Cholesterol Unbound ROR γ t Protein Enables a Sensitive Inverse Agonist Screening

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ABSTRACT

The retinoic acid-related orphan receptor gamma T (ROR γ t) plays an important role in Th17 cell proliferation and functionality. Thus, ROR γ t inverse agonists are thought to be potent therapeutic agents for Th17-mediated autoimmune diseases, such as rheumatoid arthritis, asthma, inflammatory bowel disease, and psoriasis. Although ROR γ t has constitutive activity, it is recognized that the receptor is physiologically regulated by various cholesterol derivatives. In this study, we sought to identify ROR γ t inverse agonists through a high-throughput screening campaign. To this end, we compared an apo-ROR γ t protein from *Escherichia coli* and a cholesterol-bound ROR γ t protein from insect cells. The IC₅₀ of the known ROR γ t inverse agonist T0901317 was significantly lower for the apoprotein than for the cholesterol-bound ROR γ t. Through high-throughput screening using a fluorescence-based cholesterol binding assay with the apoprotein, we identified compound 1 as a novel cholesterol-competitive ROR γ t inverse agonist. Compound 1 inhibited the ROR γ t-TopFluor cholesterol interaction, coactivator recruitment, and transcriptional activity of ROR γ t. Cell-based reporter gene assay demonstrated that compound 1 showed higher potency by lipid depletion treatment. Collectively, our findings indicate that eliminating cholesterol from the ROR γ t protein is suitable for sensitive high-throughput screening to identify ROR γ t inverse agonists.

Keywords: cholesterol, high-throughput screening, inverse agonist, retinoic acid-related orphan receptor gamma T, ROR γ t

INTRODUCTION

The retinoic acid-related orphan receptor gamma T (ROR γ t) is a member of the nuclear receptor (NR) superfamily.¹ As an isoform of ROR gamma (ROR γ), ROR γ t shares identical DNA binding domain (DBD)

and ligand binding domain (LBD) sequences with ROR γ .² However, expression of ROR γ t is restricted to several immune cell types, such as CD4⁺ Th17 cells, and is essential for the proliferation and functionality of Th17 cells.³ Th17 cells produce interleukin-17 (IL-17) and induce tissue inflammation leading to the pathogenesis of various autoimmune diseases.⁴ IL-17A expression is induced by ROR γ t, which directly binds to ROR response elements (ROREs) in the IL-17 promoter.⁵ Hence, the differentiation of naive CD4⁺ T cells to Th17 cells is predominantly regulated by ROR γ t.^{6,7}

The liver X receptor agonist, T0901317 (Fig. 1A), has been identified as a potent ROR γ inverse agonist⁸; since then, many inverse agonists have been identified, with some reportedly inhibiting Th17 cell differentiation. Among these compounds, SR1001, ursolic acid, and digoxin have been found to suppress experimental autoimmune encephalomyelitis.⁹ These recent reports strongly suggest that ROR γ t is a potential target for autoimmune diseases.

There has been considerable interest in endogenous ligands of RORs. X-ray crystal structure analysis has unexpectedly revealed that cholesterol was present in the ligand binding pocket (LBP) of ROR alpha (ROR α) protein purified from insect cells.¹⁰ Moreover, the crystal structure of the ROR α protein, in complex with cholesterol sulfate, was solved following a ligand exchange experiment.¹¹ From the crystal structure of the ROR β protein expressed in *Escherichia coli*, stearic acids were identified as a fortuitous ligand.¹² In addition, the crystal structure of the ROR γ LBD expressed in bacteria with a peptide motif of coactivator SRC1 and hydroxycholesterols has been solved.¹³ These structures revealed that the RORs in complex possess active conformations that generate a hydrophobic coactivator binding surface. Recently, several oxysterols were found to be agonistic ligands of ROR γ ,^{14–18} suggesting that cholesterol biosynthetic intermediates may modulate the transcriptional activity of ROR γ and ROR γ t.

To develop more sensitive assays for high-throughput screening (HTS), the selection of the appropriate recombinant protein is important. To date, limited reports exist about the relationship between the ROR protein and cholesterol. In 2002, Kallen *et al.*¹⁰ reported that the ROR α protein purified

Fig. 1. (A) Chemical structure of TO901317. (B) Assay principle of RORE reporter gene assay. (C) Assay principle of TopFluor cholesterol binding assay. (D) Assay principle of cofactor peptide recruitment assay. DBD, DNA binding domain; LBD, ligand binding domain; LUC, luciferase; ROR, retinoic acid-related orphan receptor; RORE, ROR response element; ROR γ T, retinoic acid-related orphan receptor gammaT; Tb, Terbium.

from Sf-9 cells was bound to cholesterol; however, the protein expressed in *E. coli* yielded only insoluble protein. They presumed that cholesterol, which was reportedly not synthesized in the bacteria, was needed for stabilizing the receptor. In addition, they reported that cholesterol could be exchanged with cholesterol sulfate in the ROR α protein, and the exchange of bound cholesterol sulfate could not be reversed with the excess of other cholesterol derivatives. In contrast, Wang *et al.*¹⁸ showed that the ROR α protein expressed in *E. coli* could be reconstituted in a lipid-free environment and had the ability to bind coactivator peptides. These data suggest that cholesterol is not important for ROR α protein stability or cofactor recruitment. In addition, these authors reported that the ROR α protein treated with excess cholesterol sulfate blocked the binding of radiolabeled hydroxycholesterol to the receptor. Thus, whether the relationship between cholesterol and the recombinant ROR α protein is applicable to ROR γ T remains unclear. In addition, there has been no evidence of the effect of endogenous cholesterol on ROR γ T inverse agonist activity for high-throughput screening.

In this study, we report that cholesterol within the LBP of the recombinant ROR γ T protein interferes with the potency of ROR γ T inverse agonist, and, by utilizing this result, a novel ROR γ T inverse agonist, compound 1, was identified through the high-throughput screening with TopFluor cholesterol binding

assay. TAK-828F identified through further optimization of compound 1 has higher potency, and selectivity against NRs, including ROR isoforms.^{19–21} The compound also inhibited IL-17 secretion from mouse splenocytes and human primary cells, and inhibited Th17 cell differentiation from naive T cells and memory CD4⁺ T cells.²² Furthermore, the compound showed significant efficacy in naive T cell transfer mouse colitis model.

MATERIALS AND METHODS

Materials

TO901317 was purchased from Tocris Bioscience (Bristol, UK). Lovastatin and cholesterol were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Cholesterol sulfate was purchased from Sigma-Aldrich Corporation (St. Louis, MO).

Establishment of Stable Cell Lines for Reporter Gene Assay

The reporter-gene construct, pGL4.28-hIL17-ROREx3-luc, was constructed as described by Ichiyama *et al.*²³ Briefly, the construct was created by inserting an oligonucleotide, comprising three copies of the RORE in the human IL17 promoter, into the pGL4.28 [luc2CP/minP/Hygro] reporter vector (Promega Corporation, Fitchburg, WI). The full-length complementary DNA for human ROR γ T was subcloned into the mammalian expression vector pMCMV-neo, which contained the cytomegalovirus promoter, to generate pMCMVneo-ROR γ T. To generate stable cell lines, pGL4.28/hIL17-ROREx3-luc and pMCMVneo-ROR γ T were transfected into the Jurkat Tet-On cell line (Takara Bio, Inc., Kusatsu, Japan) using Gene Pulser (Bio-Rad Laboratories, Inc., Hercules, CA), and stable cell lines were chosen based on luciferase activity.

Protein Expression and Purification

The DNA fragment of human ROR γ T LBD (Uniprot: P51449, aa. 261–518), amplified by PCR, was cloned into the pFast-BacHTb vector (Thermo Fisher Scientific, Waltham, MA). Recombinant baculovirus was generated using the Bac-to-Bac baculovirus expression system (Thermo Fisher Scientific). Protein expressed in Sf9 insect cells was harvested 48 hours

postinfection. Cell pellets were homogenized in a lysis buffer [50 mM Tris-HCl, pH 7.9, 200 mM NaCl, 5% glycerol, 0.25 mM Tri(2-carboxyethyl) phosphine (TCEP), and Complete™ protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland)]. Cell lysates were clarified by high-speed centrifugation at 37,000 *g* for 45 min and applied to affinity chromatography with ProBond Ni-chelating resin (Thermo Fisher Scientific). After extensive washing, the His-tagged protein was eluted by 50 mM Tris-HCl, pH 7.9, 150 mM NaCl, 200 mM imidazole, and 1 mM TCEP. The protein was then buffer exchanged into 25 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM dithiothreitol (DTT), and 1 mM TCEP before loading onto a MonoQ column (GE Healthcare, Chicago, IL). The flow-through fraction containing the LBD was collected. Protein concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific). The purity of the protein sample was verified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and liquid chromatography–mass spectrometry (LC–MS) (Micromass LCT™ Workstation; Waters Corporation, Milford, MA).

A truncated human RORγt LBD (aa. 261–508) with an N-terminal 6xHis tag, followed by a TEV cleavage site, was cloned into a modified pET vector and expressed in *E. coli* BL21 (DE3) cells. The bacterial culture was grown in LB media at 37°C. When the culture reached an OD₆₀₀ of 0.8, the cells were induced with 0.8 mM IPTG for an additional 18-h growth at 16°C before harvesting. The cell pellet was resuspended and sonicated in lysis buffer [25 mM Tris-HCl, pH 7.6, 1 M NaCl, 10 mM imidazole, 0.5 mM TCEP, 20 U/mL benzonase, 1 mg/mL lysozyme, and Complete™ protease inhibitor cocktail (Roche Diagnostics)]. The cell lysate was clarified by centrifugation (37,000 *g*, 45 min, and 4°C) and applied to a 5 mL HiTrap Talon column (GE Healthcare). After an extensive wash, the His-tagged protein was eluted from the column with buffer containing 300 mM imidazole in 25 mM Tris-HCl, pH 7.6, 1 M NaCl, 2 mM benzamidine, and 0.5 mM TCEP. The protein sample was buffer exchanged into 25 mM Tris-HCl, 200 mM NaCl, 2 mM benzamidine, 2 mM DTT, and 5% glycerol. It was then concentrated and loaded onto a size exclusion chromatography column (Hiload 16/60 Superdex 200; GE Healthcare). Peak fractions containing the truncated LBD protein were pooled and concentrated to 1 mg/mL. The purity of the final protein sample was verified by SDS–PAGE and LC–MS. The concentration of RORγt proteins were measured by Bradford Protein assay (Bio-Rad).

Quantification of Cholesterol by Mass Spectrometry

Protein samples were dissolved in 0.5% SDS solutions to a concentration of 500 nM, and were heated at 95°C for 5 min to

denature the sample completely. For this experiment, a Shimadzu HPLC system (Shimadzu Corp., Kyoto, Japan) and API 5000 triple quadrupole mass spectrometer (AB SCIEX, Framingham, MA) were used to quantify the concentration of cholesterol. The analysis of cholesterol was performed using a Unison UK-C8 30×3 mm ID column, 3 μm (Imtakt, Kyoto, Japan). The HPLC mobile phase A contained 10 mM ammonium acetate in water, while mobile phase B contained 10 mM ammonium acetate in acetonitrile. The chromatographic separation of cholesterol was conducted using a constant ratio of 93% of mobile phase B in mobile phase A at a flow rate of 0.8 mL/min. According to the previous report for determination of 7α-OH cholesterol, the [M+H]⁺ ions are unstable at high temperature in the ionization step, and its daughter ions losing a water molecule are more stable.²⁴ So, the concentration of cholesterol was determined with selected monitoring methods and precursor ion scanning of *m/z* 369.3 (in a form of [M+H–H₂O]⁺) in positive ion mode specific for cholesterol.

Reporter Gene Assay

The principle of the assay is outlined in *Figure 1B*. Stable Jurkat cells were maintained in RPMI 1640 medium (Thermo Fisher Scientific) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin G, and 100 μg/mL streptomycin sulfate (Thermo Fisher Scientific). In experiments, cells were plated in 384-well white tissue culture plates (3570; CORNING Incorporated, Corning, NY) with 1 μg/mL of doxycycline (Takara Bio) at a density of 20,000 cells per well. For cholesterol-depletion treatment, cells were maintained on RPMI 1640 containing 10% lipid-reduced FBS (GE Healthcare) and 10 μM lovastatin. Diminishing intracellular cholesterol was confirmed using a cholesterol fluorometric assay kit (Cayman, Ann Arbor, MI) (data not shown). After culturing for 3 h, cells were treated with dimethyl sulfoxide (DMSO) or test compounds and incubated at 37°C for 20 h. Luciferase activity was measured by Envision (PerkinElmer, Waltham, MA) using the Bright-Glo Luciferase Assay System (Promega). Raw data were analyzed using Prism 5 (GraphPad Software, San Diego, CA), and a three-parameter logistic fit equation [Eq. (1)] was used to determine IC₅₀.

$$Y = (\text{Top} - \text{Bottom}) / (1 + 10^{(X - \text{LogIC}_{50})}) + \text{Bottom} \quad (1)$$

“X” is the log of compound concentration and “Y” is response.

For normalization of data in *Table 3*, the cells treated with 1 mg/mL doxycycline wells were used as 100% controls (top) and the cells without doxycycline were used as 0% controls (bottom). IC₅₀ values were calculated by Equation (1) using Prism 5.

TopFluor Cholesterol/ROR γ T LBD (Time-Resolved Fluorescence Resonance Energy Transfer) Binding Assay Screening

The principle of the assay is outlined in Figure 1C, and the detailed protocol is described in Table 1. In 384-well black low-volume plates (Greiner Bio-One, Kremsmünster, Austria), 3 μ L per well of the library compounds diluted with an assay buffer [20 mM Tris-HCl (pH7.5), 100 mM NaCl, 1 mM DTT, 0.1% bovine serum albumin (BSA)] was added to a concentration of 1 μ M. Final DMSO concentration was 1%. The plates were then incubated at room temperature for 20 min after the addition of 3 μ L/well of ROR γ T protein expressed from *E. coli* at a final concentration of 60 nM. TopFluor cholesterol (3 μ M; Avanti Polar Lipids, Alabaster, AL) and anti-His Terbium (Tb) (2 nM; Thermo Fisher Scientific) were then added to each well at 3 μ L/well. An HT station 1200 (MSTechnos, Tokyo, Japan) was used to transfer the compounds, and a Multidrop Combi (Thermo Fisher Scientific) was used to transfer the ROR γ T protein, TopFluor cholesterol, and anti-His Tb. The plates were incubated at room temperature for 1 h and measured in Envision. To eliminate false positive compounds, a Fluorescein-His Tag (2 nM; Thermo Fisher Scientific) was used instead of the ROR γ T protein and TopFluor cholesterol. The Tb donor was excited at 320 nm, its emission was monitored at 486 nm, and the acceptor emission was monitored at 520 nm. The results were expressed as a ratio of Em520/Em486. Raw data were analyzed using Prism 5,

and a three-parameter logistic fit equation [Eq. (1)] was used to determine IC₅₀. For normalization of the data in Table 3 and Figure 3C, the wells containing DMSO were defined as 0% inhibition control (bottom) and the wells containing 10 μ M of TO901317 were defined as 100% inhibition control (top). IC₅₀ values were calculated in Equation (1) using Prism 5.

Cell-Free Cofactor Peptide Recruitment Assay

The principle of the assay is outlined in Figure 1D. The binding of a cofactor peptide motif to the purified ROR γ T protein was determined using the AlphaScreen Histidine (Nickel Chelate) Detection Kit (PerkinElmer). The experiments were conducted with 50 nM ROR γ T LBD and 10 nM of biotinylated SRC1-2 peptide (CPSSHSLTERHKILHRLQEGSPS, Scrum, Tokyo, Japan) in the presence of 10 μ g/mL donor and acceptor beads in buffer containing 50 mM Tris-HCl (pH7.4), 50 mM KCl, 1 mM DTT, and 0.1% BSA. Assay plates were incubated for 3 h at room temperature and measured by Envision. Raw data were analyzed using Prism 5, and a three-parameter logistic fit equation [Eq. (1)] was used to determine IC₅₀. For normalization of the data in Table 3, the wells containing DMSO were defined as 100% control (top) and the wells without the ROR γ T protein were defined as 0% control (bottom). IC₅₀ values were calculated in Equation (1) using Prism 5.

Thermal Shift Assay

In 96-well V-bottom polypropylene microplate (3363; CORNING), 20 μ L/well of the test compounds dissolved in an assay buffer [20 mM Tris-HCl (pH7.5), 100 mM NaCl, 1 mM DTT] was added with 20 μ L per well of the ROR γ T protein expressed from *E. coli* (3 μ M) and 20 μ L/well of SYPRO Orange (500-fold diluted; Thermo Fisher Scientific). Then, 8 μ L per well of the reaction was transferred to a 384-well PCR plate in quadruplicate. After centrifuging, the plates were heated at a ramp rate of 1°C/min and the fluorescence was monitored by ABI 7900 (Thermo Fisher Scientific). Data were analyzed by Prism 5 (GraphPad Software), and Boltzman sigmoidal fitting was used to determine melting temperature (T_m).

RESULTS

Selection of the Human ROR γ T Protein for HTS

To identify lead compounds of NRs, cell-based reporter gene assay, cell-free ligand binding assay, and cell-free cofactor peptide recruitment assay are general assay methods.²⁵ Cell-based assays can measure transcriptional effect of a compound; however, they detect a lot of false positive compounds such as toxic compounds or indirect regulators like kinase inhibitors. In contrast, cell-free assays are, although they do not exactly reflect the target in the cellular context, simple and more likely to

Table 1. TopFluor Cholesterol/ROR γ T Ligand Binding Domain (Time-Resolved Fluorescence Resonance Energy Transfer) Binding Assay Screening Protocol Table

Step	Parameter	Value	Description
1 ^a	Compounds/control addition	3 μ L	Controls–1% DMSO $\pm$ 10 μ M of TO901317
2	ROR γ T protein addition	3 μ L	
3 ^b	Incubation time	20 min	At room temperature
4	TopFluor cholesterol addition	3 μ L	
5	Anti-His Tb addition	3 μ L	
6 ^b	Incubation time	60 min	At room temperature
7 ^c	Assay readout	Ex320/Em520, Em486	TR-FRET mode

^aColumns 1–20, library compounds at 1 μ M; columns 21–22, 1% DMSO; columns 23–24, 1% DMSO plus 10 μ M of TO901317.

^bSpin plates for 10 s at 1,000 rpm, cover the plate before incubation.

^cRead the plate in an Envision (PerkinElmer).

DMSO, dimethyl sulfoxide; ROR γ T, retinoic acid-related orphan receptor gamma T; Tb, Terbium; TR-FRET, time-resolved fluorescence resonance energy transfer.

identify direct binding modulator. So, we developed a cell-free assay system for ROR γ t inverse agonist screening. To select the protein suitable for primary screening, we produced two types of human ROR γ t protein, expressed in *E. coli* [ROR γ t (*E. coli*)] and in insect cells [ROR γ t (Ins)], and compared the presence of cholesterol in the LBD, ligand binding activity, cofactor recruitment activity, and tool compound effect.

At first, we performed mass spectrometry analysis for measuring the cholesterol content of the proteins. As a result, more than 60% of the ROR γ t (Ins) protein was found to contain cholesterol. In contrast, the ROR γ t (*E. coli*) protein did not contain any cholesterol, although there was a possibility that the protein would contain cholesterol incorporated from the medium during the cultivation of *E. coli* cells (Table 2). Furthermore, we compared the activity of these proteins using the TopFluor cholesterol competition assay (Fig. 1C) and cofactor recruitment assay (Fig. 1D). Generally, an agonist binding to NR causes conformational change resulting in an interaction of coactivator for transcription of target genes. ROR γ t is constitutive active and can interact with a coactivator such as SRC1 without ligands.¹³ So, we used cell-free cofactor recruitment assay to determine whether the ROR γ t protein is in an active or inactive state by detecting interaction of SRC1-2 peptide with the ROR γ t LBD. As shown in Figure 2A, both ROR γ t (*E. coli*) and ROR γ t (Ins) proteins were effective in recruiting the SRC1-2 peptide motif. Exogenous cholesterol weakly promoted to the SRC1 peptide recruitment by ROR γ t (*E. coli*) and the EC₅₀ was about 2.5 μ M, while its activation was not observed in the reporter gene assay (data not shown). This observation implies that cholesterol is a very weak agonist, not affecting the transcriptional activity of ROR γ t.

To investigate cholesterol binding activity of these proteins, we developed a time-resolved fluorescence resonance energy transfer (TR-FRET) binding assay, which detects TopFluor cholesterol binding to the ROR γ t LBD (Fig. 1C). As shown in Figure 2B, the ROR γ t (*E. coli*) protein could specifically bind

TopFluor cholesterol compared to no protein control and the apparent K_d was calculated as 6.3 μ M, while the binding of the ROR γ t (Ins) was only detected at <10% of the ROR γ t (*E. coli*) binding capability. Exogenous cholesterol can displace the TopFluor cholesterol in ROR γ t (*E. coli*) LBD and its IC₅₀ was about 7.2 μ M.

We then examined whether differences in host cells affect the potency of the inverse agonist activity using TO901317, which is a known ROR γ t inverse agonist. As shown in Figure 2C, the SRC1-2 recruitment assay revealed that TO901317 was over 200 times more potent on ROR γ t (*E. coli*) (IC₅₀ = 23 nM) than on ROR γ t (Ins) (IC₅₀ = 6,700 nM). Crystal structure of the LBD of ROR γ with TO901317 showed that binding site of this compound is same as cholesterol binding site.²⁶ To corroborate this structural information, ligand competitive assay for TO901317 was conducted in TopFluor cholesterol binding assay. As expected, the IC₅₀ value of TO901317 was right-ward shifted depending on increasing TopFluor cholesterol concentration (Fig. 2D). It is difficult to increase TopFluor cholesterol concentration more than 10 μ M (about twofold higher than K_d) because of its solubility. These data demonstrate that TO901317 inhibited TopFluor cholesterol binding in a competitive manner.

To determine whether the reason was due to the difference in expression system or the presence of cholesterol, we tested the activity of ROR γ t (*E. coli*) incubated with excess cholesterol sulfate and applied to gel filtration to remove free cholesterol sulfate. Wang *et al.*¹⁸ used cholesterol sulfate for examining the effect of exogenous sterol to the ROR α LBD. So, cholesterol sulfate was used as a surrogate ligand because it had more solubility and higher affinity for ROR γ t (IC₅₀ = 46 nM data not shown) than cholesterol, and it is thought to be a nonfunctional ligand like cholesterol in cofactor recruit assay and reporter gene assay according to a previous report.¹⁸ Cholesterol sulfate-saturated ROR γ t (*E. coli*), as well as ROR γ t (Ins), blocked the binding of the TopFluor cholesterol (Fig. 2B) and inhibition by TO901317 in the cofactor recruitment assay (IC₅₀ > 10,000 nM, Fig. 2C), indicating that bound cholesterol sulfate is hardly exchanged from the ROR γ t protein. From these results, we selected the apo-ROR γ t (*E. coli*) protein for primary screening because it was expected that using the apoprotein would identify a greater variety of hit clusters of ROR γ t inverse agonists.

High-Throughput Screening

Among two established cell-free assays, we chose the TopFluor cholesterol competition assay for primary screening to identify not only inverse agonists but also antagonists, which cancel the agonistic activity of cholesterol derivatives. TopFluor cholesterol concentration was set as 3 μ M according to the apparent K_d from titration data (Fig. 2B) and assay

Table 2. Cholesterol Concentration in the ROR γ t Protein Expressed in Insect Cells or *Escherichia coli* as Measured by Mass Spectrometry

	Protein concentration (nM)	Cholesterol concentration (nM)	Bound rate (%)
ROR γ t expressed in insect cells	517	318	62
ROR γ t expressed in <i>E. coli</i>	550	0	0

Fig. 2. Cholesterol binding activity and SRC1–2 peptide recruitment activity of the three ROR γ T proteins. ROR γ T (Ins), ROR γ T (*Escherichia coli*), and cholesterol sulfate-saturated ROR γ T (*E. coli*) were analyzed by cofactor peptide recruitment assay and TopFluor cholesterol binding assay. **(A)** The interaction of ROR γ T (Ins) (triangle), ROR γ T (*E. coli*) (diamond), and cholesterol sulfate-saturated ROR γ T (*E. coli*) (black circle) with the biotin-SRC1-2 peptide was monitored using AlphaScreen assay. Nonspecific signals were detected without ROR proteins (white circle). Data are shown as the mean $\pm$ SEM of duplicate wells. **(B)** Binding of TopFluor cholesterol to ROR γ T (Ins) (triangle), ROR γ T (*E. coli*) (diamond), and cholesterol sulfate-saturated ROR γ T (*E. coli*) (black circle) was detected using the TR-FRET system. Nonspecific signals were detected without ROR proteins (white circle). Data are shown as the mean $\pm$ SEM of duplicate wells. K_d values were determined with GrahPad Prism 5 using a nonlinear regression method, a one-site fitting model. **(C)** The recombinant protein of ROR γ T (Ins) (white circle), ROR γ T (*E. coli*) (triangle), and cholesterol sulfate-saturated ROR γ T (*E. coli*) (diamond) was incubated with 10 nM biotin-SRC1-2 peptide and various concentration of TO901317 for 3 h at room temperature. Data are shown as the mean $\pm$ SEM of duplicate wells. IC_{50} values were calculated as described in the Materials and Methods section by using raw data. **(D)** Inhibition of TopFluor cholesterol binding by TO901317 with 1 (white circle), 3 (triangle), or 10 μ M TopFluor cholesterol. Data are shown as the mean $\pm$ SEM of duplicate wells. IC_{50} values were calculated as described in the Materials and Methods section without normalization of the data. DMSO, dimethyl sulfoxide; SEM, standard error of the mean; TR-FRET, time-resolved fluorescence resonance energy transfer.

robustness. An assay validation such as duplicate (N2) correlation and batch reproducibility was conducted, and test concentration was set as 1 μ M based on a hit rate of pre-screening (data not shown).

A high-throughput screening was conducted on the 860,000 compounds of the Takeda compound library at 1 μ M as outlined in Figure 3A. From a scatterplot of 1% DMSO controls and TO901317 controls in a single representative data from the primary screening (Fig. 3B), Z' -factor was calculated as 0.86. The average Z' -factor of all plates tested was 0.80 ± 0.05 [average $\pm$ standard deviation (SD)] with an average plate S/B ratio of 10.80 ± 1.23 . The primary screening data in histogram format (Fig. 3C) showed a normal distribution curve with an average inhibition value of 10.9% and an SD of 17.2%. Since TO901317 showed over 15-fold less activity in cell-based assay

[IC_{50} (reporter gene assay) = 1,500 nM] (Table 3) than cell-free assay [IC_{50} (TopFluor cholesterol binding assay) = 91 nM, IC_{50} (cofactor recruitment assay) = 23 nM] (Table 3), we set a cut-off criteria of 70% inhibition at 1 μ M to identify cellular active compounds directly from HTS campaign. The remaining compounds above 70% at 1 μ M from primary screening were tested in a counter assay using a Fluorescein-His tag to eliminate false positive compounds due to the inhibition of the TR-FRET signal. In total, 229 compounds passed the counter assay, and from these, 68 compounds showed >70% inhibition against the apo-ROR γ T-SRC1 peptide interaction at 1 μ M. Following ROR γ T-ROR γ reporter gene assay, 23 clusters (41 compounds) were identified as hit compounds.

Of these hit compounds, compound 1 (Fig. 3D) was selected as a lead compound because the compound had 15-fold greater

Fig. 3. Primary screening results. **(A)** Flowchart of screen procedure. **(B)** Scatterplot of 1% DMSO controls (white circle) and 10 μ M TO901317 + 1% DMSO controls (black square) from a single representative data in primary screening. Z'-factor was calculated from these 16 replicated controls using the TopFluor cholesterol/ROR γ t (*E. coli*) TR-FRET binding assay. **(C)** Histogram of % inhibition of the Takeda Library compounds from primary screening. **(D)** Chemical structure of compound 1.

selectivity against ROR α and ROR β ,¹⁹ has good physical properties, including water solubility and cLogP of 2.8, and did not have cytotoxicity and CYP inhibition. Table 3 summarizes the IC₅₀ values of compound 1 and TO901317 for ROR γ t from three functional assays, demonstrating that compound 1, similar to TO901317, was effective in the TopFluor cholesterol binding assay (IC₅₀ = 200 nM), the SRC1-2 recruitment assay (IC₅₀ = 91 nM), and the ROR γ t-RORE reporter gene assay (IC₅₀ = 1,600 nM), although the potency of compound 1 was slightly weaker than TO901317.

To confirm the direct interaction between compound 1 and ROR γ t, we performed the thermal shift assay (TSA). TSA is a common method to confirm direct binding of small molecule to the target protein, including ROR γ .^{14,27} TSA monitors the thermal stability of the target protein with fluorescent dye such as SYPRO Orange, which emits fluorescence when hy-

drophobic residues of the protein are exposed to the dye. A ligand-induced conformational stabilization of the target protein can be judged by comparing T_m with or without ligands. As shown in Figure 4A and B, T_m value of TO901317 (51.6°C at 30 μ M) and compound 1 (51.7°C at 30 μ M) was higher compared with the apo-form of ROR γ t (*E. coli*) (T_m = 45.3°C). T_m shift by compound 1 at 3 μ M (2.4°C) was lower than by TO901317 (3.6°C), consistent with their order of IC₅₀ in cell-free assay. So direct binding of compound 1 was confirmed in TSA.

The Effect of Cholesterol on the Potency of Compound 1 in Cell-Free and Cell-Based Assay

To determine the effect of cholesterol on the inhibition of compound 1, cell-free SRC1-2 recruitment assay and cell-based reporter gene assay were conducted. In the SRC1-2 recruitment assay, similar to TO901317, the IC₅₀ of compound 1

Table 3. IC₅₀ Values of Compound 1 and T0901317 in ROR γ T Reporter Gene Assay, TopFluor Cholesterol Binding Assay, and Cofactor Recruitment Assay Using Apo-ROR γ T (*Escherichia coli*)

Compound	TopFluor cholesterol binding IC ₅₀ (95% CI) nM	SRC1 peptide recruitment assay IC ₅₀ (95% CI) nM	Reporter gene assay IC ₅₀ (95% CI) nM
Compound 1	200 (160–250)	91 (67–130)	1,600 (1,200–2,200)
T0901317	91 (64–130)	23 (16–33)	1,500 (1,200–1,900)

For reporter gene assay, compounds were tested in duplicate in medium containing lipid-reduced FBS with 10 μ M lovastatin. IC₅₀ values were calculated as described in the Materials and Methods section using normalized data from a single experiment performed in duplicate.

CI, confidence interval; FBS, fetal bovine serum.

was over 100-times lower for apo-ROR γ T (*E. coli*) than for ROR γ T (Ins) and cholesterol sulfate-bound ROR γ T (*E. coli*), as shown in Figure 5A. These results revealed that compound 1 could not have been identified if ROR γ T (Ins) was used for primary screening. Figure 5B showed that compound 1 inhibited TopFluor cholesterol binding of ROR γ T protein in a competitive manner, same as T0901317, and further structural analysis of compound 1 derivative revealed that this chemotype binds to cholesterol binding site.¹⁹

To confirm the effect of cholesterol for ROR γ T transcriptional activity, we used RORE reporter gene assay (Fig. 1B), detecting inhibition of ROR γ T's constitutive active transcriptional activity by inverse agonist. Compound 1 was then tested with normal medium, cholesterol-depleted medium (lipid-reduced FBS and 10 μ M of lovastatin), or cholesterol-depleted medium containing cholesterol sulfate. The result showed that cholesterol depletion and cholesterol sulfate treatment had almost no effect

against ROR γ T transcriptional activity, suggesting that cholesterol and cholesterol sulfate are nonfunctional ligands (Fig. 5C), while, as shown in Figure 5D, the IC₅₀ value of compound 1 was 3.1 μ M with cholesterol depletion treatment, which was lower than the IC₅₀ value of 14 μ M without cholesterol depletion. Furthermore, increasing amounts of cholesterol sulfate in the cholesterol-depleted medium induced a rightward IC₅₀ shift, although the transcriptional activity without compound 1 did not increase with cholesterol sulfate. These results strongly suggest that the IC₅₀ shift in the inverse agonist activity by lovastatin arose from the intracellular cholesterol concentration, not an indirect effect of lovastatin.

From these results, in cell-free-assay, we concluded the mechanism of action of ROR γ T inverse agonists (Fig. 6). T0901317 and compound 1 bind to LBP of apo-ROR γ T protein to induce the inactive form of the protein. That results in inhibiting the recruitment of SRC1 peptide to the protein. In contrast, cholesterol-bound ROR γ T protein is hardly exchanged by these ROR γ T inverse agonists and sustains the active form of the protein and the recruitment of SRC1 peptide. Furthermore, in the cell-based

Fig. 4. Direct binding of T0901317 and compound 1 by thermal shift assay. Representative melting curves of ROR γ T (*E. coli*) with 0, 3, 10, or 30 μ M T0901317 (A) and compound 1 (B) are shown with calculated T_m . The dashed lines are represented as T_m for apoprotein. T_m is reported as mean $\pm$ SD from three independent experiments ($n=4$). SD, standard deviation.

Fig. 5. The effect of cholesterol on the potency of compound 1 in SRC1-2 peptide recruitment assay and reporter gene assay. **(A)** The recombinant protein of ROR γ t (Ins) (white circle), ROR γ t (*E. coli*) (triangle), and cholesterol sulfate-saturated ROR γ t (*E. coli*) (diamond) was incubated with 10 nM biotin-SRC1-2 peptide and various concentrations of compound 1 for 3 h at room temperature. Data are shown as the mean $\pm$ SEM of duplicate wells. IC_{50} values were calculated as described in the Materials and Methods section without normalization of the data. **(B)** Inhibition of TopFluor cholesterol binding by compound 1 with 1 (white circle), 3 (triangle), or 10 μ M (diamond) TopFluor cholesterol. Data are shown as the mean $\pm$ SEM of duplicate wells. IC_{50} values were calculated as described in the Materials and Methods section without normalization of the data. **(C)** Effect of cholesterol depletion treatment and addition of cholesterol sulfate (starting at 1 μ M; 1:2 serial dilutions) against ROR γ t transcriptional activity in cell-based reporter gene assay. The data are shown as the mean $\pm$ SD of duplicate wells. **(D)** The ROR γ t stable cell line was incubated for 18 h with the indicated concentration of compound 1 in medium containing normal FBS (white circle) or lipid-reduced FBS with 10 μ M lovastatin and 0 (triangle), 0.25 (diamond), 0.5 (black circle), and 1 μ M (white square) cholesterol sulfate. Data are shown as the mean $\pm$ SEM of quadruplicate wells. IC_{50} values were calculated as described in the Materials and Methods section without normalization of the data. FBS, fetal bovine serum.

assay, the intracellular cholesterol level affected the potency of the ROR γ t inverse agonist. Therefore, it is noteworthy that reducing cholesterol levels in both cell-free and cell-based assay is necessary for the sensitive screening of ROR γ t inverse agonists.

DISCUSSION

We have shown using cell-free assays that T0901317 was more potent on the ROR γ t (*E. coli*) protein than on the ROR γ t (Ins) protein due to the difference in their cholesterol content. In addition, compound 1 was identified as a ROR γ t inverse agonist through TopFluor cholesterol binding screening with the apo-ROR γ t protein. In the cell-based reporter gene assay, reducing intracellular cholesterol levels lowered the IC_{50} of compound 1. Although the apo-ROR γ t protein expressed in *E. coli* was already used in *in vitro* functional assays, and cholesterol depletion treatment has been used in cell-based

assays by different authors,^{14,18} the results obtained in this study show that the effect of cholesterol on the potency of ROR γ t inverse agonists is large. Thus, excluding cholesterol is important for sensitive assays to conduct high-throughput screening. In fact, high-throughput screening using SRC1-2 recruitment assay with a small-scale library with the ROR γ t (Ins) protein yielded no positive compounds (data not shown).

Although our data using LC/MS showed that $\sim 60\%$ of ROR γ t (Ins) is bound to cholesterol, TopFluor cholesterol could bind $<10\%$ of the ROR γ t (Ins). This discrepancy is thought to be attributed to the presence of other cholesterol derivatives because we detected the specific peak for cholesterol. This observation is similar to the report by Bitsch *et al.*²⁸ showing that 77% of cholesterol and 18% of 7-dehydrocholesterol were bound with ROR α LBD protein expressed in Sf9 cells. Like ROR γ t (Ins) protein, cholesterol sulfate-bound ROR γ t (*E. coli*)

Fig. 6. The effect of cholesterol against the recombinant ROR γ t protein and ROR γ t inverse agonist. Cholesterol-free apoprotein has constitutive activity and can interact with SRC1 peptide (A). Cholesterol competitive inverse agonists such as TO901317 and compound 1 bind to the LBP inducing a conformational change, leading to a blockade of SRC1 peptide interaction (B). Cholesterol-bound protein is an active form inducing the recruitment of SRC1 peptide (C). The inverse agonists seldom replace the cholesterol in the LBP of cholesterol-bound protein, and the interaction between ROR γ t and SRC1 peptide is still sustained (D). LBP, ligand binding pocket.

blocked the binding of fluorescence-labeled cholesterol and the inverse agonists, which is consistent with the previous report by Wang *et al.*¹⁸ illustrating that cholesterol sulfate-saturated ROR α -LBD inhibited the binding of ³H-25OHC to the receptor.

We showed that the presence of cholesterol within the ROR γ t (Ins) interfered with binding of the ROR γ t inverse agonist; however, IC₅₀ of cholesterol in TR-FRET binding assay was higher (about 7.2 μ M) than we expected. According to this result, we assumed that cholesterol interacts with ROR γ t in a time-dependent manner. To prove the possibility of slow binding kinetics of cholesterol against ROR γ t, we tried to perform SPR experiment by using Biacore biosensor, but it did not work because of sticky profile of cholesterol. Further experiments are required to investigate the binding mode of cholesterol against ROR γ t.

As for the cell-free assay, we showed that compound 1 and TO901317 could not exchange the endogenous cholesterol bound to the ROR γ t protein (IC₅₀ of compound 1 was >10 μ M), while in the cell-based ROR γ t reporter gene assay, they inhibited the ROR γ t transcriptional activity (IC₅₀ of compound 1 was 14 μ M) in normal FBS medium, in which intracellular cholesterol is abundant, and IC₅₀ ratio of normal condition versus cholesterol depletion treatment in the reporter gene assay was lower than cell-free assay. In addition, various inverse ag-

onist compounds, including ursolic acid, and digoxin are effective in *in vivo* assay, although endogenous cholesterol is present,²⁹ suggesting that LBP of ROR γ t in mammalian cells is exchangeable by inverse agonists. We speculated one reason that the intracellular turnover of ROR γ t is faster compared with other cellular proteins. In fact, our SILAC analysis showed that the half-life of ROR γ t was ~6.2 h (data not shown). On the time scale of the cell-based assay, ROR γ t inverse agonists may bind to ROR γ t before cholesterol binds the protein generated in the cell body. Another possible reason is that cholesterol derivatives bound to ROR γ t in mammalian cells can be exchanged by the inverse agonists because of weaker affinity. This observation in which ligand bound with purified protein acts as a potency tuner is supported by the previous reports with hepatocyte nuclear factor 4 (HNF4) family, which show high constitutive activity. Crystallographic analysis of HNF4 α and HNF4 γ LBD protein identified a mixture of fatty acids^{30,31} and the endogenous fatty acids bound with the recombinant protein did not readily exchange with radiolabeled palmitic acid. On the other hand,

affinity isolation/mass spectrometry showed that endogenously expressed HNF4 α in mammalian cells was occupied by linoleic acid. This ligand was silent ligand and linoleic acid-bound HNF4 α protein was exchangeable by another ligand in mammalian cells.³² These reports suggest that composition of ligands bound with the NR protein is different in host cells, and further investigation of the factual form of the ROR γ t LBD in mammalian cells should be conducted.

In summary, we identified compound 1 as a cholesterol-competitive ROR γ t inverse agonist and showed that the inverse agonist effect of compound 1 depends on whether cholesterol is saturated in the receptor or not. Further compound optimization study of compound 1 has been reported by Shirai *et al.*¹⁹ This study emphasizes the importance of characterizing the recombinant protein and the optimization of cell-based assay conditions for high-throughput screening, and should impact the investigation of other orphan NRs for obtaining new lead compounds.

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