in hepatocytes via modulation of the expression of key genes such as *G6Pase* and *PEPCK*.<sup>2</sup> REV-ERB $\alpha$  suppresses the expression of these genes in a heme-dependent manner, and the synthetic REV-ERB $\alpha$  ligand, GSK4112, also suppresses the expression of these genes (Figure 4A and B).<sup>2,11</sup> In HepG2 cells, endogenous levels of heme acting on REV-ERB $\alpha$  maintain a tonic suppression of these genes,<sup>2</sup> and thus we expected that addition of SR8278 would result in an increase in expression of these genes due to blocking of the action of the endogenous agonist. As shown in Figure 4C and D, this is exactly what we observed. Treatment of HepG2 cells with SR8278 resulted in a significant increase in the expression of either *G6Pase* or *PEPCK* mRNA expression.

In summary, we report the identification of the first REV-ERB $\alpha$  antagonist in this study. Treatment of HepG2 cells with SR8278 results in increased expression of REV-ERB $\alpha$  target genes consistent with the compound blocking the action of the endogenous agonist, heme. In a cell-based assay, SR8278 is considerably more potent than the other synthetic REV-ERB $\alpha$  ligand, GSK4112, which functions as an agonist. Like GSK4112,<sup>11</sup> SR8278 displayed poor pharmacokinetic properties that will likely limit its use to biochemical and cell-based assays (data not shown). Nevertheless, SR8278 represents a novel chemical tool that can be used to probe the function of REV-ERB $\alpha$  in cell-based models and may be used as a point to initiate optimization of more potent and efficacy REV-ERB $\alpha$  antagonists as well as *in vivo* probes.



**Figure 4.** Modulation of the expression of REV-ERB $\alpha$  target genes, *G6Pase* and *PEPCK*, by a REV-ERB $\alpha$  agonist, GSK4112, and antagonist, SR8278. HepG2 cells were treated with 10  $\mu\text{M}$  drug or vehicle (DMSO) for 24 h prior to harvesting the cells for mRNA. (A) Suppression of *PEPCK* mRNA expression by GSK4112. (B) Suppression of *G6Pase* mRNA expression by GSK4112. (C) Stimulation of *PEPCK* mRNA expression by SR8278. (D) Stimulation of *G6Pase* mRNA expression by SR8278. Note that the endogenous REV-ERB $\alpha$  agonist, heme, is present in cells, and thus there is basal suppression of REV-ERB $\alpha$  target genes. Addition of a REV-ERB $\alpha$  antagonist would be expected to block this leading to apparent stimulation of gene expression due to blocking heme action. Gene expression was normalized to cyclophilin mRNA. \* denotes  $p < 0.05$  via Student's *t* test.

## METHODS

**Synthesis of SR8278.** To a mixture of 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (2 g) in anhydrous ethanol (50 mL) was added H<sub>2</sub>SO<sub>4</sub> (1 mL). The resulting solution was refluxed for 18 h, cooled to RT, and then concentrated in vacuo to remove the ethanol. The residue was dissolved in ethyl acetate and saturated aqueous NaHCO<sub>3</sub> solution, and the layers were separated. The organic layer was washed with satd aqueous NaHCO<sub>3</sub> (2×) and brine (2×), dried (MgSO<sub>4</sub>), and concentrated to afford ethyl 1,2,3,4-tetrahydroisoquinoline-3-carboxylate as a pale yellow oil, which slowly crystallized. The compound was used without further purification.

To the crude aminoester in methylene chloride (50 mL) at 0 °C was added triethylamine (1.5 equiv). A solution of 5-(methylthio)thiophene-2-carbonyl chloride (1.2 equiv) in methylene chloride was added dropwise, and the reaction was allowed to warm to RT overnight. After 18 h, the reaction was concentrated to remove the solvent. The crude residue was resuspended in ethyl acetate and washed with 1 M HCl (2×), NaHCO<sub>3</sub> (2×), and brine (1×), dried (MgSO<sub>4</sub>), and concentrated. The crude product was purified by chromatography on silica gel to afford SR8278 as a near colorless oil >95% pure as judged by reverse-phase analytical HPLC analysis.

**Cell Culture and Cotransfections.** HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C under 5% CO<sub>2</sub>. HepG2 cells were maintained and routinely propagated in minimum essential medium supplemented with 10% fetal bovine serum at 37 °C under 5% CO<sub>2</sub>. Twenty-four hours prior to transfection, HepG2 cells were plated in 96-well plates at a density of 15 × 10<sup>3</sup> cells/well. Transfections were performed using Lipofectamine 2000 (Invitrogen). Sixteen hours post-transfection, the cells were treated with vehicle or compound. Twenty-four hours post-treatment, the luciferase activity was measured using the Dual-Glo luciferase assay system (Promega). The values indicated represent the means ± SE from four independently transfected wells. The experiments were repeated at least three times. The REV-ERB $\alpha$  and reporter constructs have been previously described.<sup>9,12</sup>

**cDNA Synthesis and Quantitative PCR.** Total RNA extraction and cDNA synthesis as well as the QPCR were performed as previously described.<sup>10,12</sup>

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