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Discovery of [1,2,4]Triazolo[1,5-*a*]pyridine Derivatives as Potent and Orally Bioavailable RORγt Inverse Agonists

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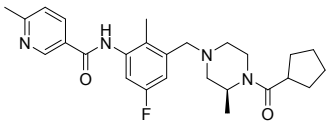
ABSTRACT: The retinoic acid receptor-related orphan nuclear receptor gamma t (RORγt), a promising therapeutic target, is a major transcription factor of genes related to psoriasis pathogenesis such as interleukin (IL)-17A, IL-22, and IL-23R. Based on the X-ray cocrystal structure of RORγt with **1a**, an analog of a known piperazine RORγt inverse agonist (**1**), triazolopyridine derivatives of **1** were designed and synthesized, indicating analog **3a** as a potent RORγt inverse agonist. The structure-activity relationships (SAR) studies in **3a**, focusing on the treatment of its metabolically unstable cyclopentyl ring and the central piperazine core, led to a novel analog, namely 6-methyl-*N*-(7-methyl-8-(((2*S*,4*S*)-2-methyl-1-(4,4,4-trifluoro-3-(trifluoromethyl)butanoyl)piperidin-4-yl)oxy)-[1,2,4]triazolo[1,5-*a*]pyridin-6-yl)nicotinamide (**5a**), which exhibited strong RORγt inhibitory activity and a favorable pharmacokinetic profile. Moreover, the *in vitro* and *in vivo* evaluation of **5a** in a human whole-blood assay and a mouse IL-18/23-induced cytokine expression model revealed its robust and dose-dependent inhibitory effect on the IL-17A production.

The retinoic acid receptor-related orphan nuclear receptor gamma t (RORγt) is a major transcription factor of genes related to psoriasis pathogenesis such as interleukin (IL)-17A, IL-22, and IL-23R.[1-2] Therapies blocking IL-17A or IL-23R have successfully improved skin lesions in patients with moderate-to-severe psoriasis,[3-7] thus rendering the RORγt inhibition a promising therapeutic target. After T0901317 has been reported for its effective, albeit unselective binding to RORγ,[8] many RORγt antagonists (inverse agonists) have been developed,[9-23] while several are already being clinically investigated as promising targets for the treatment of autoimmune diseases[24]. Generally, nuclear receptors are proteins with highly conserved ligand binding domains (LBDs), which are structurally composed of alpha helices that form a large lipophilic pocket responsible for binding small lipophilic ligands such as retinoid derivatives, fatty acids, cholesterol, and other lipophilic hormones and vitamins.[25] Thus, one of the main challenges for drug delivery in this target class is the lipophilicity balance, which is required for strong LBD binding potency, while the metabolism associated with lipophilic small-molecule ligands should be minimized to afford favorable drug-like properties.



1

Molecular Weight = 453

$cLogD_{7.4}$ = 3.78

Figure 1. A representative RORγt ligand, used as reference in the current study.

Based on a previous report describing a series of piperazine RORγt ligands,[26] compound **1** was selected as a starting point for further investigation, mainly due to its moderately low molecular weight (453) and lipophilicity

(cLogD$_{7.4}$ = 3.78). The optimization of **1** resulted in a new triazolopyridine derivative (**3a**) (Table 1) that exhibited inverse RORγt agonistic activity. Further optimization of **3a** by modifying its metabolically unstable cyclopentyl ring and the central piperazine ring led to a new derivative, 6-methyl-*N*-(7-methyl-8-(((2*S*,4*S*)-2-methyl-1-(4,4,4-trifluoro-3-(trifluoromethyl)butanoyl)piperidin-4-yl)oxy)-[1,2,4]triazolo[1,5-*a*]pyridin-6-yl)nicotinamide (**5a**)(Table 3), which was indicated as the best candidate for further studies. Specifically, compound **5a** exhibited potent RORγt inhibitory activity, good pharmacokinetic (PK) profile in a mouse cassette dosing study, and dose-dependent inhibition of the IL-17A production in a mouse IL-18/23-induced cytokine expression model. Herein, the results of these studies are described.

An X-ray cocrystal structure of human RORγt (PDB ID 6O3Z) with **1a** (Figure 2C), an analog of the piperazine RORγt ligand **1**, was successfully obtained[27-28]. As illustrated in Figure 2A, the cyano group of **1a** formed hydrogen bonds with Arg367 and Leu287, while the amide NH group with Phe378. Furthermore, a hydrogen bond was developed between the fluorobenzene ring and the hydroxyl group of Ser404, where a small space around the positions 4 and 5 of the ring was detected (Figure 2B). Considering this additional space in the pocket, the phenyl ring of compound **1** could be replaced by a nitrogen-containing bicyclic ring to simultaneously decrease the overall lipophilicity and facilitate the hydrogen bond interactions with Ser404, allowing thus a more detailed investigation of this residue. The previous report by Hintermann and co-workers described a low lipophilic triazolopyridine derivative having *N*-([1,2,4]triazolo[4,3-*a*]pyridin-6-yl)amide moiety, which displayed moderate inhibitory activity in the reporter gene assay and good liver microsomal stability[29]. This triazolopyridine derivative demonstrated a nitrogen-containing bicyclic ring was tolerable for the RORγt inhibitory activity, which lead us into detailed investigation with other chemo types of triazolopyridine analogs.

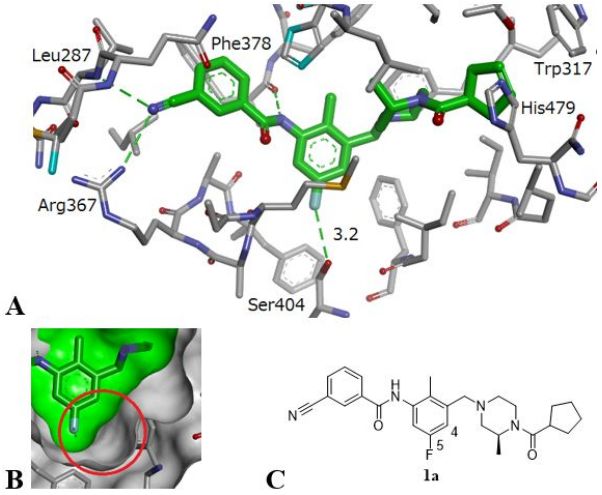
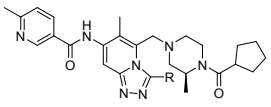


Figure 2. X-ray cocrystal structure of RORγt (PDB ID 6O3Z) with compound **1a**. **A:** RORγt-LBD and compound **1a** are depicted in gray and green, respectively. Green dashes represent the hydrogen bond interactions. **B:** The red circle indicates the small space around the positions 4 and 5 of the fluorobenzene ring of **1a**. The surface of the RORγt-LBD site (gray) and the surface of **1a** (green) were calculated from the X-ray structure. **C:** Molecular structure of **1a**.

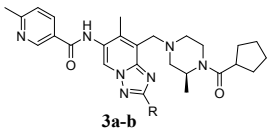
To that end, two series of analogs bearing *N*-([1,2,4]triazolo[4,3-*a*]pyridin-7-yl)amide and *N*-([1,2,4]triazolo[1,5-*a*]pyridin-6-yl)amide moieties were synthesized. Since it has been reported that RORγt is constitutively active in the absence of an endogenous ligand,[30] all compounds were evaluated in a luciferase

reporter gene assay without a control agonist ligand to assess their RORγt inverse agonistic activity.

Table 1. Structure-activity relationships (SAR) of the triazolopyridine series.



2a-b



3a-b

Compd	R	cLogD$_{7.4}$[a]	Luc IC$_{50}$ (nM)[b]	Human LM CL$_{int}$ (mL/min/mg)[c]
1	-	3.78	14	0.088
2a	H	2.57	590	0.059
2b	Me	2.53	8000	
3a	H	2.37	41	0.032
3b	Me	2.32	3200	

[a]Calculated by Pipeline Pilot 17.2. [b]Luciferase reporter gene assay used for the evaluation of the RORγt transcriptional activity inhibition. The employed HEK293T cells were transfected with GAL4-NR-luciferase plasmids and the activity was evaluated using the Dual-GLOTM Luciferase Assay System. [c]Metabolic stability in liver microsome (LM).

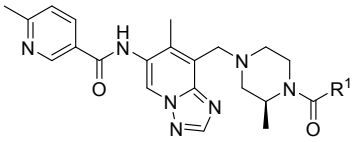
Moreover, all the synthesized triazolopyridine analogs were designed to achieve a lower lipophilicity than compound **1** (cLogD$_{7.4}$ = 3.78). As outlined in Table 1, the cLogD$_{7.4}$ values of the novel analogs were successfully decreased by approximately one unit compared to **1**. However, the [1,2,4]triazolo[4,3-*a*]pyridine derivative **2a** displayed reduced inhibitory activity in the reporter gene assay (IC$_{50}$ = 590 nM), whereas the [1,2,4]triazolo[1,5-*a*]pyridine derivative **3a** retained an excellent inhibitory activity (IC$_{50}$ = 41 nM), comparable to that of compound **1**, indicating that the nitrogen atoms in the [1,2,4]triazolo[1,5-*a*]pyridine ring were well tolerated for the inhibition of the RORγt transcriptional activity. Furthermore, although **3a** improved the human LM stability (human CL$_{int}$ = 0.032 mL/min/mg), the methyl-substituted triazolopyridine derivatives **2b** and **3b** exhibited a lower *in vitro* activity, probably due to steric repulsions in the binding pocket. Moreover, the X-ray analysis results implied that only unsubstituted triazolopyridine rings are acceptable, because the available space at the 4 and 5 positions of the fluorobenzene ring of **1a** is small. Therefore, compound **3a**, which exhibited the most improved LM stability and high potency, was further optimized.

To elucidate the PK profile of **3a**, this compound was incubated in human hepatocytes and its metabolites were explored by mass spectrometry (MS). During the MS analysis, no glutathione adducts were detected, suggesting a low tendency toward the formation of reactive metabolites, while no metabolic soft spots on the central triazolopyridine core could be observed, supporting thus the suitability of the triazolopyridine moiety as a lead scaffold. In contrast, oxidative adducts were detected on the cyclopentyl ring, which prompted us to further optimize analog **3a**.

A series of **3a** analogs were designed by exploring cyclopentyl ring alternatives and acyclic chains bearing fluorine atoms that could potentially block the metabolism. The SAR results of the cyclopentyl ring modifications in analog **3a** are summarized in Table 2. Although the substitution of the cyclopentyl ring by two fluorine atoms (**4a**) improved the cLogD$_{7.4}$ and the LM stability, the reporter gene inhibitory activity of RORγt was slightly decreased (IC$_{50}$ = 130 nM). Similarly, the replacement of

the cyclopentyl ring by a pyran ring (**4b**) significantly decreased the inhibitory activity. Moreover, the phenyl derivative **4c** had similar LM stability (human CL_{int} = 0.021 mL/min/mg) compared to **3a**, but its inhibitory activity was slightly lower (IC_{50} = 79 nM). The cyclopentyl ring of **3a** was then replaced by branched or unbranched alkyl chains, resulting in derivatives **4d**-**4g**. The inhibitory activity of the isopropyl analog (**4d**) was slightly decreased (IC_{50} = 230 nM), whereas the isobutyl derivative **4e** had comparable inhibitory activity (IC_{50} = 59 nM) and LM stability to **3a**. Given that the trifluoromethyl group is often used instead of a methyl group to decrease the oxidative metabolism or to increase the steric size, the two methyl groups of **4e** were replaced by trifluoromethyl groups (**4f**), resulting in similar inhibitory activity (IC_{50} = 50 nM) and improved LM stability. Moreover, based on the results of the mono-trifluoromethyl derivative **4g** with an IC_{50} value of 240 nM, it was proven that the double substitution was more favorable for the inhibitory activity. Hence, compound **4f** was selected for further investigation

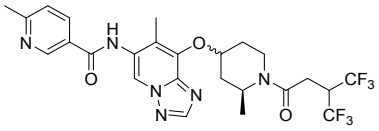
Table 2. SAR of the 3a derivatives.

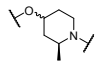
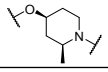
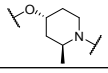


Compound	R^1	$cLogD_{7.4}$[a]	Luc IC_{50} (nM)[b]	Human LM CL_{int} (mL/min/mg)[c]
1	-	3.78	14	0.088
3a		2.37	41	0.032
4a		1.61	130	<0.010
4b		1.05	10000	<0.010
4c		2.27	79	0.021
4d		1.82	230	0.24
4e		2.07	59	0.020
4f		2.61	50	<0.010
4g		2.07	240	<0.010

[a]Calculated by Pipeline Pilot 17.2. [b]Luciferase reporter gene assay to assess the RORγt transcriptional activity inhibition. The employed HEK293T cells were transfected with GAL4-NR-luciferase plasmids and the activity was evaluated using the Dual-GLOTM Luciferase Assay System. [c]Metabolic stability in liver microsome.

Table 3. RORγt inhibitory activity and lipophilicity of analogs 5a and 5b.



Compound		$cLogD_{7.4}$[a]	Luc IC_{50} (nM)[b]	Human LM CL_{int} (mL/min/mg)[c]
1	-	3.78	14	0.088
4f	-	2.61	50	<0.010
5a		2.67	51	0.010
5b		2.67	1500	0.028

[a]Calculated by Pipeline Pilot 17.2. [b]Luciferase reporter gene assay to assess the RORγt transcriptional activity inhibition. The employed HEK293T cells were transfected with GAL4-NR-luciferase plasmids and the activity was evaluated using the Dual-GLOTM Luciferase Assay System. [c]Metabolic stability in liver microsome.

To improve the activity of **4f**, the 2-methylpyridine moiety was replaced by substituted pyridines or other heteroaryl rings (data not shown). However, this structural modification did not improve either the inhibitory activity or the lipophilicity of the obtained analogs. Therefore, the piperazine moiety of **4f** was replaced by piperidine to retain low lipophilicity values, while introducing an ether linkage between the piperidine ring and the triazolopyridine ring. *Cis*- (**5a**) and *trans*-piperidine (**5b**) analogs were thus obtained with $cLogD_{7.4}$ values of 2.67 (Table 3). Interestingly, **5a** maintained good RORγt inhibitory activity (IC_{50} = 51 nM) and LM stability (human CL_{int} = 0.010 mL/min/mg) compared to **4f**, whereas the inhibitory activity of **5b** was insignificant. Furthermore, the *in vitro* absorption, distribution, metabolism, and excretion (ADME) profiles of compounds **1**, **3a**, **4f**, and **5a** were assessed (Table 4). Compounds **3a**, **4f**, and **5a** displayed better microsomal stability than compound **1** (human CL_{int} = 0.032, <0.010, and 0.010 mL/min/mg, respectively) along with decreased plasma protein binding (PPB) rates (77.3%, 86.1%, and 91.8%, respectively). Moreover, compounds **4f** and **5a** were sufficiently soluble in an aqueous medium. Although **3a** and **4f** exhibited low Caco-2 permeability in the apical-to-basolateral (A to B) direction, **5a** displayed efficient permeability in the same evaluation system.

Table 4. *In vitro* ADME profiles of compounds 1, 3a, 4f, and 5a.

Compound	LM CL_{int} (mL/min/mg)[a]		PPB (%)[b]		Solubility (μM)[c]	Caco-2 P_{app} ($\times 10^{-6}$cm/s)[d]	
	human	mouse	human	mouse		A to B	B to A
1	0.088	0.11	99.7	95.7[e]	71	40	55
3a	0.032	0.011	77.3	78.8	190	2.5	46
4f	<0.010	< 0.010	86.1	76.7	200	9.6	36
5a	0.010	0.030	91.8	86.5	170	31	51

[a]Metabolic stability in liver microsome. [b]Plasma protein binding using human or mouse plasma. [c]The second fluid of the Disintegration Test of the Japanese Pharmacopoeia (pH 6.8) was used. [d]Permeability in the apical-to-basolateral (A to B) direction and *vice versa* (B to A). [e]Compound **1** might be unstable in the mouse PPB assay.

Table 5. PK profile of compounds 1, 3a, 4f, and 5a in a mouse cassette dosing study.[a]

Compound	BA (%)	AUC p.o. (nM*h)	CL/CL_u (L/h/kg)	VD_{ss} (L/kg)	$t_{1/2}$ p.o. (h)	$t_{1/2}$ i.v. (h)
1	48	490	2.0/46	1.6	0.7	0.7
3a	47	840	1.1/5.3	0.77	1.2	1.4
4f	110	2300	1.1/4.5	1.1	1.1	1.0
5a	120	15000	0.15/1.1	0.80	3.6	3.9

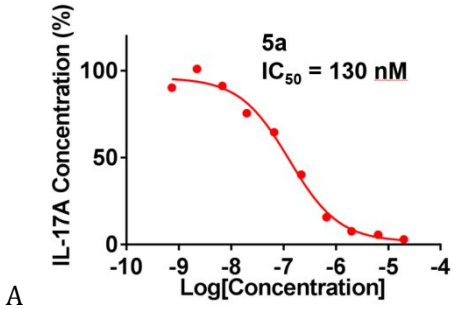
[a]2 μmol/kg p.o. dose and 1 μmol/kg i.v. dose.

The *in vivo* PK profile of compounds **1**, **3a**, **4f**, and **5a** was investigated in mice using cassette dosing (Table 5). Compared to compound **1**, compounds **4f** and **5a** showed improved bioavailability (BA > 100%), notably lower unbound clearance (CL_u = 1.1 L/h/kg for **5a**), and higher area under the curve (AUC) (around 31-fold higher than **1** in the case of **5a**) and $t_{1/2}$ (>3 h for **5a**). Owing to the favorable *in vitro* activity and ADME profile, analog **5a** was further investigated.

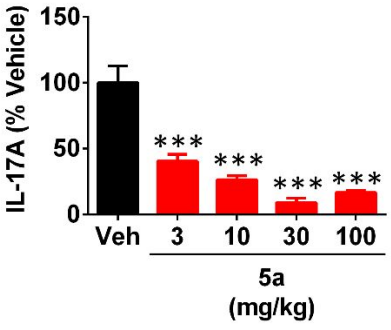
A human whole-blood assay of compound **5a** was conducted to assess its inhibitory activity against the T-cell receptor-dependent production of IL-17A in human whole blood. It was proven that compound **5a** suppressed the IL-17A production in a dose-dependent manner with an IC_{50} value of 130 nM (Figure 3A). The *in vivo* potency of **5a** toward the modulation of the cytokine production through the suppression of the differentiation and activation of Th17 and Th1/17 cells was assessed using an *in vivo* mouse IL-18/23-induced cytokine expression model. Compound **5a** was orally administered once 3 h prior to the IL-18/23 injection, and the IL-17A level in blood was measured 4 h after the injection of IL-18/23. As illustrated in Figure 3B, **5a** achieved a dose-dependent inhibition of the IL-17A production in 3, 10, 30, and 100 mg/kg doses.

In summary, a new triazolopyridine derivative (**3a**) was identified as a potent RORγt inhibitor. Optimization of the

metabolic soft spots on the cyclopentyl ring of **3a**, followed by optimization of the piperazine ring, led to a novel analog, namely 6-methyl-*N*-(7-methyl-8-(((2*S*,4*S*)-2-methyl-1-(4,4,4-trifluoro-3-(trifluoromethyl)butanoyl)piperidin-4-yl)oxy)-[1,2,4]triazolo[1,5-*a*]pyridin-6-yl)nicotinamide (**5a**). **5a** exhibited potent RORγt inhibition in a luciferase reporter gene assay as well as in a human whole-blood assay measuring IL-17A release. Moreover, a robust PK profile allowed the *in vivo* evaluation in a IL-18/23-induced cytokine expression mouse model, where **5a** significantly inhibited the IL-17A production in a dose-dependent manner.



A



B

mean ± SEM (n=6), ***P<0.001, vs Vehicle (Dunnett test)

Figure 3. A: Human whole-blood assay (n = 2). Human whole blood was diluted in half with RPMI 1640™ and was stimulated using anti-CD3 and anti-CD28 monoclonal antibodies, IL-18, and IL-23, followed by treatment with **5a**. The level of IL-17A in the culture medium was measured after two days. B: *In vivo* mouse IL-18/23-induced cytokine expression model. Mice were administered orally once with a vehicle (0.5% MC400) or **5a**. After 3 h, the mice were administered intraperitoneally with 2 μg of mIL-18 (Medical & Biological Laboratories) and 1 μg mIL-23 (R&D Systems). A blood sample was collected 4 h later and the IL-17A concentration in the plasma was measured.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Synthetic experimental, compound characterization, crystallography studies and assay descriptions (PDF).

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

BA, bioavailability; RORγt, retinoic acid receptor-related orphan receptor γt.

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Discovery of [1,2,4]Triazolo[1,5-*a*]pyridine Derivatives as Potent and Orally Bioavailable RORγt Inverse Agonists

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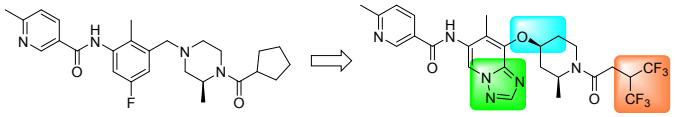

Compound 1

Reporter Gene IC_{50} = 14 nM
$cLogD_{7.4}$ = 3.78
mouse CDPK CL_u (L/hr/kg) = 46

5a

Reporter Gene IC_{50} = 51 nM
$cLogD_{7.4}$ = 2.67
mouse CDPK CL_u (L/hr/kg) = 1.1