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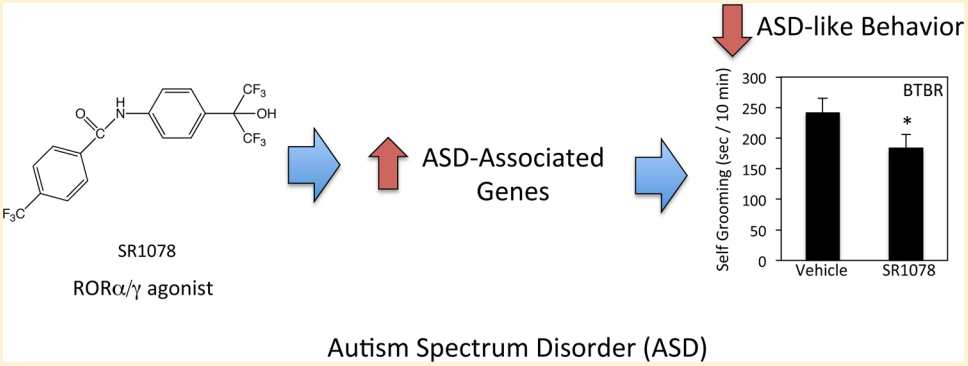
Therapeutic Effect of a Synthetic RORα/γ Agonist in an Animal Model of Autism

Yongjun Wang, Cyrielle Billon, John K. Walker, and Thomas P. Burris*

Department of Pharmacology & Physiology, Saint Louis University School of Medicine, St. Louis, Missouri 63104, United States



ABSTRACT: Autism is a developmental disorder of the nervous system associated with impaired social communication and interactions as well excessive repetitive behaviors. There are no drug therapies that directly target the pathology of this disease. The retinoic acid receptor-related orphan receptor α (RORα) is a nuclear receptor that has been demonstrated to have reduced expression in many individuals with autism spectrum disorder (ASD). Several genes that have been shown to be downregulated in individuals with ASD have also been identified as putative RORα target genes. Utilizing a synthetic RORα/γ agonist, SR1078, that we identified previously, we demonstrate that treatment of BTBR mice (a model of autism) with SR1078 results in reduced repetitive behavior. Furthermore, these mice display increased expression of ASD-associated RORα target genes in both the brains of the BTBR mice and in a human neuroblastoma cell line treated with SR1078. These data suggest that pharmacological activation of RORα may be a method for treatment of autism.

KEYWORDS: *Autism, drug discovery, nuclear receptor, orphan receptor, behavior*

Autism is a developmental disorder affecting the nervous system that is associated with impaired social interactions and communication, and repetitive behaviors. Approximately 1.5% of children in the United States are diagnosed with autism spectrum disorder (ASD). Only two drugs are approved for the treatment of autism, the antipsychotics resperidone and aripiprazole. These drugs are used to treat irritability associated with autism rather than the core symptoms, and thus, there is a clear unmet medical need for drugs that effectively target this disease.[1]

Nuclear receptors are ligand regulated transcription factors and many members of this superfamily are validated targets for drugs to treat a range diseases in humans. The retinoic acid receptor-related orphan receptors (RORα, β, and γ) are nuclear receptors that were originally identified as constitutively active orphan receptors, but several studies, including many from our laboratory, have demonstrated that activity of these receptors can be regulated by natural and synthetic ligand that bind directly to their ligand binding domains.[2−13] RORs regulate a range of physiological processes including the circadian rhythm, immunity, metabolism, and development. RORα and RORγ are widely expressed whereas RORβ expression is much more restricted and is primarily found in the central nervous system. A clear role for RORα in neural development has been demonstrated based on a murine strain (Staggerer) that contains a spontaneous deletion within the gene encoding RORα (*Rora*).[14] These mice display ataxia and hypotonia and are small relative to wild type mice. The major neural deficit is underdevelopment of the cerebellar cortex where there is a deficiency in granule and Purkinje cells.[14]

The RORs have been linked to autism in human in several studies. In 2010, Nguyen and co-workers reported that RORα protein expression was significantly reduced in the brains of autistic patients and this decrease in expression was attributed to epigenetic alterations in the *RORA* gene.[15] Additional work from this group demonstrated that multiple genes associated with autism spectrum disorder are direct RORα target genes and suggested that reduction of RORα expression results in reduced expression of these genes associated with the disorder leading to the disease.[16] Independently, Devanna and Vernes demonstrated that miR-137, a microRNA implicated in neuropsychiatric disorders, targets a number of genes associated with autism spectrum disorder including *RORA*.[17] There are

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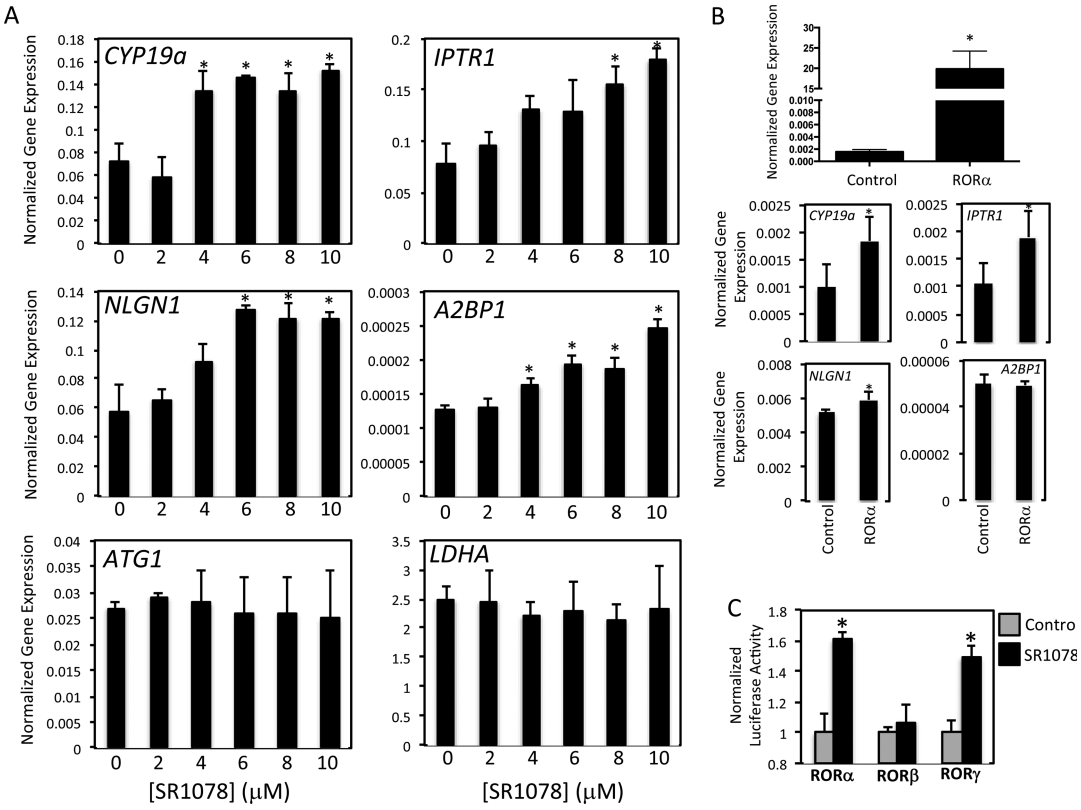

Figure 1. SR1078 increases the expression of ASD-associated genes in SH-SY5Y cells. (A) SH-SY5Y were treated with SR1078 (multiple doses), and after 24 h cells were harvested and cDNA prepared to assess the expression of ASD-associated genes by quantitative PCR. *$p < 0.05$, one-way ANOVA followed by Dunnett tests. (B) Overexpression of RORα in SH-SY5Y cells leads to increased expression of ASD associated genes. *$p < 0.05$, Student's t test. (C) SR1078 (10 μM) does not increase RORβ transcriptional activity in a cotransfection assay in HEK293 cells but does significantly increase the transcriptional activity of RORα and RORγ. Student's t test.

also additional links between RORα and autism. Deficiency of Purkinje cells is one of the most consistently identified neuroanatomical abnormalities in brains from autistic individuals[18,19] and RORα is critical in development of the Purkinje cells.[20−23] Significant circadian disruptions have also been recognized in autistic patients,[24−27] and RORs play a critical role in regulation of the circadian rhythm.[7,28] Additionally, the staggerer mouse displays behaviors that are associated with autism including abnormal spatial learning, reduced exploration, limited maze patrolling, and perseverative behavior relative to wt mice.[29−33]

Based on this data, we hypothesized that enhancing RORα activity with a synthetic agonist may be efficacious in treating autism by restoring RORα activity to natural levels by activating the limited RORα that is present. We previously described the discovery of a synthetic ROR agonist, SR1078, with mixed RORα/γ activity.[8] SR1078 directly binds to the ligand binding domain of RORα and RORγ and increases the transcriptional activity of these receptors, leading to stimulation of RORα/γ target gene transcription.[8,34,35] SR1078 also functions in vivo effectively increasing the expression of RORα/γ target genes.[8,35] We first tested the ability of SR1078 to increase the expression of putative RORα target genes that are also ASD-associated genes. The ASD-associated genes, *A2BP1* (*ataxin 2-binding protein 1*), *CYP19A1* (*aromatase*), *ITPR1* (*inositol 1,4,5-trisphosphate receptor type 1*), and *NLGN1* (*neuroligin-1*) identified by Sarachana and Hu as RORα target genes that may be associated with the link between the dysregulation of RORα expression and the pathobiology of ASD.[16] These authors used the human neuroblastoma cell line SH-SY5Y to demonstrate that knock-down of RORα expression resulted in decreased expression of these genes.[16] We treated SH-SY5Y cells with various concentrations of SR1078 and observed a dose-dependent increase in expression of *A2BP1*, *CYP19A1*, *NLGN1*, and *IPTR1* (Figure 1). EC_{50}'s were in the range of 3−5 μM, which is consistent with the previously reported EC_{50} of SR1078 for activation of RORα target genes.[8] There was no increase in "global" gene expression since we observed that genes such as *autophagy related 1* (*ATG1*) and *lactate dehydrogenase* (*LDHA*) were not affected by SR1078 treatment (Figure 1) We also overexpressed RORα in these cells and examined the expression of these four genes. Given that RORα has constitutive transcriptional activation activity, we expected an increase in the expression of these four genes and indeed we observed an increase in three of the four genes (*CYP19A*, *IPTR1*, and *NLGN1*) (Figure 1B). A2BP1 expression did not change with overexpression of RORα and this may be due to a situation where RORE sites regulating A2BP1 may be already saturated with RORα whereas the sites regulating the other three genes are not. Thus, increasing RORα would increase expression of some of the genes but not others. Another point to address is that RORα overexpression was substantial and not matched by the level of increase observed in the responsive ASD-associated genes. Again, this is likely due to circumstances that once the regulatory RORE sites are occupied there is no further ability to increase the expression of the target genes. It is key to emphasize that loss of RORα resulted in decreased expression

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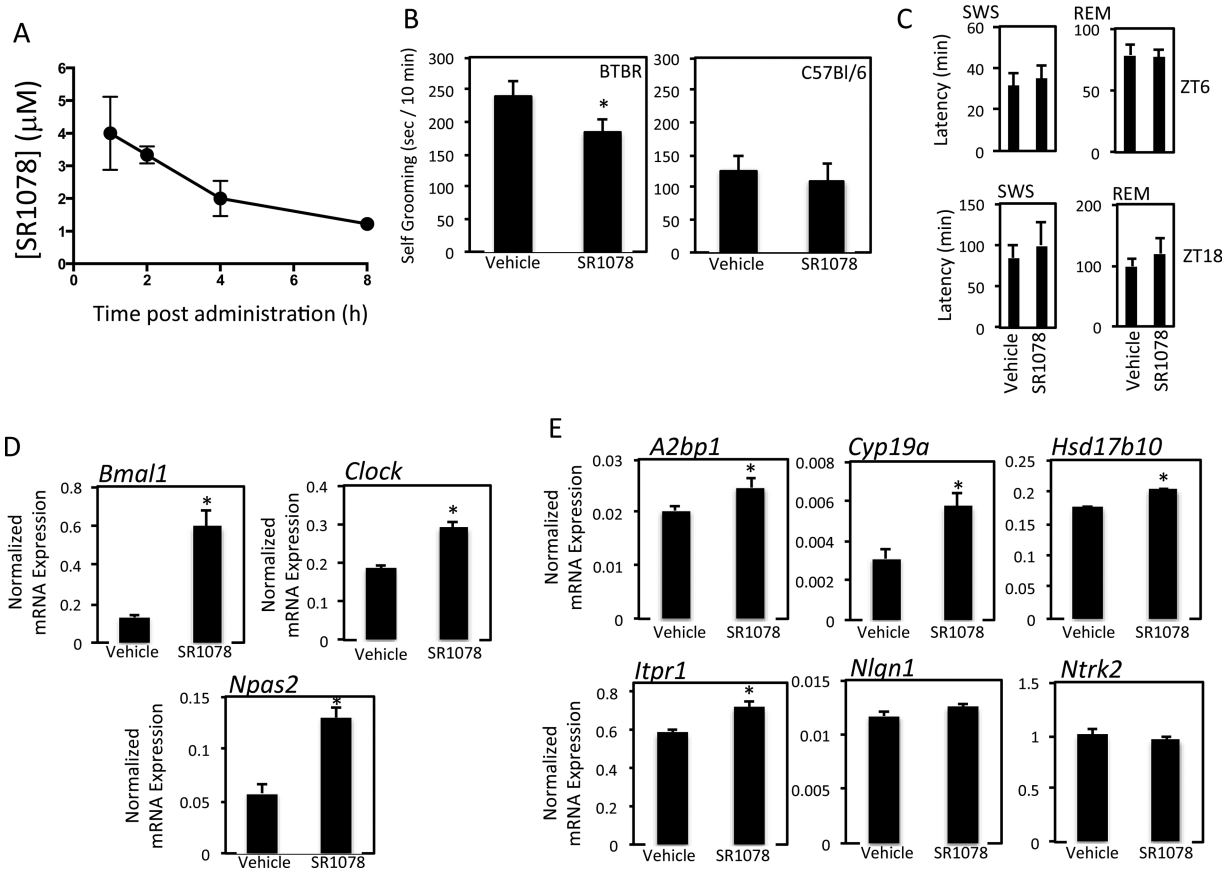

Figure 2. Therapeutic effect of pharmacological activation of RORα in autism. The RORα/γ agonist, SR1078, increases the expression of genes that are known to be downregulated in brains of autistic individuals and significantly reduces repetitive behavior in a mouse model of autism (BTBR mice). (A) Brain levels of SR1078 after 10 mg/kg i.p. administration. (B) SR1078 treatment in BTBR mice (a mouse model for autism) results in reduced repetitive behavior, suggesting potential therapeutic utility of RORα agonists in treatment of autism (left panel). SR1078 treatment in C57Bl/6 mice does not alter their grooming behavior (right panel). (C) Administration of SR1078 at either ZT6 or ZT 18 does not alter latency to enter slow wave sleep (SWS) or REM sleep as assessed by EEG/EMG indicating that SR1078 does not sedate the mice. (D) Treatment of BTBR mice with SR1078 leads to increased expression of well-characterized RORα target genes in brain tissue confirming target engagement. (E) Expression of genes that are known to be downregulated in human autistic brains is upregulated by SR1078 treatment in BTBR mice. *$p < 0.05$, Student's t test, $n = 8$.

of these genes[16] providing important data supporting a physiological role for natural levels of RORα maintaining the expression of these ASD-associated genes.

All three RORs (RORα, RORβ, and RORγ) are expressed in the brain and recognize identical DNA response elements, thus it is likely that they have overlapping roles. A recent study has also characterized dysregulation of RORβ expression in the brains of autistic individuals.[36] Thus, it was considered whether SR1078 was exerting at least some of its activity via targeting RORβ. We assessed the potential for SR1078 to target RORβ using a cotransfection assay similar to those used in our original description of SR1078 to characterize the ability of the compound to activate RORα and RORγ.[8] In HEK293 cells transfected with an expression vector for RORβ and a reporter responsive to this receptor (*Bmal1* promoter driven luciferase), we observed no activity of SR1078 at a concentration of 10 μM while we observe significant enhancement of gene expression when RORα or RORγ are transfected into the cells (Figure 1C).

Based on the data indicating that modulation of RORα levels in SH-SY5Y alters the expression of ASD-associated gene expression as well as data illustrating that we could pharmacological target this pathway, we believed that examination of the potential efficacy of SR1078 in an animal model of autism was warranted. The inbred BTBR mouse strain is commonly used to examine the efficacy of potential therapeutics for autism, as it displays behavioral phenotypes relevant to all three diagnostic symptoms of autism.[37−47] First, the mice display long bouts of repetitive self-grooming observed to be similar to the stereotyped and repetitive patterns of behavior seen in autistic patients. Second, BTBR mice display low levels of social interactions relative to other strains of mice. Third, these mice exhibit altered communication patterns (ultrasonic vocalizations in response to social cues) relative to other strains. The BTBR mouse model of autism has become a standard model for assessment of potential efficacy of drugs that would be used to target autism in the clinic. A range of drugs, both clinically approved and in development, have been examined including mGluR5 allosteric modulators, fluoxitene, GABA receptor agonists, oxytocin, and 5HT-2A receptor antagonists.[45,48−52]

We dosed BTBR mice or C57Bl/6 mice (as a control) with 10 mg/kg SR1078 (Q.D., i.p.) for 2 weeks followed by monitoring repetitive grooming behavior of single housed mice as described by other investigators.[45,50] Our previous studies determined that this dose was effective activating RORα in

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vivo[8,35] and as shown in Figure 2A, brain levels of SR1078 1h after injection are ~4 μM and are maintained above 1 μM for at least 8 h post administration (10 mg/kg i.p.). We observed a significant 25% reduction in repetitive grooming behavior in the BTBR mice as illustrated in Figure 2B whereas there was no significant effect in C57Bl/6 mice. As expected, the C57Bl/6 mice displayed significantly lower levels of repetitive grooming behavior relative to the BTBR mice (Figure 2B). A decrease in grooming behavior may be associated with sedative action of a particular drug, and although we did not expect this to be the case based on the strain selective decrease in grooming action, we also examined the effect of SR1078 on sleep using EEG/EMG. We administered SR1078 (10 mg/kg, i.p.) at either ZT6 or ZT18 (zietgeber time 6 or 18 with ZT0 as lights on) and observed no alteration in the latency to enter either slow wave sleep or REM sleep indicating that there was no sedative activity (Figure 2C). We confirmed target engagement by observation that well characterized RORα target genes *Bmal1*, *Clock*, and *Npas2*[7,28] were upregulated (in brain tissue) following the SR1078 treatment (Figure 2D) in the BTBR mice. In the BTBR mice, we also examined the expression of the genes outlined in Figure 1 as putative RORα target genes that are downregulated in autistic individuals. As illustrated in Figure 2E, we observed that expression of three of the four genes (*A2bp1*, *Cyp19a*, and *Itpr1*, but not *Nlgn1*) were significantly increased in response to treatment with SR1078 in the brain. Thus, the ROR agonist-dependent decrease in repetitive grooming behavior in this BTBR mice correlates with an increase in expression in the ASD-associated genes that are regulated in a ROR-dependent manner.

SR1078 is a relatively low potency compound with limited RORα efficacy (3−5 μM EC_{50} E_{max} ~ 40%),[8] but the efficacy compares favorably to other classes of compounds that have been optimized such as a 38% decrease in the same model induced by the mGluR5 allosteric modulator GRN-529[49] and a 47% reduction by the mGluR5 antagonist MPEP.[45] Both of these compounds have been optimized and display high potency (single digit nanomolar range at mGluR5) and strong efficacy.[53,54] Thus, we believe that focused optimization of RORα ligands will provide compounds that will have improved efficacy in this model. It should also be noted that SR1078 has both RORα and RORγ agonist activity[8] and a RORα selective agonist has not yet been developed. Thus, it is possible that the RORγ activity of this compound may also play a role in its efficacy in this model of autism. In summary, we have demonstrated that a synthetic RORα/γ agonist is able to increase the expression of key genes whose decrease in expression is associated with ASD both in cell culture and in vivo. Furthermore, the agonist decreases repetitive behavior in an animal model of autism suggesting that it is possible that ROR agonists may hold utility in treatment ASD.

METHODS

SR1078. SR1078 was prepared as previously described using standard medicinal chemistry methodology.[8]

Animals. Male BTBR T^{+} $Itpr3^{tf}$/J (BTBR) and C57Bl/6 mice were purchased from Jackson Laboratory and housed in groups of three to four mice per cage with food and water available ad libitum. The housing room was maintained at 23 °C on a 12 h light/dark cycle (lights off at 6 p.m.). BTBR or C57Bl/6 mice were administered 10 mg/kg SR1078 or vehicle (Q.D., i.p.) at 8 weeks of age, and behavior testing was performed 2 weeks after drug administration. The whole brains of the mice were harvested after behavior testing. RNA isolation and cDNA synthesis were performed as described below and as previously performed.[55,56] Pharmacokinetic studies were performed as previously described in C567Bl6 mice.[8] EEG/EMG studies were performed as previously described[57] using C57Bl/6 mice. Mice were administered 10 mg/kg SR1078 at either ZT6 or ZT18 (lights on at ZT0). All experimental procedures were approved by the Institutional Animal Care and Use Committees of Saint Louis University and/or The Scripps Research Institute.

Repetitive Grooming. Each subject was placed individually in a clean standard mouse cage with a video camera 15 cm away from the cage. The behavior of the mice was recorded for 20 min. The first 10 min was acclimation time, and the second 10 min was used to analyze the behavior. Two investigators watched the video and scored the cumulative time spent in self-grooming. The average cumulative time of each mouse was used for statistical analysis.

Cell Based Experiments. The human neuroblastoma cells SH-SY5Y (ATCC, Manassas, VA) were maintained in 1:1 modified Eagle's medium (MEM) and Ham's F12 media supplemented with 15% (v/v) fetal bovine serum at 37 °C with 5% CO2. At 24 h prior to treatment, SH-SY5Y cells were plated at density of 25×10^4 cells/well in a 24-well plate. The cells were treated with SR1078 at 0, 2, 4, 6, 8, and 10 μM concentration for 24 h. For RORα overexpression assay, SH-SY5Y cells were plated in 24-well plates at a density of 1×10^5/well 24 h prior to transfection. The cells were cotransfected with 1 μg pcDNA3.1_RORα or pcDNA_LacZ for 24 h using Lipofectamine 2000 (Invitrogen) and then harvested for RNA isolation. RNA isolation and cDNA synthesis were performed as described below.

RNA Isolation and cDNA Synthesis. Total RNA from SH-SY5Y cells was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. For brain from mice, the total brain of mice was homogenized using The Bullet Blender homogenizer (Next Advance, Averill Park, NY) and RNA was isolated using using TRIzol RNA extraction. cDNA synthesis was performed using the qScript cDNA Synthesis Kit (Quanta, Gaithersburg, MD) according to the manufacturer's instructions.

Cotransfection Assay. HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum at 37 °C under 5% CO2. At 24 h prior to transfection, HEK293 cells were plated in 96-well plates at a density of 15×10^3 cells per well. Transfections were performed with 50 ng of pcDNA3.1_RORα (or RORβ or RORγ) or pcDNA_LacZ, 100 ng pGL3-Bmal1 and 25 ng of pGL4.73 [*hRluc*/SV40] (Promega, Madison, WI) using Lipofectamine 2000 (Invitrogen). At 24 h post-transfection, the cells were treated with vehicle or 10 μM SR1078. At 24 h post-treatment, the luciferase activity was measured using the Dual-Glo luciferase assay system (Promega). The values indicated represent the means and SE from four independently transfection.

QPCR. Quantitive PCR was performed on QuantStudio 7 Flex (ABI) platform as previously described.[55,56] The amplification conditions were as follows: 50 °C for 2 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min. Relative mRNA expression of each target gene was normalized to cyclophilin B using standard curve method. Ct values for the various amplicons were as follows: mouse genes (*Cypb* 21−22, *Bmal1* 21−23, *Clock* 24−25, *Npas2* 21−23, *Iptr1* 22−23, *Cyp19a* 28−30, *A2bp1* 26−28, *Nlgn1* 27−28, *Ntrk2* 21−22, *Hsd17b10* 23−24); human genes (*CYPB* 19−21, *A2BP1* 30−32, *CYP19A* 23−24, *NLGN1* 22−24, *RORA* 11−25).

Statistical Analysis. All data are expressed as the mean ± SEM (*N* as indicated in the figure legends). The statistical test used to determine significant differences between treatment groups is indicated in the figure legends. For in vivo experiments, the treatment groups were blinded to the investigators. Mice were randomized to treatment groups, to control for potential effects of cage mates. Statistical tests for significant differences within dose response experiments were performed by one-way ANOVA followed by Dunnett tests. Statistical tests for significant differences within assays that compared a control/vehicle group to a single dose of SR1078 or RORα overexpression were performed by a Student's *t* tests.

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AUTHOR INFORMATION

Corresponding Author

*Phone: 314-977-6400. E-mail: burristp@slu.edu.

Author Contributions

T.P.B. conceived of the study. T.P.B., C.B., and Y.W. designed the study. C.B. and Y.W. conducted the experiments. J.K.W. synthesized the compound. T.P.B., C.B., and Y.W. analyzed the data, and wrote and edited the manuscript.

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Notes

The authors declare no competing financial interest.

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