Identification of an allosteric binding site for RORγt inhibition

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RORγt is critical for the differentiation and proliferation of Th17 cells associated with several chronic autoimmune diseases. We report the discovery of a novel allosteric binding site on the nuclear receptor RORγt. Co-crystallization of the ligand binding domain (LBD) of RORγt with a series of small-molecule antagonists demonstrates occupancy of a previously unreported allosteric binding pocket. Binding at this non-canonical site induces an unprecedented conformational reorientation of helix 12 in the RORγt LBD, which blocks cofactor binding. The functional consequence of this allosteric ligand-mediated conformation is inhibition of function as evidenced by both biochemical and cellular studies. RORγt function is thus antagonized in a manner molecularly distinct from that of previously described orthosteric RORγt ligands. This brings forward an approach to target RORγt for the treatment of Th17-mediated autoimmune diseases. The elucidation of an unprecedented modality of pharmacological antagonism establishes a mechanism for modulation of nuclear receptors.

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Nuclear receptors (NRs) modulate transcription of particular sets of genes on binding of small lipophilic ligands and thereby regulate physiological parameters of cellular functions. NRs are also important pathological regulators in diseases such as cancer, diabetes and autoimmune disorders. This combination of characteristics of NRs has given rise to some of the most notable pharmaceutical agents of the past century. The retinoic-acid-receptor-related orphan receptor (ROR) is a NR subclass that demonstrates great therapeutic potential.

In particular, RORγt, whose activity is required for the proliferation and functionality of immune Th17 cells, is the subject of intense investigation to modulate its activity to achieve clinical benefit. Th17 cells exert an inflammatory, pathological role in autoimmune diseases and on stimulation produce pro-inflammatory cytokines. Antibodies directed against the cytokine IL17 have been clinically successful, proving the potential of targeting the Th17/IL17 axis. Active RORγt is a prerequisite for the differentiation of T cells into Th17 cells. Small-molecule inhibition of RORγt has therefore been brought forward as a novel strategy for the treatment of autoimmune diseases.

NRs are characterized by the ability to bind small ligands at a highly conserved hydrophobic orthosteric-binding pocket located within the protein’s ligand-binding domain (LBD). A typical NR LBD exhibits a three-layered fold of ~12 alpha helices and 2–3 β-strands. Ligand binding in this pocket can activate or inhibit the receptor to various degrees. Helix 12 (H12, also called activation function-2, AF-2) can adopt distinct conformations in response to ligand binding, regulating the interaction of the LBD with cofactor proteins with resulting changes in gene transcription at a particular locus. Typically, on the binding of an agonist, H12 is stabilized in a conformation that facilitates the binding of a coactivator. Conversely, antagonist binding induces a different H12 conformation unsuitable for coactivator binding. NR drugs thus bind to this orthosteric-binding pocket and act as molecular ‘switches’ that control NR transcriptional activity due to the positioning of H12. This canonical ligand binding is associated with selectivity issues and mutation-induced antagonist/agonist switches for different NRs and therefore molecules that occupy allosteric-binding sites on NRs are highly sought after. Such allosteric modulation might be expected to induce conformational effects that are not dependent on competition with endogenous ligands and could provide enhanced potency/efficacy or greater specificity over canonical ligands.

We previously identified a novel series of RORγt inhibitors. Here we characterize the mode of action of these inhibitors to guide an optimization program and surprisingly find a novel binding mode, thereby identifying the first allosteric-binding pocket for a highly potent, cellular active small NR ligand. Structural, biochemical and cellular data reveal that the unprecedented allosteric-binding modality confers both high potency and selectivity to RORγt for these novel antagonists.

Results

Helix 12 repositionings to generate a novel binding pocket.

Literature suggests that the RORS feature ligand-independent transcription, with their LBDs partially in a conformation promoting coactivator binding. Biological data and the crystal structures of RORγt LBD bound to hydroxycholesterols, and synthetic inverse agonistic ligands such as T0901317 have shown that the RORγt LBD is still structurally responsive to ligands. For RORγt, reports suggest that multiple small molecules affect antagonism via binding to the canonical orthosteric site. A high-throughput screen for molecules that disrupt the interaction of the RORγt-LBD with steroid receptor coactivator-1 (SRC-1) cofactor peptide, followed by a hit optimization program, led to the identification of indazoles as a novel class of RORγt inhibitors, typified by MRL-871 (Fig. 1a). To elucidate the molecular basis of RORγt modulation by MRL-871, we performed co-crystallization studies with an equimolar complex of RORγt-LBD and MRL-871. Co-crystals grew in two different space groups (Table 1). Crystals in space group R32:H had unit cell dimensions of \(a = b = 173.8, c = 67.2\) Å, diffracted to 2.3 Å resolution and contain one molecule per asymmetric unit. Crystals in space group P6122 had unit cell dimensions of \(a = b = 108.5, c = 104.7\) Å, diffracted to 2.2 Å resolution and also contain one molecule per asymmetric unit. The experimental electron density maps from both conditions showed clear density for all features of the protein, including MRL-871. The overall structures from both sets were identical in all the features and the ligand conformation. The RORγt LBD crystallized with the typical NR arrangement of helices 1–11; but with the H12 positioned in a conformation unprecedented for all NR LBD complexes reported to date (Fig. 1c, green). On top of this, the crystal structure revealed the binding site of MRL-871 to be different from the canonical orthosteric NR ligand-binding site. MRL-871 binds instead to a previously unidentified allosteric pocket in the RORγt LBD, located distal to the classical binding site (Fig. 1c, orange). The crystal structure analysis shows that this allosteric pocket, absent in the classical NR-folding motif, is formed by helices 4, 5, 11 and the reoriented flexible H12 (Fig. 1b). The ortho-substituted trifluoromethyl and chloro moieties are spatially clearly positioned and impart a specific rotation to the phenyl group of MRL-871 and address hydrophobic sites in the allosteric pocket (Fig. 1b,d). Hydrogen-bonding interactions exist between the molecule’s carboxylic acid group and the side chain of RORγt residue Q329 as well as the main-chain amide hydrogen atoms of residues A497 and F498. Apart from these distinct polar interactions, the newly generated allosteric pocket is predominantly hydrophobic, because of the amino-acid side chains of residues on helices 4, 5, 11 and, notably, H12 and the activation function loop between helices 11 and 12 (AF-2 domain). The arrangement of the RORγt LBD in the presence of MRL-871 generates a druggable molecular-binding site critically regulated by favourable interactions of MRL-871 with the AF-2 domain. These interactions reposition the highly flexible H12 in a conformation unique among reported NR ligand structures, of either the agonist or antagonist type, and distinct from the previously reported RORγt agonist-bound state. The unique conformation induced by the binding of MRL-871 prevents interaction with cofactor peptides, which typically bind RORγt through a conserved LXXL motif, at the AF-2.

Functional RORγt inhibition via allosteric inverse agonism.

To determine the functional effect of the binding of these novel modulators and the resulting RORγt conformational changes, two variants of MRL-871 (MRL-058 and MRL-003) were also prepared and tested alongside an agonist and canonical inverse agonist in an AlphaScreen cofactor peptide recruitment assay. Indazoles MRL-871 and its derivatives MRL-058 and MRL-003 all inhibited coactivator binding in a dose-dependent manner (Fig. 2a) with half-maximum inhibitory concentration (IC\(_{50}\)) values of 7 ± 1 nM (MRL-871), 98 ± 23 nM (MRL-058) and 280 ± 117 nM (MRL-003), showing the antagonistic profile of the series. Cholesterol, binding as agonist to the LBD, functioned as a weak activator, further enhancing the interaction of the RORγt-LBD with a cofactor peptide. The inverse agonist T0901319 demonstrated an IC\(_{50}\) value of 24 ± 13 nM in this assay, which is in good agreement with previously reported values, but...
less potent than MRL-871 (refs 26,27). The data thus show that the allosteric-binding mode of the novel modulators as observed in the crystal structures translates into potent inhibition of coactivator peptide binding to RORγt-LBD. Furthermore, these data also indicate that both ortho-substituents on the benzamide moiety are required to enhance RORγt-binding affinity, which suggest that the observed binding mode in the crystal structure is most optimal.

To corroborate our structural findings and gain more insight in the mode of action for these compounds, competitive binding assays for both MRL-871 (Fig. 2c) and T0901317 (Fig. 2d) were performed against fixed concentrations of cholesterol. On the basis of the structural data, we hypothesized that MRL-871 should not compete with cholesterol for the orthosteric ligand-binding pocket, because they bind into different pockets. As such, the IC_{50} value of MRL-871 for inhibition of the RORγt coactivator interaction should be independent of cholesterol concentration. The competitive cofactor-binding assay for MRL-871 showed no significant change in IC_{50} value when performed in the presence of different concentrations of cholesterol (Fig. 2c). In contrast, the inverse agonist T0901317 competes for the same binding site as cholesterol, resulting in a competitive displacement and a cholesterol concentration-dependent increase of the IC_{50} value for T0901317 (Fig. 2d); that is, RORγt binding and inhibition by T0901317 becomes less potent in the presence of agonistic ligands. Together, these data demonstrate that the allosteric inhibition of coactivator binding to RORγt by MRL-871 is both potent and independent of orthosteric site occupancy.

RORγt mediates IL17a gene expression in EL4 cells\(^4\). The EL4 murine lymphoblast cell line constitutively expresses RORγt, which drives production of IL17a (ref. 28). To confirm that allosteric RORγt modulation has also functional relevance at the cellular level, EL4 cells were treated with 10 μM of modulators MRL-871, MRL-058 and MRL-003 for 24 h and IL17a messenger RNA (mRNA) levels were measured by quantitative reverse transcriptase PCR (RT–PCR) (Fig. 2b). Treatment of EL4 cells with the more potent MRL-871 and MRL-058 significantly reduced the IL17a mRNA levels, while the weaker MRL-003 did not reduce the mRNA levels, consistent with the lower
biochemical activity observed for this compound. This result thus demonstrates that functional modulation of the allosteric pocket by small molecules results in cellular responses (that is, reduced gene transcription).

### Structural basis for ligand potency

The cofactor recruitment assay described above provides an indirect assessment of modulator potency since the site of cofactor interaction does not directly overlap with the binding site of the indazoles. Therefore, an orthogonal assay that directly and selectively probes the novel allosteric ROR$_{gt}$-binding site is desirable for screening, characterization and optimization purposes. We used the co-crystal structure of ROR$_{gt}$ and MRL-871 (Fig. 1c) to rationally design a synthetic ligand analogue containing a time-resolved fluorescence resonance energy transfer (TR-FRET) acceptor. AlexaFluor 647 was connected to the six position of the indazole ring and one at the phenyl substituent. MRL-673 contains additional polar functionality—the phenyl substituent contains a hydroxyl substituent ortho to the carboxylic acid, and the C-4 of the indazole is replaced by nitrogen. In the case of MRL-673, the benzoic acid functionality is saturated, and the carbon atom adjacent to the carboxylate is alkylated, resulting in a tetrasubstituted carbon centre. This modification induces a displacement of the carboxyl group and highlights the importance of the polar interactions in the binding site.

The additional fluorine groups (MRL-299) and the polar functionalities (MRL-367) were both important for ROR$_{gt}$-binding potency and functional inhibition (Table 2). In particular, MRL-367 exhibited enhanced potency in the cofactor displacement and direct binding assay, as well as increased inhibitory activity in a luciferase reporter assay and in functional inhibition of IL-17 production. To insure that the functional activity of the indazole series is attributable to inhibition of ROR$_{gt}$, MRL-299 was tested against a commercially available panel of cell-based NR reporter assays (Supplementary Table 1). Across this panel of NR assays, MRL-299 was >100-fold selective for ROR$_{gt}$. The only significant off-target activity was against PPAR$_{gamma}$ (PPAR$_{gamma}$ activity was also recently reported for a structurally similar series of molecules$^{29}$), which was de-risked (Supplementary Note 1).

Structural elucidation of these compounds reveals that all antagonists occupy the same allosteric-binding pocket as MRL-871 (Fig. 3). Detailed comparison of, for example, MRL-871 and MRL-367 (Fig. 3b) reveals that both polar molecular additions to the scaffold are fully tolerated and lead only to very minor changes in the surrounding amino-acid orientations. MRL-673, in contrast, exhibits 10-fold weaker

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**Table 1 | Data collection and refinement statistics (molecular replacement).**

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<th>MRL-871</th>
<th>MRL-367</th>
<th>MRL-299</th>
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R.m.s., root mean squared.

*Values in parentheses are for highest-resolution shell.
potency in both the biochemical and cellular assays relative to MRL-367. Structural comparison of, for example, MRL-367 and MRL-673 (Fig. 3c) reveals that, while these compounds bind the same allosteric site, the position of the carboxylic acid ring of MRL-673 distorts the binding pocket. The modification induces a displacement of the carboxyl group and highlights the importance of the polar interactions in the binding site. Although the carboxylate of MRL-673 makes the same number of polar contacts with RORγt as MRL-367, the methylation induces the reorientation of the side chains of Phe498 and Tyr502. This shift in the side chains and the accompanying shifts in the backbone of the C-terminal residues of Ala497 and Phe498, explains the lower receptor affinity and functional inhibitory potency.

**Discussion**

NR drug development has been based primarily on the ability of NRs to bind ligands at the highly conserved hydrophobic orthosteric pocket of the LBD, which is also the binding site of endogenous ligands. Compared with unbound NRs, the binding of an agonist induces a conformational change that stabilizes the positioning of H12 and AF-2 domain, resulting in coactivator recruitment. The binding of antagonist or inverse agonist ligands typically destabilizes H12 and the AF-2 domain (possibly unfolding it) resulting in a lack of coactivator or enhanced corepressor recruitment, ultimately resulting in transcriptional inhibition at specific loci. Numerous successful drugs targeting NRs have been developed, all working via this mechanism, but increasing resistance against certain cancer therapy-oriented NR antagonists, as well as the challenge of targeting orphan receptors has increased the need for alternative site modulators of NRs. Similar to, for example, the efforts to target allosteric sites in protein kinases, allosteric NR modulators may differentiate favourably versus orthosteric ligands. Potential advantages of allosteric NR inhibitors are enhanced selectivity, no competition with increasing endogenous ligands during pathological conditions, and no sensitivity to agonist/antagonist switching due to mutations.

The recently reported crystal structure of RORγt with a potent tertiary amine orthosteric agonist revealed the coactivator-binding site on the LBD, in a classical NR H12 switch agonist mode and bound to a cofactor peptide motif. The new crystal structures of RORγt reported here with MRL-871 and its analogues (Fig. 3) reveal that these indazole modulators bind to a receptor position normally occupied by H12 in the non-ligated or agonist ligand-bound conformation. The final orientation of H12 in the presence of the novel indazole modulators is such that the classical binding surface for the cofactor LXXLL motif is not only modified, but actively blocked. This overall orientation effectively antagonizes cofactor binding to RORγt. The consequence of this allosteric modulator-mediated refolding of RORγt is functional inhibition as evidenced by biochemical and cellular studies. The unique mode of allosteric binding reported thus provides a structural rationale for targeting NRs with small molecules in an orthogonal manner that does not require competition with canonical ligands.
The X-ray structures demonstrate that these indazoles all occupy the novel allosteric binding pocket. Reported crystal structures with orthosteric inverse agonists, binding to the canonical site of RORγt, lack clear structural data for the position of the H12 and the resulting cofactor-binding site (for example, Fig. 1c with T0901317 and Fig. 4b). Synthetic RORγt inverse agonists T0901317 (ref. 27) and tertiary sulfonamides26 bind at the canonical orthosteric NR ligand-binding pocket. Their binding distorts the structure of the LBD, leading to helices 11’ and 12 being unstructured in the crystallized protein (Fig. 4b). A superposition of the RORγt structures with an inverse agonist in the orthosteric site and an indazole modulator in the allosteric site reveals the additional benefit of the novel site (Fig. 1c; Supplementary Fig. 4). Whereas both compounds induce the partial unfolding of helix 11’ (ref. 32), MRL-871 subsequently also stabilizes the folding of H12 via direct interactions. The unfolded helix 11’ spans the distance to the displaced H12 N terminus (Fig. 4d).

Table 2 | Potency (mean ± s.d.) of selected RORγt allosteric inverse agonists in biochemical and cellular assays.

<table>
<thead>
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<th>Compound ID</th>
<th>SRC1 Cofactor FRET IC₅₀ (nM)</th>
<th>MRL-871 AlexaFluor Probe Competition IC₅₀ (nM)</th>
<th>GAL4 Luciferase Reporter IC₅₀ (nM)</th>
<th>hPBMC IL17A IC₅₀ (nM)</th>
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<tr>
<td>MRL-871</td>
<td>6.1 ± 2.0</td>
<td>5.8 ± 1.6</td>
<td>73.6 ± 15.4</td>
<td>39.6 ± 18.0*</td>
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<td>MRL-299</td>
<td>3.8 ± 0.9</td>
<td>5.4 ± 3.5</td>
<td>33.4 ± 9.4</td>
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<td>MRL-367</td>
<td>1.9 ± 0.7</td>
<td>4.1 ± 1.8</td>
<td>42.4 ± 9.7</td>
<td>13.0 ± 7.6</td>
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<td>MRL-673</td>
<td>21.8 ± 6.7</td>
<td>26.4 ± 13.0</td>
<td>279 ± 137</td>
<td>142 ± 64</td>
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FRET, fluorescence resonance energy transfer; IC₅₀, half-maximal inhibitory concentration; SRC1, steroid receptor coactivator 1.

*For a representative dose-response curve see Supplementary Fig. 2.
This results in RORγt taking on a stably folded antagonistic state and directly blocking cofactor peptide binding (Fig. 4e). These antagonists thus induce a conformational change in the RORγt LBD, which blocks cofactor binding through the stabilization of the H12 subdomain in an unprecedented folded state (Fig. 5).

In summary, we demonstrate the structural and functional elucidation of an unprecedented NR allosteric inhibitory mechanism, based on highly potent small-molecule modulators for RORγt. This is the first allosteric binding pocket with highly potent small drug-like molecules, functionally active in Th17 differentiation/IL17a production inhibition. This allosteric inhibitory mechanism offers great advantages for NR drug development, including selectivity, independence of endogenous ligands and less responsive to point mutations within the orthosteric ligand binding site. RORγt regulates a variety of physiological processes and has emerged as a highly promising drug target for autoimmune diseases. The discovery of this novel allosteric NR pocket and concomitant selective molecules that target this site offers a new strategic opportunity to develop agonist-independent therapeutics for Th17-mediated autoimmune disorders.
Apo RORγt | RORγt-LBD SRC-1 cofactor FRET assay. RORγt-LBDs of from human (GenBank accession number NP_003051.2 aa259–518) and mouse (GenBank accession number NP_053511.1 aa356–516) were amplified by PCR and cloned into pET-28a(+) and were expressed in BL21(DE3) E. coli grown in LB media. Expression was induced with 0.1 mM IPTG and allowed to continue for 16 h at 16 °C. Cells were lysed in 50 mM Tris, 300 mM NaCl, 10 mM imidazole, 5% glycerol, 10 mM beta-mercaptoethanol, 1% Triton X-100 and 0.2 mM PMSF, pH 8.5 using a microfluidizer. After removal of cellular debris by centrifugation, lysates were loaded onto Ni-NTA resin and washed with 50 mM Tris, 300 mM NaCl, 20 mM imidazole, 5% glycerol and 10 mM beta-mercaptoethanol at pH 8.5. The column was washed with the same buffer containing 60 mM imidazole, and protein was eluted with the above buffer containing 250 mM imidazole. The proteins were dialysed into 50 mM Tris, 100 mM NaCl, 5% glycerol and 1 mM DTT, pH 8.5 for storage at −80 °C. The potency of small-molecule RORγt ligands was then assessed by monitoring their effect on the association of a LXXLL-motif-containing SRC-1 peptide. Compound (10 mM dimethyl sulfoxide (DMSO)) signal was serially diluted in threefold steps using an Agilent 8150 liquid handler. Diluted compound or DMSO (25 nL) was transferred into a black Greiner 384-well plate (Cat#781076) using a LabCyte Echo acoustic dispenser. To each well of the plate was added 15 μL of 3.75 nM RORγt-LBD in receptor buffer (50 mM Tris-HCl pH 7.0, 30 mM potassium chloride, 1 mM EDTA, 0.1% delipidated bovine serum albumin, 1 mM DTT, 1.25 nM Anti-His W1024 Europium chelate antibody (PerkinElmer) and 3% (v/v) of lyvate from ~24,000 S9 cells. Compounds were allowed to incubate with receptor for 15 min and then 5 μL of peptide in detection buffer or detection buffer alone were added. Detection buffer (5 x) consists of 50 mM Tris-HCl pH 7.0, 30 mM potassium chloride, 1 mM EDTA, 0.1% delipidated bovine serum albumin, 1 mM DTT and 20 mM streptavidin-alumion (PerkinElmer). When peptide (Biotin-SPS-SSHSLETIKHLRRLQEGSP) was included, its concentration in the 5 x stock was 250 nM. The plate was then incubated overnight at 4 °C. The following morning the plate was warmed to room temperature and read using an Envision plate reader (PerkinElmer). TR-FRET signal was serially diluted as the ratio of the florescent signal in the presence of RORγt was corrected for background and the 1/KD determined using the One-site specific binding equation in Graph Pad Prism. To assess the potency of unlabelled compounds, a similar assay format was utilized except that 100 nM probe was preincubated with the RORγt-LBD for 15 min before addition to serially diluted compounds. IC50 values were determined by fitting per cent activity data to a four-parameter logistic dose–response equation in GraphPad Prism.

RORγt-LBD Alexa Fluor-647-labelled MRL-871 competitive binding assay. As an alternate assessment of inhibitor potency MRL-871 was covalently labelled with Alexa Fluor-647. To determine the intrinsic affinity of the probe, the compound (5 mM DMSO stock) was serially diluted in threefold steps using an Agilent Bravo liquid handler. Diluted probe or DMSO (25 nL) was transferred into a black Greiner 384-well plate (Cat#781076) using a LabCyte Echo acoustic dispenser. To each well of the plate was added 15 μL of RORγt-LBD at 1.6, 3.33 and 6.65 nM in receptor buffer or receptor buffer alone. The plate was then incubated for 15 min at room temperature. Subsequently, 5 μL of receptor buffer with 1.2 nM Anti-His W1024 Europium chelate antibody (PerkinElmer) was added and the TR-FRET signal measured using the Anti-His Alexa Fluor-647-labeled RORγt-LBD assay described above. TR-FRET intensity in the presence of RORγt was corrected for background and the KD determined using the One-site specific binding equation in Graph Pad Prism. To assess the potency of unlabelled compounds, a similar assay format was utilized except that 100 nM probe was preincubated with the RORγt-LBD for 15 min before addition to serially diluted compounds. IC50 values were determined by fitting per cent activity data to a four-parameter logistic dose–response equation in GraphPad Prism.
Chimeric RORγt–GAL4 reporter assay. The coding sequence of RORγt a97–518 was cloned in frame with the DNA-binding domain of the yeast GAL4 protein within the CMV-promoter-driven pCDNA3.1 vector. This vector, along with the GAL4 UAS-luciferase reporter vector pGL4.31 (Promega), was used to transfect HEK293T cells. Briefly, 1 × 106 cells in 10 ml of DMEM high-glucose media with 10% fetal bovine serum (FBS) were transfected with a mixture consisting of 10 μg of each plasmid and 60 μl of Transit-T293 (Mirus Bio) in 1.5 ml of OptiMEM (Invitrogen). Following transfection, cells were transferred to one T75 flask and incubated overnight at 37°C and 5% CO2. Compound dilutions are prepared as above and 50 nl was transfected to a 384-well Greiner white tissue-culture-treated plate (catalog #781080) using an Echo acoustic dispenser (LabCyte). Cells were collected and resuspended at 0.8 × 106 cells per ml in DMEM high-glucose media with 10% FBS. To each well of the plate was added 25 μl of cell suspension and the cells incubated overnight at 37°C and 5% CO2. After 20–22 h, the plates were brought to room temperature and 25 μl of Steady-Glo luciferase reagent (Promega) was added to each well. The luminescent signal was measured on an Envision plate reader.

Quantitative IL17a RT–PCR. EL4 cells (Sigma-Aldrich) were grown in DMEM ( Gibco). At 24h after the cells were seeded onto a 12-well plate, the cells were transfected with BtTG by using Lipofectamine2000 (Bio-Rad). Primer sequences used for quantitative RT–PCR were: 5′-ctctcttgctcagtgtccttgct-3′, Rev: 5′-cttcgttcagttggaggaaagctc-3′ and Gapdh: 5′-gggccacctctgctcaca-3′, Rev: 5′-ctcttgctgcttcctcg-3′. The level of IL17a mRNA expression was normalized to that of Gapdh expression. All data are expressed as the mean ± s.e.m. (n=6). Statistical analysis was performed using an one-way analysis of variance comparing against the DMSO control following Dunnett post hoc test.

PBMCD17 polarization and IL17 production assay. Test compounds were prepared as 10 mM stocks in DMSO and serially diluted 1:3 to provide an eight-concentration titration. The compounds (200 nl of each dilution) were acoustically dispensed into a 96-well Costar 3912 assay plate. Frozen human PBMCs from an anonymous healthy donor were obtained commercially and were diluted to a density of 5 × 105 cells per ml with growth media (RPMI 1640/ 10% FBS/pen/strep/ L-glutamine) and the stimulated cytokines were added to final concentrations of 25 ng/ml IL-1β, 10 ng/ml IL-23, 0.5 ng/ml IL-12 and 10 ng/ml IL-6 (all cytokines from R&D Systems). In addition, T-Acidator CD3/28 Dynabeads (Invitrogen) were added to a concentration of 100,000 beads per ml. The stimulated cells were immediately dispensed into the assay plate containing serially diluted compound at a volume of 200 μl per well. Cell plates were then incubated at 37°C and 5% CO2 for 4 days. Culture media (100 μl) was collected from each well and IL-17 expression was measured by enzyme-linked immunosorbent assay (R&D Systems) according to the manufacturer’s instructions. Cell viability was assessed by the addition of 100 μl of CellTiter-Glo (Promega) to each well of the cell assay plate followed by luminescence detection on an Envision plate reader (PerkinElmer).

References


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Author contributions

Additional information
Accession codes: Coordinates and structure factors for the RORγt bound to MRL-871, MRL-299 MRL-367 and MRL-673 have been deposited in the Protein Data Bank under accession codes 4YPQ, 5C40, 5CHU, 5CS5 and 5CAT.

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications


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