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Biochemical Properties of TAK-828F, a Potent and Selective Retinoid-Related Orphan Receptor Gamma t Inverse Agonist

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Keywords
Retinoid-related orphan receptor gamma t · Inverse agonist · Interleukin-17 · Inflammatory disease

Abstract
Background/Aims: Retinoid-related orphan receptor gamma t (RORγt) is a master regulator of T helper 17 cells that plays a pivotal role in the production of inflammatory cytokines including interleukin (IL)-17. Therefore, RORγt has attracted much attention as a target receptor for the treatment of inflammatory diseases including rheumatoid arthritis, multiple sclerosis, inflammatory bowel diseases, and psoriasis. This study aims to characterize TAK-828F, a potent and selective RORγt inverse agonist. ***Methods:*** The biochemical properties of TAK-828F were evaluated using Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET) binding assay, surface plasmon resonance (SPR) biosensor assay, cofactor recruitment assay, reporter assay, and IL-17 expression assay. ***Results:*** TR-FRET binding assay and SPR biosensor assay revealed rapid, reversible, and high affinity binding of TAK-828F to RORγt. The cofactor recruitment assay showed that TAK-828F inhibited the recruitment of steroid receptor coactivator-1 to RORγt. Furthermore, TAK-828F inhibited the transcriptional activity of human and mouse RORγt with selectivity against human RORα and RORβ. TAK-828F also suppressed IL-17 production in Jurkat cells, overexpressing human RORγt. ***Conclusion:*** These favorable properties will be of advantage in the evaluation of TAK-828F in clinical studies for inflammatory diseases. Furthermore, these findings demonstrate that TAK-828F could serve as a pharmacological tool for further studies of RORγt and inflammatory diseases.

Introduction

T helper (Th) 17 cells have been identified as a subset of effector T cells, in addition to Th1 and Th2 cells in the immune system. They are characterized by potent pro-inflammatory cytokine expression such as interleukin (IL)-17A, IL-17F, IL-22, and granulocyte-macrophage colony-stimulating factor [1–3]. Th17 cells and the cytokines they express have been implicated in the pathogenesis of many inflammatory diseases including rheumatoid arthritis, multiple sclerosis, inflammatory bowel diseases, and psoriasis [4]. Differentiation from effector T cells to Th17 cells

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and the subsequent IL-17 expression are controlled by retinoid-related orphan receptor gamma t (RORγt) [5, 6].

RORγt is a member of the nuclear receptor superfamily that is expressed mainly in cells of the immune system, such as T cells, γδ T cells, and group 3 innate lymphoid cells [7–9]. RORγt is known to function as a master regulator of IL-17 [10]. Antibody therapy against IL-17, which is downstream of RORγt pathway, has been shown to be efficacious in the treatment of inflammatory diseases in clinical trials [11, 12]. These encouraging results with anti-IL-17 antibodies have demonstrated the validity of this pathway for the treatment of inflammatory diseases. The inhibition of RORγt function by small molecule inverse agonists has also effectively suppressed the production of inflammatory cytokines in this pathway and demonstrated the efficacy in animal disease models [10, 13–18]. Therefore, RORγt has attracted much attention as a therapeutic target for inflammatory diseases to date.

In an effort to develop novel RORγt inverse agonists, we have identified TAK-828F, a potent and selective RORγt inverse agonist. In this study, we investigated the properties of TAK-828F by diverse in vitro assays, including Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET) binding assay, surface plasmon resonance (SPR) biosensing assay, cofactor recruitment assay, and some cell-based functional assays. TR-FRET binding assay and SPR biosensing assay demonstrated that TAK-828F rapidly and reversibly binds to human RORγt with high affinity. The cofactor recruitment assay indicated that TAK-828F inhibits the binding of human RORγt and steroid receptor coactivator-1 (SRC-1) peptide and TAK-828F functions as an inverse agonist for RORγt. In cell-based functional assays, TAK-828F inhibited the transcriptional activity of human and mouse RORγt with clear selectivity against human RORα and RORβ. In addition, in the assay using Jurkat cells transiently overexpressing RORγt, TAK-828F suppressed the IL-17 production. These (favorable) properties including potent binding activity, selectivity towards RORα and RORβ, and concentration-dependent inhibition of IL-17 expression support the evaluation of TAK-828F in clinical studies and provide novel insights into the relationship between RORγt signaling and inflammatory diseases.

Materials and Methods

Reagents

TAK-828F and compound A, fluorescence-labeled probe for human RORγt, were synthesized in Takeda Pharmaceutical Company Limited (Fujisawa, Japan). Tb-labeled anti His-tag antibody was obtained from Thermo Fisher Scientific (Waltham, MA, USA). About 5 mol/L NaCl was obtained from Promega Corporation (Fitchburg, WI, USA). AlphaScreen histidine detection kit was obtained from PerkinElmer (Waltham, MA, USA). Biotinylated SRC-1 peptide (Biotin-CPSSHSSLTERHKILHRLLQEGSPS) was synthesized in Scrum (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from Corning Inc. (Corning, NY, USA). Lipid-reduced FBS was obtained from GE Healthcare (Buckinghamshire, UK). Neon transfection system was obtained from Thermo Fisher Scientific. The primers and probes for human IL-17 and human GAPDH used for quantitative PCR were purchased from Thermo Fisher Scientific. Other materials were purchased from Wako Pure Chemicals Industries (Osaka, Japan).

Protein Preparation

A truncated ligand binding domain (LBD) of human RORγt (aa. 261-508) with 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. Bacterial culture was grown in LB media at 37 °C. When the culture reached an OD_{600} of 0.8, the cells were induced with 0.8 mmol/L IPTG for an additional 18 h growth at 16 °C before harvesting. Cell pellet was resuspended and sonicated in lysis buffer (25 mmol/L Tris-HCl, pH 7.6, 1 M NaCl, 10 mmol/L imidazole, 0.5 mmol/L tris-[2-carboxyethyl]phophine [TCEP], 20 U/mL benzonase, 1 mg/mL lysozyme, and protease inhibitors). Cell lysate was clarified by centrifugation and applied to a 5 mL HiTrap Talon column (GE Healthcare, UK). After an extensive wash, the His tagged protein was eluted from the column with buffer containing 300 mmol/L imidazole in 25 mmol/L Tris-HCl, pH 7.6, 1 mol/L NaCl, 2 mmol/L benzamidine, and 0.5 mmol/L TCEP. Protein sample was buffer exchanged into 25 mmol/L Tris-HCl, 200 mmol/L NaCl, 2 mmol/L benzamidine, 2 mmol/L dithiothreitol (DTT), and 5% glycerol. It was then concentrated and loaded onto a size exclusion chromatography column (Hiload 16/60 Superdex 200). Peak fractions containing the truncated LBD protein were pooled and concentrated to 1 mg/mL. Purity of the final protein sample was verified by SDS-PAGE. This LBD of RORγt was used in cell-free assays (TR-FRET assay, SPR biosensor assay, and cofactor recruitment assay).

TR-FRET Binding Assay

To determine the dissociation constant (K_d) of compound A, 10 μL of compound A diluted in various concentration by assay buffer containing 20 mmol/L Tris-HCl (pH 8.0), 100 mmol/L NaCl, 0.1% bovine serum albumin (BSA), 1 mmol/L DTT was added to the white 384 plate (784075, greiner bio-one). Then 10 μL of 2 nmol/L His-tagged human RORγt and 0.2 nmol/L Tb-labeled anti His-tag antibody mixture was added. After the plate was incubated at room temperature for 1 h, the fluorescence ratio (Ex 486 nm/Em 520 nm) was measured by plate reader Envision (PerkinElmer). For the calculation of K_d value of compound A, signal from the sample with human RORγt and Tb-labeled anti His antibody was defined as total binding, and signal from the sample with Tb-labeled anti-His antibody alone was defined as non-specific binding. Specific binding was calculated by subtracting the average value of non-specific binding from the average value of total binding. K_d value of compound A was estimated by one site – specific binding in the software GraphPad Prism (GraphPad Software, San Diego, CA, USA).

The binding activity of TAK-828F to human RORγt was evaluated by TR-FRET binding assay using compound A, fluores-

cence-labeled analog compound of TAK-828F. Around 4 μL of TAK-828F diluted in various concentrations in assay buffer was added to the white 384 plate. Then 4 μL of 3 nmol/L human RORγt and 0.3 nmol/L Tb-labeled anti-His tag antibody mixture was added. Finally, 4 μL of 120 nmol/L compound A solution was added. After the plate was incubated at room temperature for 1 h, the fluorescence ratio (Ex 486 nm/Em 520 nm) was measured by plate reader Envision. For the determination of inhibitory activity, signal from the vehicle control was defined as 0% control inhibition and signal from the sample without human RORγt was defined as 100% control inhibition.

The dissociation constant (K_d) of TAK-828F to human RORγt was calculated by using the following Cheng-Prusoff equation:

$$K_d \text{ (TAK-828F)} = IC_{50}/(1 + [L]/K_d \text{ [compound A]}),$$

where [L] and K_d (compound A) represent the concentration and the dissociation constant of compound A. In this study, [L] was 40 nmol/L and K_d of compound A was 46 nmol/L.

Kinetic Analysis Using SPR Biosensor Assay

SPR biosensing assay was performed on a Biacore S200 instrument (GE Healthcare). HBS-N (10 mmol/L Hepes, 150 mmol/L NaCl, pH 7.4) supplemented with 1 mmol/L DTT was used as the running buffer for immobilization. RORγt protein containing N-term his-tag was immobilized on a nitrilotriacetic acid (NTA) sensor chip (a carboxymethylated dextran pre-immobilized chip with NTA) through his-tag capturing and mild cross-linking. All binding experiments were performed in 20 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 0.2 mmol/L TCEP, 0.02% Surfactant P20, and 1% DMSO at 20 °C. Compounds were injected for 90 s at a flow rate of 50 μL/min, and the dissociation was thereafter followed for up to 200 s. Solvent correction was included as described in the Biacore S200 software handbook. Data processing was performed using Biacore S200 evaluation software (GE Healthcare). Sensorgrams were double referenced prior to global fitting of the concentration series to one-to-one binding models, and the on-rate constant (k_{on}) and the off-rate constant (k_{off}) values were analyzed.

The dissociation constant of TAK-828F was calculated by the following equation:

$$K_d = k_{off}/k_{on}$$

The half-time of TAK-828F was calculated by the following equation:

$$t_{1/2} = \ln 2/k_{off}$$

Cofactor Recruitment Assay

Cofactor recruitment assay was performed using the AlphaScreen histidine detection kit (Perkin Elmer) according to the manufacturer's instructions with minor modifications. Around 5 μL of TAK-828F diluted in assay buffer containing 50 mmol/L Tris-HCl, 50 mmol/L NaCl, 0.1% BSA, 1 mmol/L DTT was added to the white 384 OptiPlate (Perkin Elmer). Then 10 μL of 125 nmol/L human RORγt was added to the plate and 10 μL of AlphaScreen beads mixture containing 12.5 μg/mL Streptavidin Donor beads, 12.5 μg/mL Ni-chelate Acceptor beads, and 125 nmol/L biotin-SRC1 peptide was added to the plate. After the plate was incubated in the dark for 1 h, the AlphaScreen signal was read by plate reader Envision. To normalize the inhibition activity, signal from vehicle control was defined as 0% inhibition and signal without human RORγt was defined as 100% inhibition.

Reporter Assay

Jurkat cells (ATCC, TIB-152) were maintained in RPMI medium containing 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. For human RORs reporter assay, 5 μL of TAK-828F diluted to various concentrations by reaction medium, RPMI containing 10% lipid-reduced FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 5 μmol/L Lovastatin was added to the white 384 plate (3570, Corning, NY, USA). Lovastatin was added to the assay buffer to stop the biosynthesis of cholesterol and facilitate the detection of TAK-828F inhibitory activity. The reporter vector was prepared by inserting human IL-17 ROR response element into the upstream of luciferase of pGL 4.28 (Promega). RORs expression vectors were prepared by inserting full length RORs sequence into the downstream of CMV promoter. Jurkat cells were transiently transfected with each of 1 μg/mL RORs expression vector and 1 μg/mL reporter vector by electroporation and diluted with reaction medium to the concentration of 1.0×10^6 cells/mL. Then 20 μL of transfected cells were added to the plate. After 24 h incubation in a 5% CO_2 incubator, 25 μL of Bright-Glo (Promega Corporation, WI, USA) was added and the plate was shaken for 5 min. The luminescence of reporter gene was detected using plate reader Envision. For the determination of inhibitory activity, signal from the vehicle control was defined as 0% control inhibition and signal from the sample without human RORs expression was defined as 100% control inhibition.

Nuclear Receptor Selectivity

The evaluation of general nuclear receptor selectivity was performed by SelectScreen® Cell-Based Nuclear Receptor Profiling Service in Life Technologies. The reporter assays use a beta-lactamase as a reporter gene under transcriptional control of an Upstream Activator Sequence and HEK293 cells expressing the fusion protein of Gal4 DNA binding domain and each nuclear receptor LBD were used.

IL-17 Production in Jurkat Cells

Jurkat cells were electroporated with the expression vector of full length RORγt and diluted with RPMI medium containing 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin to the concentration of 1.0×10^6 cells/mL. About 100 μL of transfected cells were added to the white 96 well plate (3917, Corning, NY, USA) and incubated in 5% CO_2 incubator overnight. TAK-828F was diluted in the medium to various concentrations and 12.5 μL was added to the wells. Phorbol myristate acetate (PMA) and A-23187 (Ca^{2+} ionophore) were added to the cells to the final concentration of 1.6 and 500 nmol/L, respectively. After 6–24 h incubation in 5% CO_2 incubator, 75 μL of supernatant was collected and IL-17 was measured by Human IL-17 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instruction. About 150 μL of CellAmp Processing Buffer (Takara Bio, Kusatsu, Japan) was added to each well and RT-PCR was performed to quantify the amounts of human IL-17 mRNA.

Data Analysis and Statistics

Data analysis for compound A binding assay, cofactor recruitment assay, and IL-17 production assay was performed with GraphPad Prism 5 software (GraphPad, San Diego, CA, USA). The

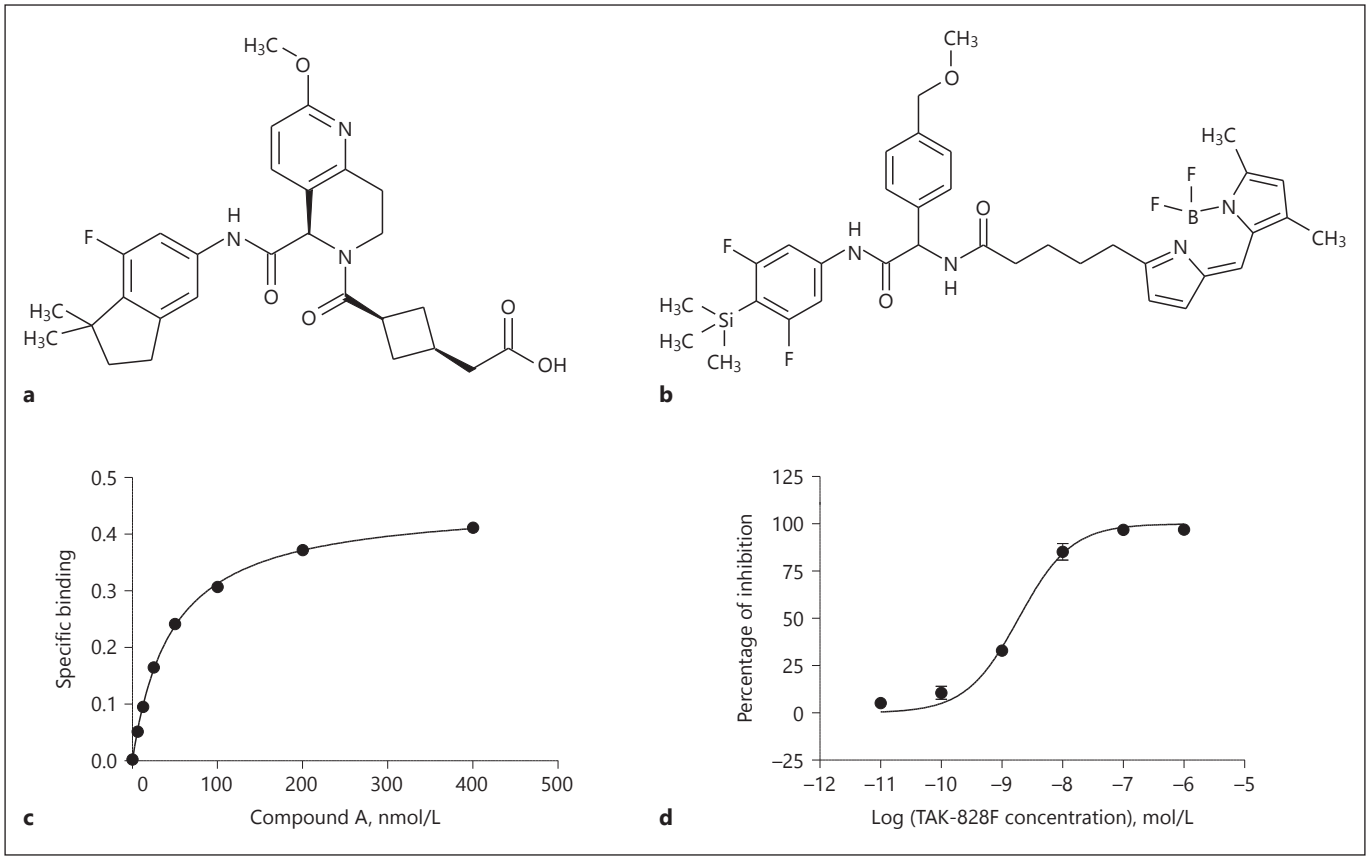

Fig. 1. TAK-828F competes with compound A for binding to human RORγt. Structure of TAK-828F (**a**) and compound A (**b**). Various concentrations of compound A were incubated with 1 nmol/L His-tagged human RORγt and 0.1 nmol/L Tb-labeled anti His-tag antibody and incubated for 1 h at room temperature (**c**). TAK-828F was incubated with 40 nmol/L compound A, 1 nmol/L His-tagged human RORγt, and 0.1 nmol/L Tb-labeled anti His-tag antibody and incubated for 1 h at room temperature (**d**). Data points are the mean ± SD of 2 values from a representative experiment of 3 separate experiments. Comparable results were obtained by each independent experiment.

data of compound A binding assay were fitted to a one-site binding equation to determine the K_d value of compound A. IC_{50} values of cofactor recruitment assay and IL-17 production assay were calculated by fitting the data to a sigmoidal dose–response equation. IC_{50} values of TAK-828F binding assay and the reporter assays were calculated by XLfit software. All graphs except SPR analysis were depicted by GraphPad Prism 5 software. IC_{50}, K_d, on-rate constant, off-rate constant, and $t_{1/2}$ values are expressed as the mean with 95% CI.

Results

TAK-828F Competes with Compound A for Binding to Human RORγt

In our effort to discover RORγt inverse agonists, we have identified the potent and selective RORγt inverse agonist, TAK-828F. For the evaluation of binding activity of TAK-828F to human RORγt by TR-FRET binding assay, we synthesized fluorescence-labeled analog compound of TAK-828F, compound A. We first performed the association assay of compound A and human RORγt by TR-FRET to determine the K_d value of compound A. As shown in Figure 1c, compound A showed the binding activity to human RORγt with a K_d value of 46 nmol/L (95% CI 43–50 nmol/L). From this result, we concluded that compound A could be used as a probe for TR-FRET binding assay.

Subsequently, we evaluated the binding activity of TAK-828F to human RORγt by competition binding assay based on TR-FRET using 40 nmol/L compound A. TAK-828F showed the binding activity to human RORγt with IC_{50} value of 1.9 nmol/L (95% CI 1.6–2.2 nmol/L; Fig. 1d) and K_d value was calculated as 1.0 nmol/L (95% CI 0.85–1.2 nmol/L) from Cheng-Prusoff equation. Table 1

Table 1. Binding properties of compound A and TAK-828F to human RORγt

Fluorescence probe	K_d (compound A) (95% CI), nmol/L	Competition study		Values calculated from Cheng-Prusoff equation
		compound A concentration, nmol/L	IC_{50} of TAK-828F (95% CI), nmol/L	K_d (TAK-828F) (95% CI), nmol/L
Compound A	46 (43–50)	40	1.9 (1.6–2.2)	1.0 (0.85–1.2)

summarizes the binding profile of compound A and TAK-828F, demonstrating TAK-828F possesses high affinity to human RORγt.

Kinetic Analysis Using SPR Biosensor Assay

To better understand the binding property of TAK-828F to human RORγt, we evaluated the kinetic parameters of TAK-828F using an SPR biosensing assay. RORγt protein containing N-term his-tag was immobilized on an NTA sensor chip through his-tag capturing and mild cross-linking. Various concentrations of TAK-828F were injected and sensorgrams were obtained. Kinetic parameters were analyzed by Biacore S200 evaluation software. As shown in Figure 2, TAK-828F is physically bound to human RORγt in a reversible manner and dissociates slowly from human RORγt. The on-rate constant (k_{on}) and the off-rate constant (k_{off}) values were calculated as 4.0×10^5 mol/L^{-1}s^{-1} (95% CI $3.8–4.2 \times 10^5$ mol/L^{-1}s^{-1}) and 2.4×10^{-3} s^{-1} (95% CI $1.6–3.2 \times 10^{-3}$ s^{-1}), respectively. The K_d of TAK-828F was calculated from the on-rate constant and the off-rate constant as 6.0 nmol/L (95% CI 4.0–8.0 nmol/L), and this value agrees very well with IC_{50} value in the TR-FRET binding assay. To evaluate how slowly TAK-828F dissociates from human RORγt, $t_{1/2}$ value was calculated from the off-rate constant to be 289 s (95% CI 217–433 s) using the equation described in Materials and Methods. Table 2 summarizes the kinetic parameters of TAK-828F for human RORγt, suggesting that TAK-828F binds to human RORγt in a reversible manner and dissociates slowly.

TAK-828F Inhibits Recruitment of SRC-1 to Human RORγt

To further understand the effect of TAK-828F on human RORγt, we next performed cofactor recruitment assay with SRC-1 peptide. RORγt is constitutively active and spontaneously binds to SRC-1 peptide. TAK-828F inhibited this binding with IC_{50} value of 59 nmol/L (95% CI 35–100 nmol/L). From this result, we concluded that TAK-828F has the inverse agonist activity for human RORγt.

Table 2. Kinetics parameters of TAK-828F

	k_{on} (95% CI) 10^5 mol/L^{-1}s^{-1}	k_{off} (95% CI) 10^{-3} s^{-1}	$t_{1/2}$ (95% CI) s
TAK-828F	4.0 (3.8–4.2)	2.4 (1.6–3.2)	289 (217–433)

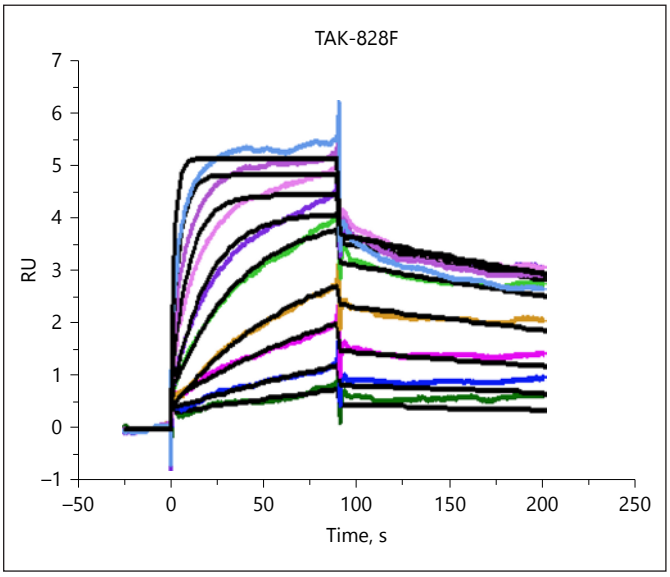

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Fig. 2. Kinetics analysis by SPR. The binding of TAK-828F to human RORγt was evaluated by SPR biosensing assay. Various concentrations of TAK-828F were injected for 90 s and the dissociation was followed up for 200 s. SPR sensorgram (colored) and global fit (black) of the interaction of TAK-828F and human RORγt. The concentration of TAK-828F was 1,000, 500, 250, 125, 62.5, 31.3, 15.6, 7.81, and 3.91 nmol/L from top.

TAK-828F Suppresses the Transcriptional Activity of Human and Mouse RORγt, Not Human RORα and RORβ

To further investigate whether TAK-828F inhibits the transcriptional activity of RORγt in cells, we developed the reporter assay with Jurkat cells and evaluated the inhibitory effect of TAK-828F on the transcriptional activity of RORs in the cells. Jurkat cells were electroporated with the

human RORγt expression vector and the reporter vector which has 3 repeated sequence of ROR response element in front of luciferase sequence and incubated with various concentrations of TAK-828F for 24 h. In this reporter assay, TAK-828F inhibited the transcriptional activity of human RORγt with IC_{50} value of 6.1 nmol/L (95% CI 3.7–10 nmol/L; Fig. 4). Subsequently, to investigate the species specificity and selectivity to other ROR subtypes, we performed the reporter assay with mouse RORγt, human RORα, and human RORβ, as described in Materials and Methods. TAK-828F showed inhibitory effect on mouse RORγt with IC_{50} value of 9.5 nmol/L (95% CI 6.4–14 nmol/L; Fig. 4), which is almost the same as on human RORγt. On the contrary, TAK-828F showed no inhibitory effect on human RORα and RORβ up to 10 μmol/L (Fig. 4). These results demonstrate that TAK-828F possesses the potent inverse agonist activity to human and mouse RORγts with selectivity against human RORα and RORβ. To elucidate the selectivity of TAK-828F against other nuclear receptors, we examined the agonistic and antagonistic effects of TAK-828F on other nuclear receptors. Table 3 summarizes the activities of TAK-828F to other nuclear receptors, demonstrating that TAK-828F is highly selective to RORγt.

TAK-828F Represses IL-17 Production in Jurkat Cells Overexpressing Human RORγt

To confirm the inhibitory effect of TAK-828F on IL-17 production, we developed the IL-17 expression assay using Jurkat cells overexpressing human RORγt to detect the IL-17 expression levels in the cells. Jurkat cells were electroporated with human RORγt expression vector, stimulated by PMA and A-23187, and incubated for 6–24 h. The amount of IL-17 secreted into the medium was detected by ELISA and IL-17 mRNA level in the cells was measured by RT-PCR. TAK-828F showed a concentration-dependent inhibitory activity to both IL-17 protein expression and IL-17 mRNA level with IC_{50} values of 19 nmol/L (95% CI 11–32 nmol/L) and 4.3 nmol/L (95% CI 1.7–11 nmol/L), respectively (Fig. 5). From the data of IL-17 expression assays along with the results of reporter assays, we conclude that TAK-828F possesses the potent inhibitory activity against IL-17 expression through inhibiting the transcriptional activity of RORγt.

Discussion

The study of small molecule inverse agonists of RORγt dates back to the discovery of the inverse agonist activity of T0901317 for RORγt. In 2010, Kumar from

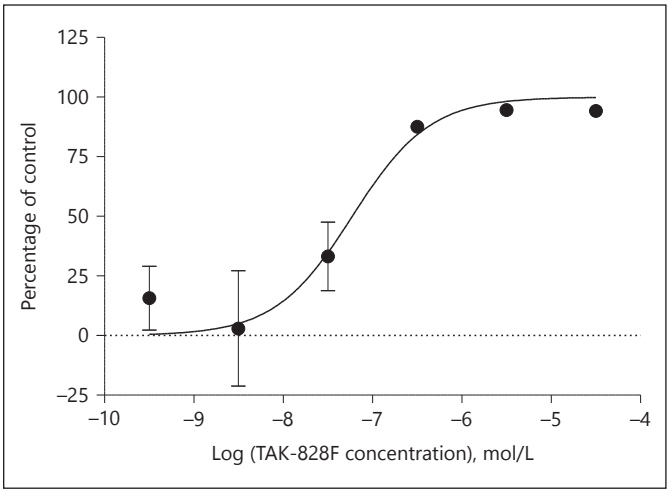

Fig. 3. TAK-828F inhibits the recruitment of SRC-1 to human RORγt. TAK-828F was incubated with 50 nmol/L human RORγt, 10 nmol/L biotin-labeled SRC-1 peptide, 5 μg/mL Streptavidin Donor beads, and 5 μg/mL Ni-chelate acceptor beads in the dark at room temperature for 1 h. Data points are the mean ± SD of 4 values from a representative experiment of 3 separate experiments and comparable results were obtained by each independent experiment.

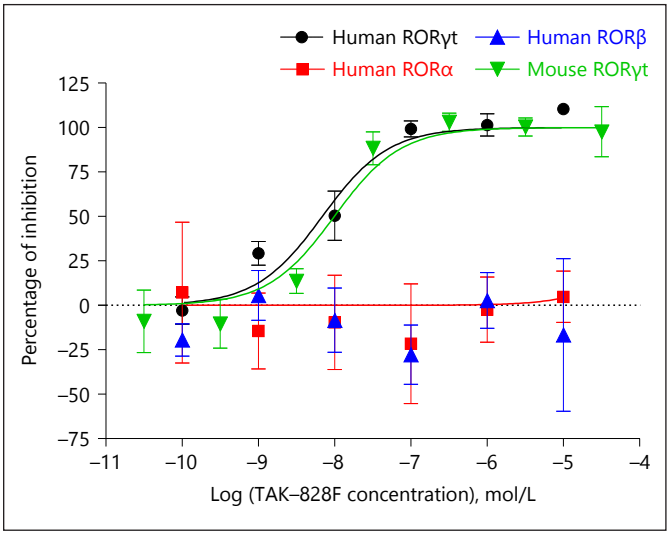

Fig. 4. TAK-828F suppresses the transcriptional activity of human and mouse RORγt, not human RORα and RORβ. Jurkat cells were transiently transfected with human or mouse RORγt, human RORα, or RORβ expression vector and the reporter vector with ROR-response element (RORE). The transfected cells were incubated with TAK-828F for 24 h and the luciferase activity was detected by Bright-Glo. Data points are the mean ± SD of 4 values from a representative experiment of 3 separate experiments. Comparable results were obtained by each independent experiment.

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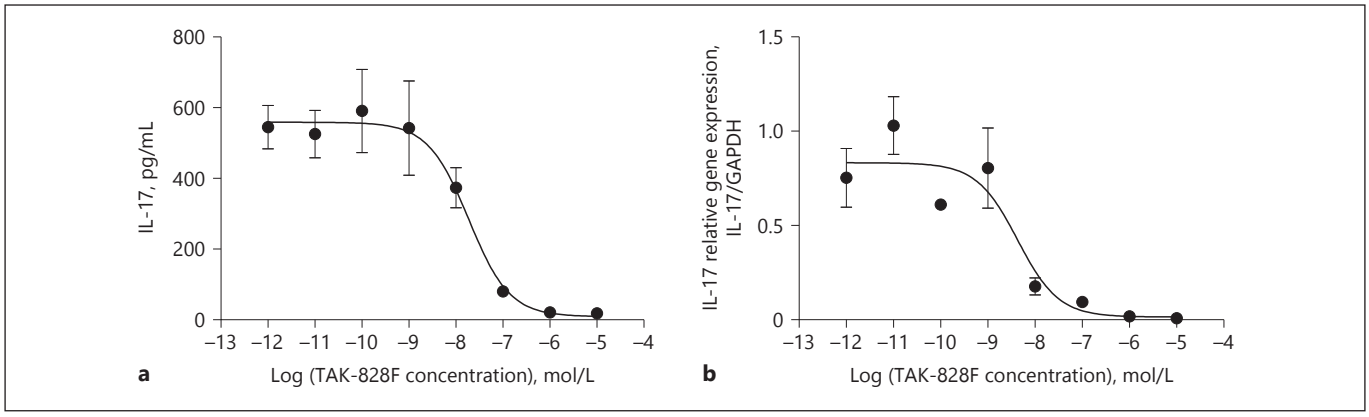

Fig. 5. TAK-828F represses IL-17 production in Jurkat cells overexpressing human RORγt. Jurkat cells were transiently transfected with human RORγt expression vector. The transfected cells were incubated with TAK-828F and stimulated with 1.6 nmol/L PMA and 500 nmol/L A-23187 for 6–24 h. The amount of IL-17 in the culture media was measured by IL-17 ELISA kit using the samples after 24 h incubation (**a**) and the amount of IL-17 mRNA was quantified by RT-PCR using the samples after 6 h incubation (**b**). Data points of IL-17 ELISA are the mean ± SD of 4 values from a representative experiment of 3 separate experiments. Data points of IL-17 mRNA are the mean ± SD of 5 values from a representative experiment of 3 separate experiments. Comparable results were obtained by each independent experiment.

The Scripps Research Institute first reported the synthetic small molecule T0901317 as an inverse agonist of RORα and RORγt [19]. However, T0901317 is also well known to have LXR agonistic activity and has been used in the LXR study [20]. Furthermore, it was also revealed that T0901317 activated FXR and PXR [21, 22]. Thus, due to the lack of selectivity, this compound is not suitable for RORγt research. In 2011, the Scripps group reported that they synthesized SR1001 by modification from T0901317 and showed that SR1001 was an inverse agonist of RORα and RORγt without LXR activity [17]. It was also shown that SR1001 inhibited Th17 cell differentiation and had the efficacy in mouse experimental autoimmune encephalomyelitis model. By this successful discovery of a small molecule inverse agonist of RORγt and its biological efficacy, RORγt attracted much attention as a promising pharmacological target for inflammatory diseases. So far many pharmaceutical companies and institutions have reported various inverse agonists of RORγt [23–27].

In this study, we discovered the potent and selective RORγt inverse agonist, TAK-828F, and investigated its properties by using various assays. In an attempt to understand the binding profile of TAK-828F, we employed TR-FRET binding assay and synthesized the BODIPY-labeled fluorescent probe for the assay using an analog compound of TAK-828F. The K_d value of synthesized probe, compound A, was 46 nmol/L (95% CI 43–50 nmol/L). TAK-828F, based on concentration, competed with compound A for human RORγt with IC_{50} value of 1.9 nmol/L (95% CI 1.6–2.2 nmol/L) and K_i value of 1.0 nmol/L (95% CI 0.85–1.2 nmol/L). This is one of the most potent RORγt inverse agonists reported so far. For example, T0901317 was reported by Scripps Research Institute to have a K_i value of 51 nmol/L to human RORγt [19], and hence TAK-828F is fifty-fold more potent compared to T0901317.

Table 3. Selectivity towards other nuclear receptors

Targets	AR, ERα, ERβ, FXR, GR, LXRα, LXRβ, MR, PPARδ, PPARγ, PR, RARα, RARβ, RARγ, RXRα, RXRβ, TRα, TRβ, and VDR
% act. @ 10 μmol/L	All <50%
% inh. @ 10 μmol/L	All <50% except: PPARδ (60%), PPARγ (86%), RARβ (52%)

Next we investigated the binding profile of TAK-828F in more detail using SPR biosensing assay. Figure 2 shows that TAK-828F dissociates slowly from human RORγt. Its on-rate constant and off-rate constant are 4.0×10^5 mol/L^{-1}s^{-1} (95% CI 3.8–4.2 $\times 10^5$ mol/L^{-1}s^{-1}) and 2.4×10^{-3} s^{-1} (95% CI 1.6–3.2 $\times 10^{-3}$ s^{-1}), respectively. These binding properties of TAK-828F indicate that it could serve as a useful tool for various pharmacological studies.

As to the binding site of TAK-828F on RORγt protein, our colleagues recently published the paper describing

the results of a crystal structure of RORγt combined with TAK-828F. According to their paper, TAK-828F binds to the LBD of RORγt, surrounded by amino acids of Phe377, Glu379, Arg364, Gln286, Phe388, and Gly380 [33].

It is well known that the activity of a nuclear receptor is regulated by various cofactor proteins. Our knockdown experiment of various cofactors in RORγt reporter assay revealed that SRC-1 is largely involved in the RORγt-induced transcription activity (data not shown). We attempted to evaluate the ability of TAK-828F to regulate the interaction between RORγt and SRC-1 peptide by AlphaScreen technology to determine whether TAK-828F functions as an agonist or an inverse agonist to RORγt. The interaction of SRC-1 peptide and RORγt was inhibited by TAK-828F in a concentration-dependent manner (Fig. 3), demonstrating that TAK-828F functions as an inverse agonist to RORγt. The results of TR-FRET binding assay and cofactor recruitment assay indicate that TAK-828F binds directly to RORγt and inhibits the binding of RORγt and SRC-1 peptide.

We then sought to assess the activity of TAK-828F to suppress the function of human RORγt in cells. We also assessed the species specificity and selectivity to other ROR subtypes in the same reporter assay system. RORα is widely expressed in various tissues, such as muscle, liver, lung, skin, thymus, kidney, and brain and RORα is also known to play an important role in the maturation of Purkinje cells [28]. The RORα knockout mice display motor ataxia such as tremor, body imbalance, small size and die between 3 and 4 weeks due to defective Purkinje cells [29]. RORβ is expressed mainly in the central nervous system, especially in the pineal gland and retina and serves as a regulator in circadian rhythm [30]. The RORβ knockout mice show a duck-like gait, transient male incapability to reproduce sexually, and a severely disorganized retina [31]. Thus, the selectivity against RORα and RORβ is of critical importance when the compound is chronically administered, and hence the compound needs to be highly selective for RORγt in the ROR family. As shown in Figure 4, it was revealed that TAK-828F inhibits human and mouse RORγt-induced reporter activity with IC_{50} values of 6.1 nmol/L (95% CI 3.7–10 nmol/L) and 9.5 nmol/L (95% CI 6.4–14 nmol/L), respectively. As for the selectivity among the ROR family, TAK-828F shows no inhibitory activity to human RORα and RORβ up to 10 μmol/L, demonstrating that TAK-828F possesses significant selectivity. To further elucidate the selectivity of TAK-828F towards other nuclear receptors, general nuclear receptor panel assays (Thermo Fisher Scientific) were performed. TAK-828F shows no agonistic activities and no or weak antagonistic activities toward 19 other nuclear receptors tested (Table 3), suggesting that TAK-828F is one of the most selective compounds reported so far [23, 26]. The specificity and selectivity profiles of these species of TAK-828F indicate that it can be adequately evaluated in both human and murine model as a RORγt inverse agonist.

Since it is well known that IL-17 expression is regulated by RORγt, we next sought to confirm the activity of TAK-828F to suppress IL-17 expression in Jurkat cells. Jurkat cells are considered to be suitable cell line for the study of IL-17 pathway because Jurkat cells are derived from human T lymphocyte and known to be activated by PMA and A-23187 stimulation. Thus, in our study, Jurkat cells were electroporated with human RORγt expression vector and stimulated by PMA and A-23187 to induce IL-17 production. TAK-828F suppressed both IL-17 production and IL-17 mRNA level with IC_{50} values of 19 nmol/L (95% CI 11–32 nmol/L) and 4.3 nmol/L (95% CI 1.7–11 nmol/L), respectively. This is almost the same activity as that of the reporter assay, suggesting that TAK-828F inhibits the transcription activity of RORγt and subsequent production of IL-17.

In addition to this study, our colleagues recently published a report about the pharmacological profile of TAK-828F and its in vivo efficacy on some disease models [32].

Taken together, our results show that TAK-828F directly binds to human RORγt in a reversible manner and functions as an inverse agonist. TAK-828F inhibits the transcriptional activity of human and mouse RORγt with selectivity towards human RORα and RORβ, and suppresses IL-17 production. With these favorable biochemical properties, TAK-828F could be a highly promising candidate for the treatment of inflammatory diseases.

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Ethics Statement

Experiments were conducted according to the Guidelines of gene recombination experiments of Japan.

Disclosure Statement

The authors state no conflict of interest.

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