Increasing Human Th17 Differentiation through Activation of Orphan Nuclear Receptor Retinoid Acid-Related Orphan Receptor γ (RORγ) by a Class of Aryl Amide Compounds[S]

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ABSTRACT

In a screen for small-molecule inhibitors of retinoid acid-related orphan receptor γ (RORγ), we fortuitously discovered that a class of aryl amide compounds behaved as functional activators of the interleukin 17 (IL-17) reporter in Jurkat cells. Three of these compounds were selected for further analysis and found to activate the IL-17 reporter with potencies of ~0.1 μM measured by EC_{50}. These compounds were shown to directly bind to RORγ by circular dichroism-based thermal stability experiments. Furthermore, they can enhance an in vitro Th17 differentiation process in human primary T cells. As RORγ remains an orphan nuclear receptor, discovery of these aryl amide compounds as functional agonists will now provide pharmacological tools for us to dissect functions of RORγ and facilitate drug discovery efforts for immune-modulating therapies.

Introduction

The retinoid-related orphan receptors (RORα, RORβ, and RORγ) are nuclear receptors that play critical roles in development, immunity, circadian rhythm, and cellular metabolism (Hamilton et al., 1996; Lau et al., 2008). The first member of the ROR subfamily of receptors (RORα) was originally identified based on sequence similarities to the retinoic acid receptor and therefore named "retinoic acid receptor-related orphan receptor" (Becker-André et al., 1993; Giguere et al., 1994). The highly similar receptors, RORβ and RORγ, were identified soon afterward (Carlberg et al., 1994; Hirose et al., 1994). Similar to all nuclear hormone receptors, the RORs display the conserved domain structure with a variable amino-terminal A/B domain; a central, highly conserved DNA-binding domain containing two zinc fingers (C domain) and hinge region (D domain); and a carboxyl-terminal ligand-binding domain (LBD; E domain). The three RORs display significant sequence similarities, and each ROR gene generates multiple isoforms based on alternative promoter usage and splicing. The RORs exhibit distinct patterns of expression. RORα is expressed in the liver, skeletal muscle, skin, lungs, adipose tissue, kidney, thymus, and brain (Hamilton et al., 1996). RORβ (NR1F2) exhibits the most restricted pattern of expression and is limited to the central nervous system (André et al., 1998a,b). RORγ is mostly expressed in the thymus, but significant expression is also found in the liver, skeletal muscle, adipose tissue, and kidney (Medvedev et al., 1996). All RORs recognize and bind to specific sequences of DNA termed ROR-response elements as monomers, and these ROR-response elements typically consist of an AGGTCA "half-site" with a 5'-AT-rich extension (Giguere et al., 1994; Hirose et al., 1994). The isoform of RORγ known as RORγt, is expressed exclu-

ABBREVIATIONS: ROR, retinoid acid-related orphan receptor; LBD, ligand binding domain; Th17, T-helper 17; IL, interleukin; CNS, conserved noncoding sequence; PBS, phosphate-buffered saline; SRC, steroid receptor coactivator; FRET, fluorescence resonance energy transfer; TR, time-resolved; APC, allophycocyanin; DMSO, dimethyl sulfoxide; T0901317, N-(2,2,2-trifluoroethyl)-N-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]-benzenesulfonamide; SR1078, N-(4-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)phenyl)-4-(trifluoromethyl)benzamide; inhibitor Y, N-(5-benzoyl-4-phenylthiazol-2-yl)-2-(4-(ethylsulfonyl)phenyl)acetamide; compound 1b, N-(4,6-dimethylbenzo[d]thiazol-2-yl)-3-methylthiophene-2-carboxamide; compound 1c, N-(2-(4-ethylphenyl)-2H-benzo[d][1,2,3]triazol-5-yl)propionamide; CD, circular dichroism; h, human; ELISA, enzyme-linked immunosorbent assay; inh%, percentage of inhibition.

sively in the immune system and is important to the development of the T-helper (Th) 17 cell subset implicated in many autoimmune diseases as well as the formation of secondary lymphoid tissues (Yang et al., 2008). A number of small-molecule inhibitors of RORγt have demonstrated modulation of Th17 functions in cell and animal models, thus validating RORγt as a therapeutic target for Th17-mediated diseases such as multiple sclerosis (Huh et al., 2011; Solt et al., 2011).

Binding of ligands to the LBD of nuclear receptors induces the recruitment of coactivators or corepressors to modulate transcriptional activities in the cell. Structural studies suggest that cholesterol, 7-dehydrocholesterol, and cholesterol sulfate are ligands for RORα and that *trans*-retinoic acid is the ligand for RORβ (Kallen et al., 2002, 2004). However, the LBD of RORγ is structurally different from those of RORα and RORβ, and the native agonist for RORγ is still not clearly identified. A key intermediate in bile acid metabolism, 7-α-hydroxycholesterol was shown to bind both RORα and RORγ with high affinity and suppress transcriptional activities of RORα and RORγ (Wang et al., 2010b). Kumar et al. (2010) discovered synthetic compounds that not only behave as ligands to RORα and RORγ but also suppress their transcriptional activities. A nonspecific RORγ synthetic agonist, SR1078, was identified by Wang et al. (2010a) but with a low potency at ~10 μM. In the course of compound screening looking for small-molecule inhibitors of RORγ, we fortuitously discovered small-molecule compounds that are capable of activating RORγ with ~0.1 μM potency, up to 100-fold more potent than *N*-(4-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)phenyl)-4-(trifluoromethyl)benzamide (SR1078). These agonists can be used as surrogate ligands in screening assays and may greatly assist the development of selective RORγ antagonists. Most importantly, these agonists were found to enhance human Th17 differentiation, and therefore have the potential to modulate human immune responses.

Materials and Methods

Plasmids. Human RORγ-LBD (Kallen et al. 2002, 2004) was expressed in *Escherichia coli* strain BL21 as an amino-terminal polyhistidine-tagged fusion protein. The plasmid encoding this recombinant protein was subcloned into a modified pET21a expression vector (Novagen, Billerica, MA). A modified polyhistidine tag (MKKHHHHHHHLVPRGS) was fused in-frame to residues 263 to 518 of the human RORγ sequence.

A luciferase reporter plasmid was generated by cloning human IL-17-3kb-CNS (10) promoter into pGL4-Luc2/hygro reporter plasmid (Promega, Madison WI). Human IL-17-3kb-CNS promoter was amplified by polymerase chain reaction from human genomic DNA from 293T cells using primers as follows: forward primer, 5′-GGTAC-CTGCCCTGCTCTATCCTGAGT-3′ (KpnI site is underlined) and reverse primer, 5′-AAGCTTGGATGGATGAGTTTGTGCCT-3′ (HindIII site is underlined). The 3-kb DNA band was excised, purified, and inserted into KpnI/HindIII-digested pGL4.31[luc2P/GAL4UAS/Hygro] (Promega) to generate the pIL-17-3kb-luciferase reporter construct. To overexpress RORγ in reporter cell line, the full-length cDNA of human RORγ identical to the published sequence GenBank
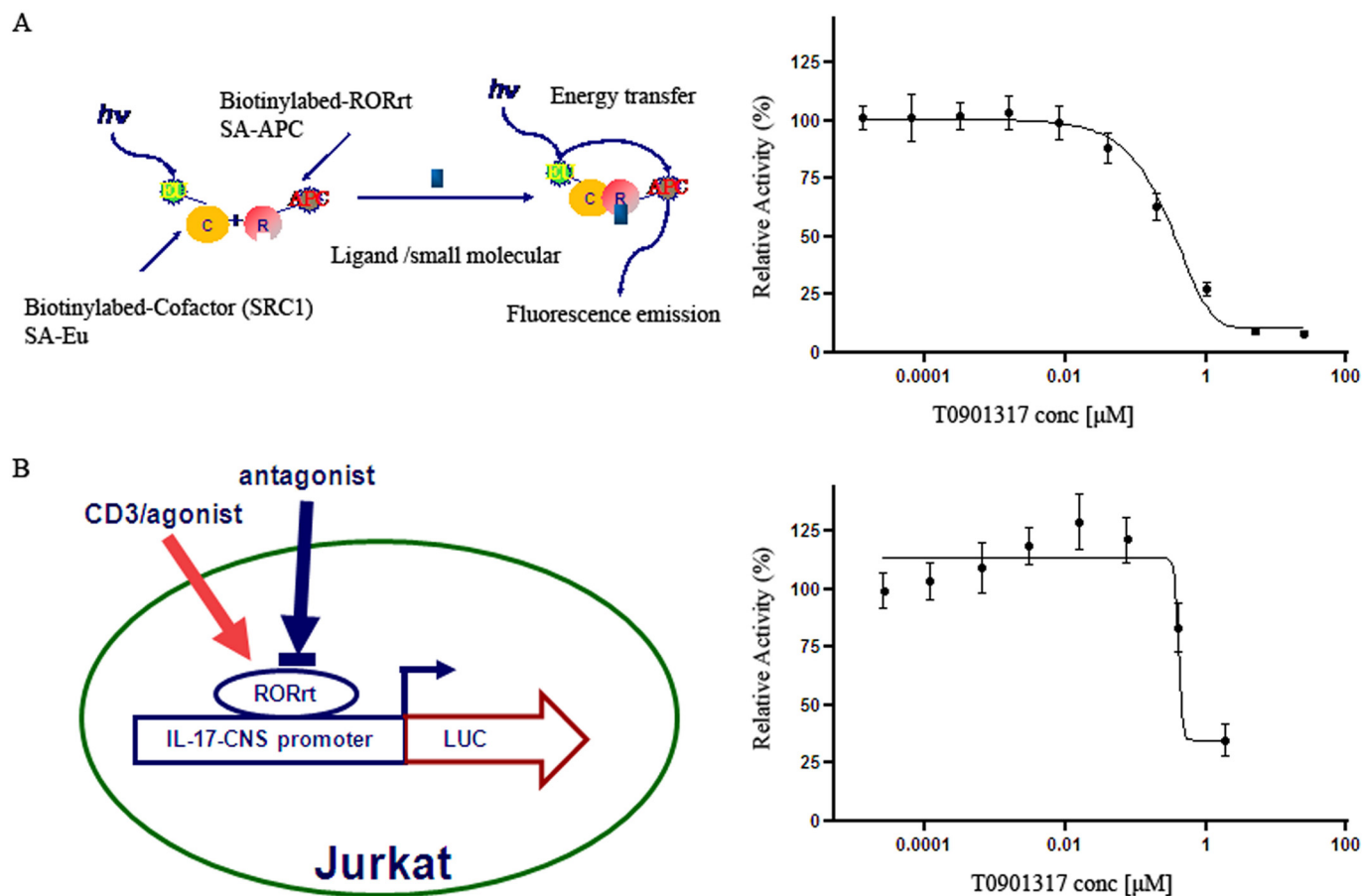
Fig. 1. Assay principles for RORγ. A, TR-FRET cofactor recruitment assay and validation by a tool compound. B, Jurkat IL-17 reporter assay and validation by a tool compound.

accession number NM_001001523 was cloned into pcDNA3.1 at the KpnI-NotI cloning sites to generate the RORγ overexpression plasmid.

RORγ-LBD Protein Purification. Approximately 50 g of *E. coli* cell pellet was resuspended in 300 ml of lysis buffer consisting of 30 mM imidazole and 150 mM NaCl, pH 7.0. Cells were lysed by sonication, and cell debris was removed by centrifugation for 30 min at 20,000*g* at 4°C. The cleared supernatant was loaded onto a column packed with ProBond nickel-chelating resin (Invitrogen, Carlsbad, CA), pre-equilibrated with 30 mM imidazole and 150 mM NaCl, pH 7.0. After washing to baseline absorbance with the equilibration buffer, the protein was eluted with a gradient from 30 to 500 mM imidazole, pH 7.0. Column fractions containing the RORγ-LBD protein were pooled and loaded onto a Superdex 200 column pre-equilibrated with 20 mM Tris-Cl and 200 mM NaCl, pH 7.2. The fractions containing the desired RORγ-LBD protein were pooled together.

RORγ-LBD Biotinylation. Purified RORγ-LBD was dialyzed with three changes of at least 100 times of the volume of PBS (100 mM sodium phosphate and 150 mM NaCl, pH 8.0). The concentration of RORγ-LBD was approximately 30 μM in PBS. A 5-fold molar excess of *N*-hydroxysuccinimide-long chain-biotin (Thermo Fisher Scientific, Waltham, MA) was added and incubated with occasional gentle mixing for 60 min at ambient room temperature. The resulted biotinylated RORγ-LBD was dialyzed with two buffer changes (Tris-buffered saline containing 5 mM dithiothreitol, 2 mM EDTA, and 2% sucrose, pH 8.0), each with at least 20 times of the volume. The biotinylated RORγ-LBD was distributed into aliquots, frozen on dry

ice, and stored at −80°C. The biotinylated RORγ-LBD was subjected to mass spectrometric analysis; approximately 95% of the protein had at least a single site of biotinylation, and the overall extent of biotinylation followed a normal distribution of multiple sites that ranged from one to five. A biotinylated peptide (CPSSHSSLTERH-KILHRLLQEGSPS) corresponding to amino acids ~676 to 700 of steroid receptor coactivator (SRC1) was generated using a similar method.

RORγ FRET Assay. The principle of the assay is outlined in Fig. 1A. The assays were performed in an assay buffer consisting of 50 mM NaF, 50 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.4, 50 μM 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate, 0.1 mg/ml bovine serum albumin, and 10 mM dithiothreitol in 384-well plates (Greiner Bio-One, Longwood, FL). The total volume was 10 μl/well. The europium-labeled SRC1 solution was prepared by adding an appropriate amount of biotinylated SRC and europium-labeled streptavidin (PerkinElmer Life and Analytical Sciences, Waltham, MA) into assay buffer, with final concentrations of 80 and 10 nM, respectively. The APC-labeled RORγ-LBD solution was prepared by adding an appropriate amount of biotinylated RORγ-LBD and APC-labeled streptavidin (CR130-100; PerkinElmer Life and Analytical Sciences) at a final concentration of 100 nM each. After 15 min of incubation at room temperature, a 20-fold excess of biotin was added to block the remaining free streptavidin. Equal volumes of europium-labeled SRC- and APC-labeled RORγ-LBD were then mixed with 0.6 μM (a compound previously found to enhance recruit-
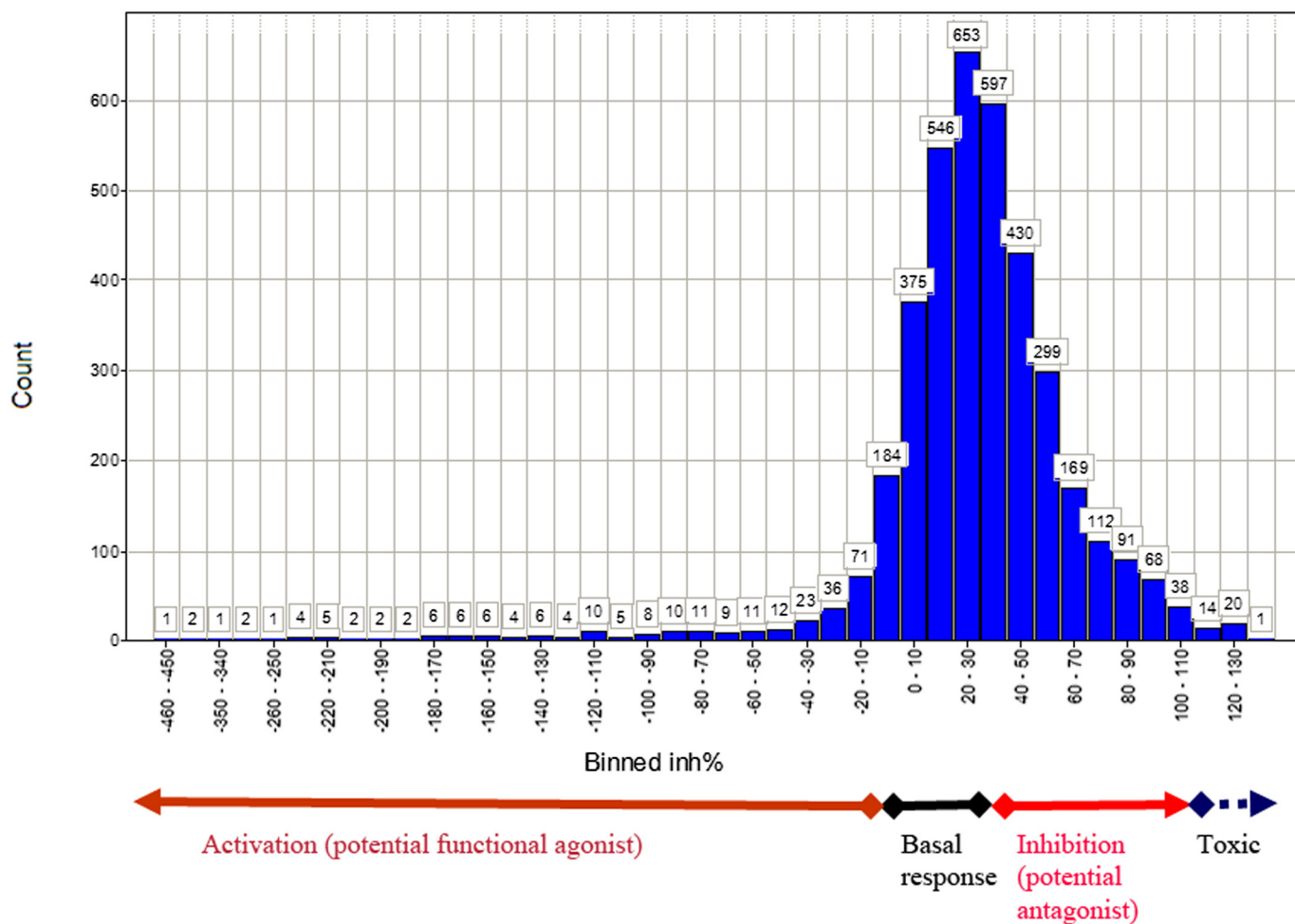

Fig. 2. A fraction of TR-FRET cofactor recruitment inhibitors behave as functional agonists in Jurkat cellular reporter assay. Approximately 4000 FRET screen hits were examined at 0.1, 1, and 10 μM for potential antagonist/agonist effects in the reporter assay. The inh% was defined as 1-(unknown-unstimulated)/(CD3 stimulated-unstimulated). Thus, <0% inh% represented activation effect of potential agonist (brown); ~0 to 20% inh% represented no significant effect on RORγ activity (black); ~20 to 100% inh% represented inhibition effect of potential antagonist (red); and >100% inh% is probably attributed to cellular toxicity of tested compounds (blue).

ment of SRC peptide; W. Zhang and Z. Zhang, unpublished results; see Supplemental Materials for structure and description of synthesis) and dispensed into 384-well assay plates at 10 μl volume/well. The 384-well assay plates had 100 nl of test compound in DMSO predispensed into each well. The plates were incubated for 1 h at room temperature and then read on ViewLux (PerkinElmer Life and Analytical Sciences) in LANCE mode configured for europeum-APC labels.

Jurkat Reporter Cell Line. The pIL-17-3kb-CNS promoter-luciferase reporter plasmid and the pcDNA3.1-RORγ overexpression plasmid were transfected into Jurkat cell line by electroporation. The

transfected cells were cultured in RPMI 1640 medium with 10% dialyzed fetal bovine serum in the presence of 800 μg/ml G418 (Geneticin) and 400 μg/ml hygromycin to select for both RORγ and reporter-expressing cells. Colonies of surviving stable cells were picked, and a reporter assay was used to select the final stable clones for assay development.

IL-17 Reporter Assay. The principle of the assay is outlined in Fig. 1B. Jurkat cells described above were counted and resuspended in assay medium (phenol red-free RPMI 1640) at 1×10^6/ml and then plated in 384-well plates, which contained 40 nl of test compound per well in DMSO at a density of 4×10^4 cells/well. Cells were either
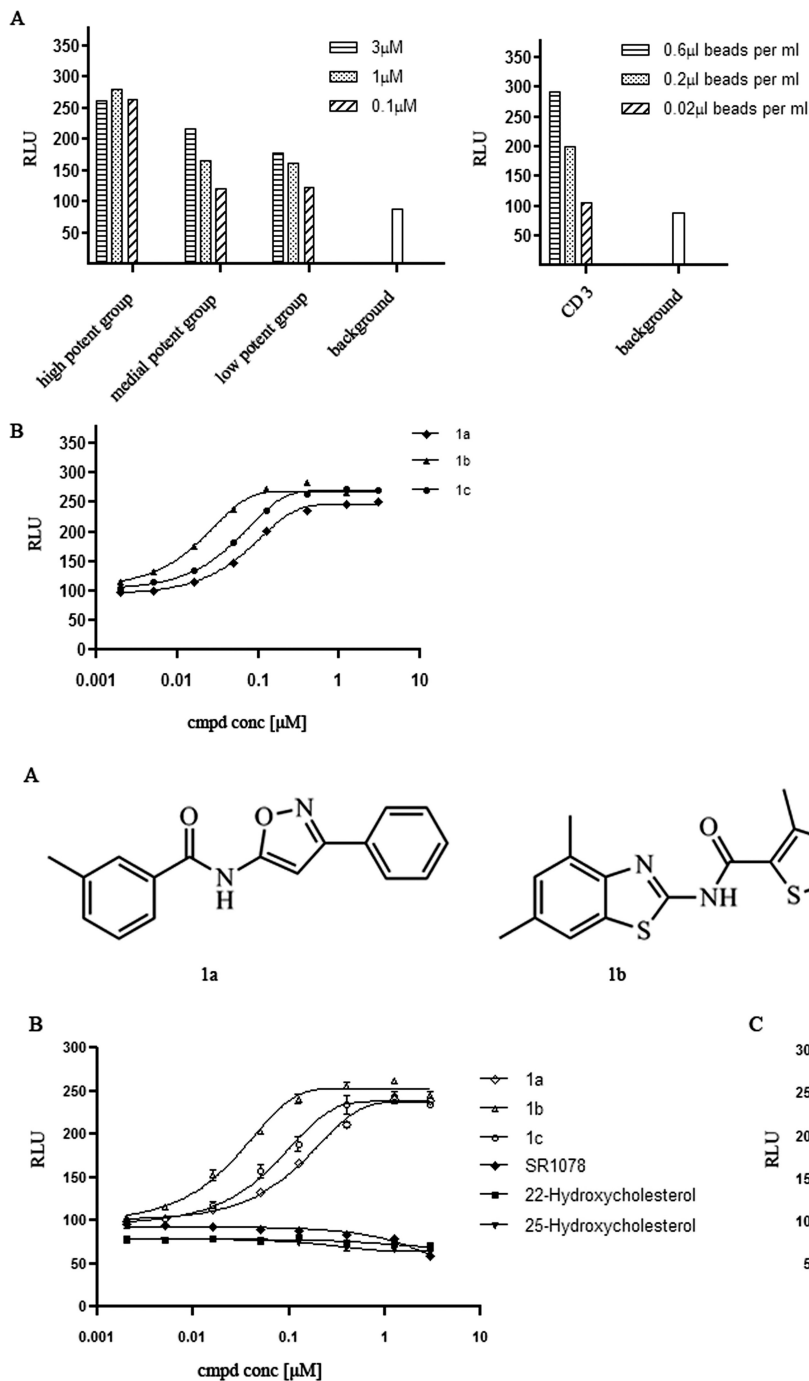
Fig. 3. Confirmation of functional agonists' activity in reporter assay. A, some representative compounds were selected based on their consistent activation effect observed in cellular functional assay at all three doses. Samples with CD3 beads alone at three doses (0.6 μl, 0.2 μl, and 0.06 μl/ml) were used as positive control. B, full dose-response analyses of the compounds in the absence of CD3 stimulation were used to confirm their agonist effect.

Fig. 4. Agonists' structures and their activities in cellular assay. Three representative compounds (1a, 1b, and 1c) from above activators were resynthesized in and tested to activate RORγ with pEC$_{50}$ of $\sim$0.1 μM. A, chemical structure of the three compounds. B, previously claimed agonists, SR1078 and 22- and 25-hydroxycholestrols, were tested and showed no effect in our assay. C, CD3 beads dose-response curve as positive control.

untreated or treated with CD3 microbeads (Miltenyi Biotec, Auburn, CA) at 1 μl of bead solution per 500,000 cells. After overnight incubation, luciferase levels were assayed by one-step addition of 40-μl substrate (Promega). Data were collected by ViewLux (using luciferase setting).

Compounds. T0901317 from Merck KGaA (Darmstadt, Germany) was used as the reference RORγ antagonist (Kumar et al., 2010). 22-Hydroxycholestrol and 25-hydroxycholestrol were purchased from Sigma-Aldrich (St. Louis, MO). SR1078 (Wang et al., 2010a) was synthesized according to published procedures.

Thermal-Shift Assay by Circular Dichroism. Circular dichroism (CD) data were obtained using a Jasco J-815 CD spectrometer (Jasco, Tokyo, Japan). The purified RORγ-LBD was diluted 1:10 into PBS buffer, pH 7.2, containing 10% glycerol at final concentration of 0.1 mg/ml in the absence or presence of 50 μM compounds (diluted from 10 mM DMSO stock). Wavelength scans were recorded from 280 to 190 nm with a bandwidth of 1 nm, response time of 1 s, and scanning speed at 50 nm/min. The protein unfolding was monitored at 224 nm with a bandwidth of 1 nm and response time of 8 s. The thermal unfolding was recorded from 20 to 85°C, with the a ramping rate of 5°C/min, sampling interval of 3°C, and equilibrium time of 5 s. The transition T_m was determined by fitting the CD signal versus temperature using a two-state unfolding model.

Th17 Differentiation Assay. Human peripheral mononuclear cells (PBMC) were prepared from buffy coats from healthy donors using Ficoll gradient separation, and CD4+ cells were purified by a human CD4+ T-cell isolation kit (Miltenyi Biotec) according to the manufacturer's instruction. Ninety-six-well plates were precoated with anti-CD3 antibody (eBioscience, San Diego, CA). CD4+ cells were resuspended in RPMI 1640 complete medium and added to 96-well plates at 10^5 cell/well in a total volume of 80 μl. One hundred microliters of a 2× cytokine cocktail and 20 μl of compounds (100×)
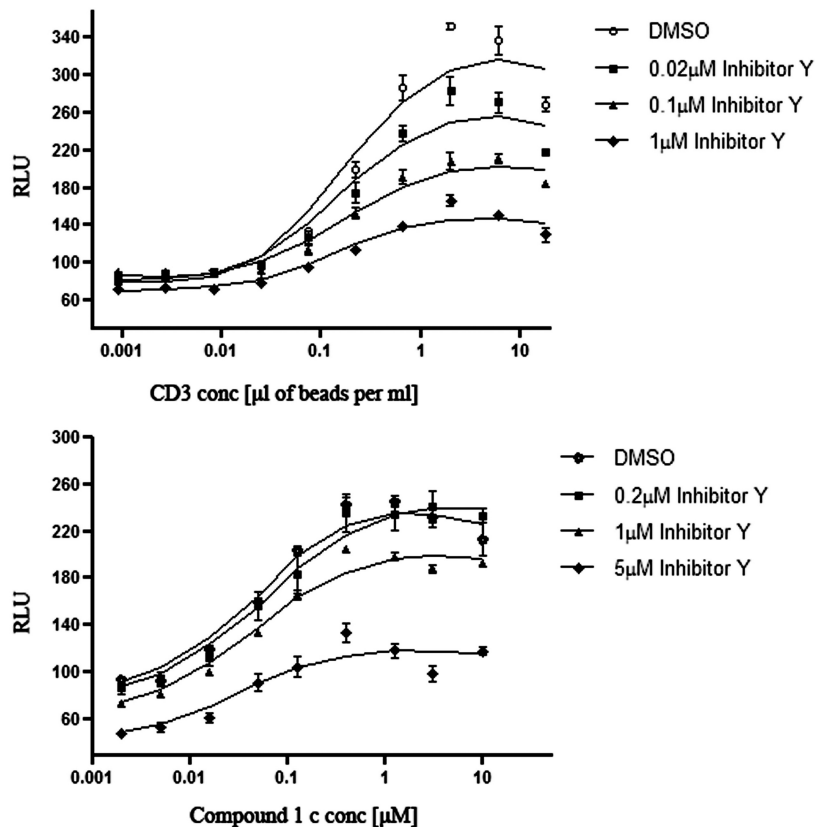
were added to the well. The final concentrations of antibodies and cytokines (all from R&D Systems, Minneapolis, MN) were as follows: anti-hCD3, 5 μg/ml; anti-hCD28, 2 μg/ml; anti-human interferon-γ, 10 μg/ml; anti-hIL4, 10 μg/ml; hIL-6, 50 ng/ml; hIL-23, 20 ng/ml; and hIL-1β, 10 ng/ml. The culture was incubated in 37°C for 3 days, and supernatants were collected for ELISA. The hIL-17 ELISA was performed according to the manufacturer's instruction (R&D Systems).

IL-17 Intracellular Staining. CD4+ cells were cultured for 5 days, and intracellular cytokine staining (BD Pharmingen, San Diego, CA) for IL-17 (anti-human CD4 fluorescein isothiocyanate and anti-human IL-17 phycoerythrin; eBioscience) was performed after stimulation with phorbol 12-myristate 13-acetate (50 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Sigma-Aldrich) for 5 h in the presence of GolgiPlug according to manufacturer's protocol (BD Bioscience).

Statistical Analyses. All values were acquired from two to four independent experiments, using the Prism 5 (GraphPad Software, San Diego, CA), and expressed as the mean ± S.E.M.

Results

Activation of IL-17 Promoter by a Class of Aryl Amide Compounds. We carried out a screen for inhibitors of RORγ recruitment of cofactor SRC1. The assay measured binding of the ligand-binding domain of RORγ with a peptide derived from the coactivator SRC1 by TR-FRET to indirectly assess RORγ activity (Fig. 1A). Given that activating T-cell receptors through anti-CD3 treatment in Jurkat cells are known to activate the IL-17 promoter through RORγ (Liu et al., 2004; Gomez-Rodriguez et al., 2009), we used a cell-based reporter assay as a filter to confirm compounds selected in

	EC50 of CD3 (μl of beads per ml)
DMSO	0.27
0.02μM Inhibitor Y	0.23
0.1μM Inhibitor Y	0.18
1μM Inhibitor Y	0.27

	pEC50 of 1c
DMSO	7.3
0.2μM Inhibitor Y	7.2
1μM Inhibitor Y	7.1
5μM Inhibitor Y	7.1

Fig. 5. Agonist induction of RORγ reporter activity is inhibited by RORγ inhibitor. A, dose-dependent activation curves of anti-CD3 (top) and agonist 1c (bottom) in Jurkat reporter assay in the presence of different concentrations of inhibitor Y. Chemical structure and the effects of inhibitor Y on the potency of compound 1c are summarized in tables.

the SRC1 recruitment assay. Although many compounds (e.g., T0901317) that were inhibitory in the recruitment assay, also inhibited the IL-17 reporter assay (Fig. 1B), we discovered a portion of compounds that surprisingly enhanced the IL-17 reporter activity stimulated by anti-CD3 treatment (Fig. 2, left, minus inh%).

If these compounds are RORγ agonists, they are expected to activate the IL-17 reporter without the need of anti-CD3 treatment. Subsequent experiments performed to test this hypothesis demonstrated that these compounds (Fig. 3A) indeed activated IL-17 promoter activity in the absence of anti-CD3 (Fig. 3B). Three representative aryl amide compounds (Fig. 4A) were selected and resynthesized to confirm their activities. The results showed that they are active in the IL-17 reporter assay with an EC_{50} of ~0.1 μM (Fig. 4B), achieving a similar extent of promoter activity compared with anti-CD3 treatment at full doses (Fig. 4C). SR1078 and 22- and 25-hydroxycholestrol, previously published RORγ agonists (Wang et al., 2010a), had no effect when tested in this assay format (Fig. 4B).

We further validated these RORγ activators (e.g., compound 1c [*N*-(2-(4-ethylphenyl)-2*H*-benzo[*d*][1,2,3]triazol-5-yl) propionamide]) by counteracting their agonistic effects using a RORγ inhibitor Y [*N*-(5-benzoyl-4-phenylthiazol-2-yl)-2-(4-(ethylsulfonyl)phenyl)acetamide] identified from the same screen. Inhibitor Y decreased the anti-CD3-stimulated luciferase reporter activity in the Jurkat assay in a dose-dependent manner (Fig. 5, top). As expected, inhibitor Y at 0.2, 1, and 5 μM repressed the agonistic effects of compound 1c (Fig. 5, bottom).

Binding of the Functional Agonists to RORγ. To demonstrate that compounds directly interact with RORγ, the thermal stability shift in the presence of compounds was measured for RORγ LBD by CD. In this assay, the loss of protein secondary structure (unfolding or melting) caused by increasing temperature was monitored by CD signal changes. Upon binding of the compound to the native protein, the protein thermal stability is increased resulting higher T_m, which is the midpoint of thermal unfolding. The CD scan of the RORγ protein in the far UV area showed a typical folded structure (Fig. 6A). The thermal unfolding was monitored at 224 nm, which is the characteristic minimum of RORγ protein. In the presence of reference antagonist compound T0901317, the T_m was shifted ~6°C (Fig. 6B). The addition of the three agonist compounds also showed increased thermal stability by ~3°C, suggesting binding interactions between these compounds and RORγ. It is noteworthy that SR1078 has 0.62°C and that 22-hydrocholesterol compound has 7.32°C T_m shifts in the same assay, suggesting their different interactions with RORγ.

RORγ Agonists Enhance Th17 Differentiation. Because RORγ plays a critical role in Th17 differentiation, it is expected that RORγ agonists will enhance Th17 differentiation. Therefore, the identified RORγ agonists were evaluated in Th17 differentiation experiments. Under differentiation conditions (see *Materials and Methods*), ~12% of human CD4+ cells became IL-17-secreting cells identified by intracellular staining of an anti-IL-17 antibody, and the percentage of IL-17-positive cells was increased in the presence of the agonist (Fig. 7a). Production of IL-17 in the cultures could be measured by ELISA, and the result showed that the agonist 1b [*N*-(4,6-dimethylbenzo[*d*]thiazol-2-yl)-3-methylth-
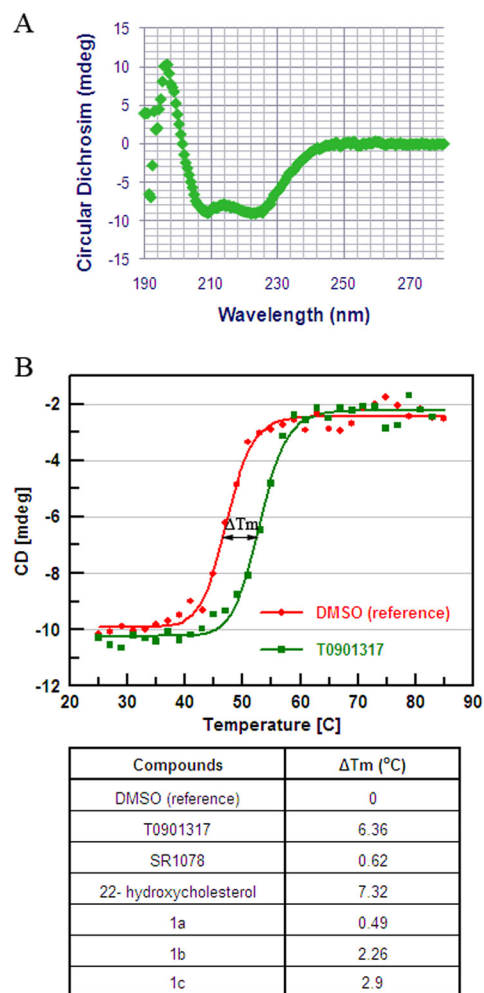

Fig. 6. Thermal stability assay by CD (circular dichrosim). A, CD wavelength scan of RORγ protein. B, thermal unfolding of RORγ (0.1 mg/ml) in the absence and presence of compounds (50 μM).

Compounds	ΔTm (°C)
DMSO (reference)	0
T0901317	6.36
SR1078	0.62
22- hydroxycholesterol	7.32
1a	0.49
1b	2.26
1c	2.9

iophene-2-carboxamide] augmented IL-17 production in a dose-dependent manner, with the maximal effect reaching 220% at 3 μM, further confirming the activation of RORγ-dependent downstream biological events by these compounds (Fig. 7b). Similar to the result observed in the Jurkat reporter assay, inhibitor Y repressed the agonistic activity of compound 1b in promoting IL-17 production (Fig. 7c). The agonist 1b also promoted other Th17-related genes, including *IL23R*, *IL22*, *CCL20*, and *CXCL3* in the Th17 differentiation culture, indicating that the enhancement effect was not limited to IL-17 alone (Fig. 7d).

Discussion

Here we described a series of aryl amide compounds that bind to the ligand binding domain of RORγ and behave as agonists for RORγ. We have the paradoxical results that a fraction of RORγ-SRC1 FRET inhibitors actually stimulated RORγ-transcriptional activity on the IL-17 promoter at the cellular level. In addition, a large percentage (~40%) of the FRET inhibitors seems to have no effect on the IL-17 reporter assay. Although the lack of inhibition on the reporter assay can be potentially attributed to a lack of cell permeability of some of these FRET inhibitors, the activation of IL-17 pro-

A

B

C

D
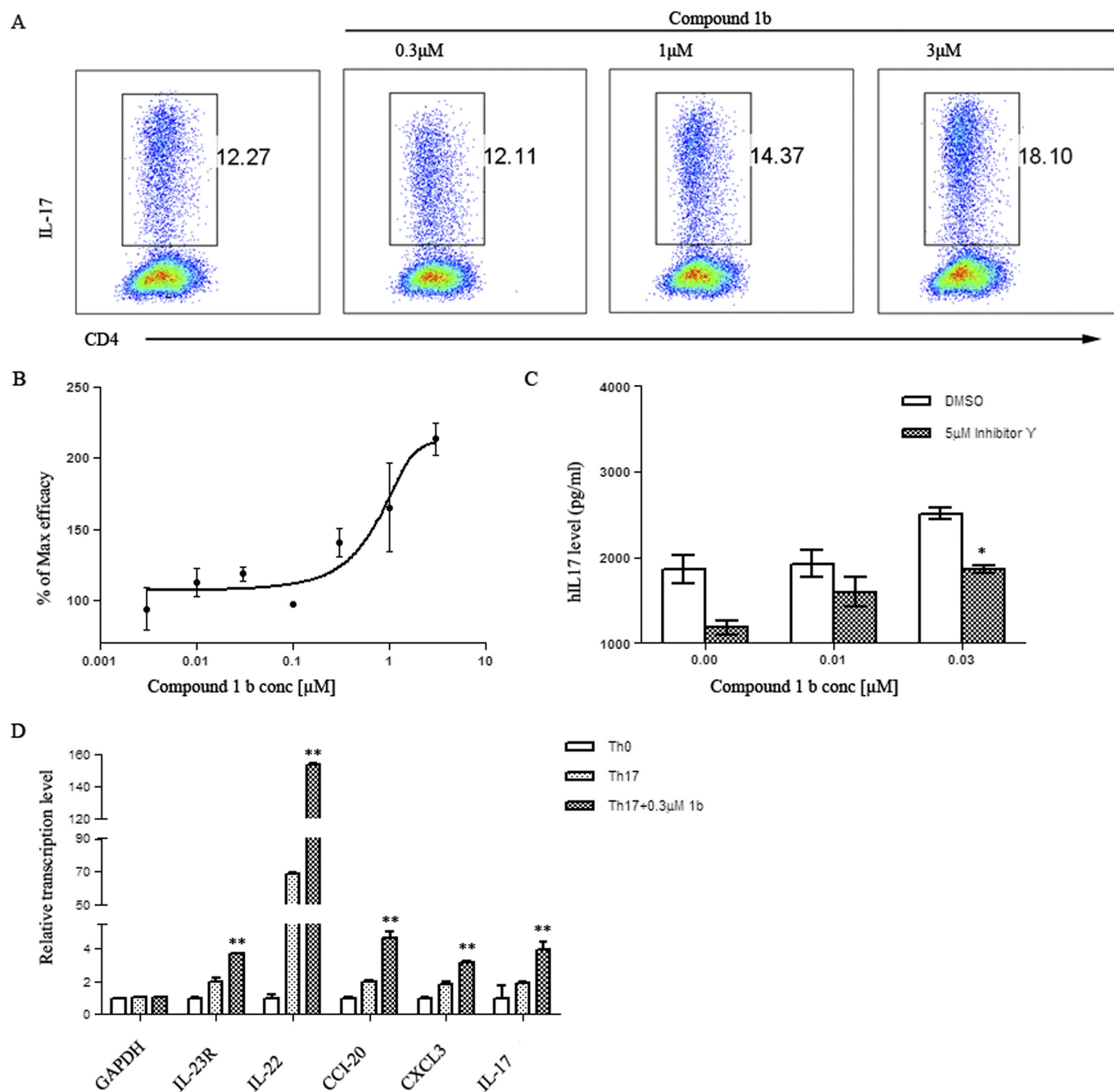
Fig. 7. Effect of agonist on in vitro Th17 differentiation. Human CD4+ T cells were differentiated in vitro as described under *Materials and Methods*. A, IL-17 production was measured by intracellular staining in the presence of compound 1b at the indicated concentrations. B, IL-17 production was measured by ELISA. The effects of compound 1b at different concentrations in augmenting Th17 differentiation were determined as the percentage of IL-17 production relative to full differentiation condition (100%). C, the effects of 5 μM inhibitor Y on the promotion of IL-17 production by compound 1b as indicated were determined. D, the effects of 0.3 μM compound 1b in promoting expression of the indicated genes in Th17 differentiation culture were determined.

moter by these FRET inhibitors suggests that the FRET assay might not fully recapitulate the cellular activity of RORγ. It is important to point out that the FRET assay uses the LBD domain of RORγ and a peptide fragment of SRC1 rather than the full-length RORγ and SRC1. It is conceivable that conformational differences between the full-length protein and fragments led to this discrepancy. In general, it happens that the agonists were initially identified as "inhibitors" because this is how SR1078 was identified. It was first found to be an inhibitor in a gal4-chimeric RORγ assay, but

in full-length assays and other assays, it functions as an agonist. The fact that the aryl amide compounds interfered with the LBD recruitment of SRC1 peptide does suggest that these compounds interacted directly with RORγ, which was also supported by the thermal stability analysis.

Another surprising result is the lack of effects of hydroxyl cholesterols in the cellular assay. It has been suggested that high basal level of hydroxyl cholesterols in cells leads to constitutive activity of RORγ and that adding additional agonists might not further increase the reporter activity. Two

sets of data contradict this hypothesis. First, the aryl amide compounds in this article seem to be quite effective in activating the IL-17 promoter. Second, treating cells with a statin does not seem to endow cells with the ability to respond to hydroxyl cholesterol (data not shown) with IL-17 promoter activity.

A lack of knowledge of native agonists has limited the study and drug discovery efforts targeting RORγ. RORγ seems to be an essential transcription factor for Th17 differentiation, yet how that is linked to the signaling pathways initiated by transforming growth factor-β and IL6, cytokines known to promote Th17, is not clear. A native ligand might provide such a link, if proven to be increased in quantity after these cytokine treatments.

It is interesting to note that in our experimental paradigm of in vitro Th17 differentiation, our synthetic agonists promote Th17 efficiency by ~2.5-fold. To the best of our knowledge, this is the first time an RORγ modulator was shown to enhance Th17 differentiation.

Authorship Contributions

Participated in research design: W. Zhang, Leung, Zhong, Y. Wang, and Zhou.

Conducted experiments: J. Zhang, Fang, S. Wang, and Xiang.

Contributed new reagents or analytic tools: An, Li, and Wisely.

Performed data analysis: W. Zhang, J. Zhang, Leung, and Zhou.

Wrote or contributed to the writing of the manuscript: W. Zhang, J. Zhang, G. Zhang, Leung, Xiang, Y. Wang, and Zhong.

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