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Identification of potent RORβ modulators: Scaffold variation

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ABSTRACT

We sought to develop RORβ-selective probe molecules in order to investigate the function of the receptor *in vitro* and *in vivo* and its role in the pathophysiology of disease. To accomplish this, we modified a potent dual RORβ/RORγ inverse agonist from the primary literature with the goal of improving selectivity for RORβ vs RORγ. Truncation of the Western portion of the molecule ablated activity at RORγ and led to a potent series of RORβ modulators. Continued exploration of this series investigated alternate replacement cores for the aminothiazole ring. Numerous suitable replacements were found during the course of our SAR investigations and are reported herein.

The retinoic acid receptor-related orphan receptors (RORα, RORβ and RORγ) are members of the NR1F subfamily of NRs and they play an important regulatory role in the maintenance of a variety of physiological and pathological processes. The RORs have been implicated in the pathology of diseases associated with dysregulation of the circadian rhythm including sleep disorders, bipolar disorder and schizophrenia. Like all NRs, the RORs display the conserved domain structure with a variable amino-terminal A/B domain, a central highly conserved two zinc finger DNA-binding domain (C domain), a hinge (domain D) and a carboxy-terminal ligand-binding domain (LBD; E domain). The three RORs display significant sequence similarities but display distinct patterns of expression. RORα is expressed in the liver, skeletal muscle, skin, lungs, adipose tissue, kidney, thymus and brain.[1-2] RORγ is most highly expressed in the thymus, but significant expression is also found in the liver, skeletal muscle, adipose tissue and kidney.[3] The RORγt isoform, which has garnered much attention lately due to its role in T_H17 cells, is exclusively expressed in key cells within the immune system.[4] RORβ has a more restricted pattern of expression and is limited to the central nervous system as well as in bone.[5] RORβ is expressed in regions of the CNS that are involved in processing of sensory information and components of the mammalian circadian clock, the suprachiasmatic nuclei, the retina, and the pineal gland. RORβ$^{-/-}$ mice show defects in circadian rhythmicity,[5] exhibit increased exploration activity and reduced anxiety behaviour.[6] Aberrant circadian rhythms are associated with numerous ailments in humans including bipolar disorder, major depressive disorder, and seasonal affective disorder.[7-8] Moreover, *RORB* genes are associated with bipolar disorder, epilepsy and schizophrenia.[6, 9-10]

RORβ is also expressed in retinal progenitor cells during development and genetic deletion of RORβ results in retinal degeneration implicating its role in vision. RORβ appears to play a role in the maturation of photoreceptors as RORβ null mice are born blind.[5, 11] Most recently, it was discovered that RORβ plays a role in osteogenesis by inhibiting Runx2 activity.[7] Levels of RORβ inversely correlated with osteogenic potential suggesting that suppression of RORβ may drive osteoblast mineralization. Additionally, RORβ and a subset of RORβ-regulated genes were increased in bone biopsies from post-menopausal women compared to pre-menopausal women suggesting a role for RORβ in human age-related bone loss.[12-13]

Although much of the work in the field has focused on development of RORγ inverse agonists for modulation of immune function including hundreds of citations, little effort has focused on RORβ, most likely due to the lack of available chemical starting points for structure-activity relationship (SAR) studies. Given the receptors specific tissue distribution and important physiological functions, the identification of RORβ-selective small molecules would be valuable chemical probes and pharmacological tools. Prior to 2014, only all trans retinoic acid (ATRA) and a synthetic analog (ALRT 1550) had been reported to bind to RORβ and function as inverse agonists.[14-15] Unfortunately, these ligands bind several other NRs including the RXRs (co-receptors for many other NRs) and the RARs.

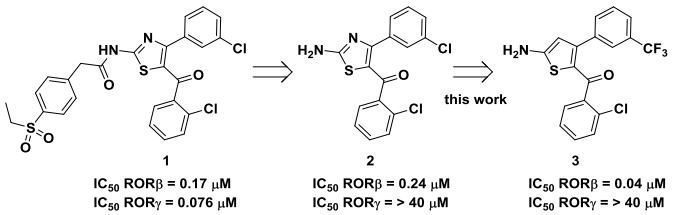


Figure 1. Aminothiophenes as potent RORβ modulators

Recently, a modestly potent dual RORβ/γ ligand was described by Fauber B. et al. with an EC_{50} of 1.2 μM and an EC_{50} of 0.41 μM respectively.[16] A more potent modulator was identified around the same time by a group from Phenex, first as a RORγ inverse agonist,[17] then as a dual RORβ/γ inverse agonist (Figure 1, **1**).[18]

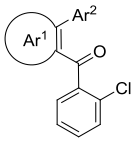
In our previous Communication,[19] we reported truncation of the sulfone containing side chain in the dual RORβ/γ ligand **1** led to RORβ-selective compounds (Figure 1, **2**). Optimization of the aminothiazole scaffold led to a series of more potent RORβ-selective ligands. Herein, we continue our SAR investigations of this scaffold and our efforts to improve the potency while maintaining the excellent selectivity for RORβ vs RORγ. The binding potency of the analogs was determined in a scintillation proximity assay (SPA) using ^{3}H-T0901317 and recombinant human RORβ and RORγ Ligand Binding Domains (LBDs). This assay measures the affinity of the compounds for RORβ vs RORγ and is tabulated in Tables 1-4.

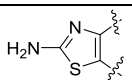
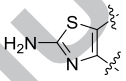
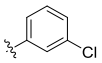
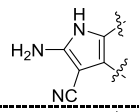
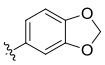
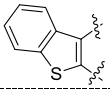
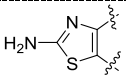
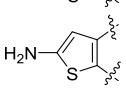
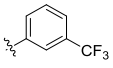
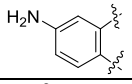
Our initial efforts to modify the core (Ar^1) were based on our lead molecule **2** (Table 1), wherein we maintained the (3-chlorophenyl-5-yl)(2-chlorophenyl) methanone substitution. Attempts to replace the 2-aminothiazole ring with this substitution pattern were not very successful. Removal of the 2-amino group led to a 10-fold loss in affinity (**4**). The thiophene (**5**) was 2-fold less potent than the aminothiazole. The aminothiazole isomer (**6**) was even less potent indicating the ring sulfur atom is likely more important than the nitrogen atom towards potency. A 2-pyridyl ring was also not a viable replacement (**8**) nor were some more esoteric heterocycles (**7**, **9**).

Given 4-substitution (Ar^2) was shown to be important for potency in the aminothiazole ring,[19] we investigated two other modifications (Table 1). With the benzo[d][1,3]dioxole ring, furan, oxazole, and benzothiophene ring systems were not potent (**11**, **12**, **13**). The thiophene ring, however, was tolerated (**10**), with similar activity to compound **5**. The 3-CF_3-phenyl ring was an alternate substitution at the 4-position on the aminothiazole ring (**14**). The corresponding thiazole (**15**) and 2-aminothiophene ring substitutions (**3**) showed 6-fold improvements in potency. Compound **3** was slightly more selective for RORβ vs RORγ than **15** (1000-fold vs 300-fold, respectively). The simple thiophene (**16**) was about 10-fold less potent than the corresponding aminothizaole (**3**), and subsequently less selective for RORγ as well. Substituting a methyl group (**17**) for amino (**3**) in the thiophene series also led to complete loss of activity. Phenyl (**18**) or aminophenyl (**19**) ring replacements were modestly more potent on RORβ than **14**, but also considerably less selective against RORγ.

Based on this first round of core replacements, three viable substitutions for aminothiazole were found giving the most potent and selective RORβ-modulators, including thiazole, thiophene, and aminothiophene.

Table 1. Core thiazole replacements



Cmpd	Ar^1	Ar^2	[a]RORβ IC_{50} (μM)	[b]RORγ IC_{50} (μM)
2			0.24±0.05	>40
4			2.4±1.6	15%
5			0.57±0.12	5%
6			30%	35%
7			30%	40%
8			2.6±1.7	25%
9			50%	0%
10			0.38±0.06	30%
11			15%	n.t.
12			5%	n.t
13			50%	n.t.
14			0.61±0.06	>40
15			0.034±0.017	10±1
3			0.040±0.011	>40
16			0.23±0.04	14±3
17			10%	n.t
18			0.18±0.03	2.6±0.8
19			0.090±0.028	3.8±1.1

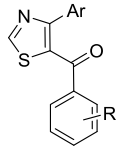
[a] Displacement of [^{3}H]-T09 from human RORβ LBD. Values are the mean ± SEM of at least three replicates. IC_{50} or displacement of [^{3}H]-T09 at 1 μM);
[b] Displacement of [^{3}H]-T09 from human RORγ LBD. Values are the mean ±

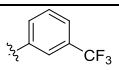
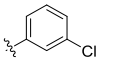
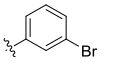
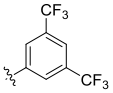
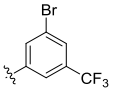
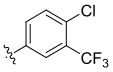
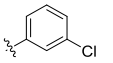
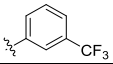
SEM of at least three replicates. IC_{50} or displacement of [^{3}H]-T09 at 1 μM); n.t. = not tested.

Hence, a second round of SAR was investigated with each of these core substitutions (Tables 2-4).

A quick scan of 4-phenylthiazole aryl substitutions did not identify compounds more potent than **15** (Table 2). Most, in fact, showed less potency on RORβ with the exception of **22**. The 2-methoxy benzoyl variant of **15** also showed good affinity for RORβ with good selectivity vs RORγ (**25**).

Table 2. SAR of 4-substitution on the thiazole ring



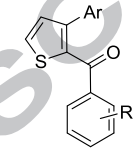
Cmpd	Ar	R	[a]RORβ[a] IC_{50} (μM)	[b]RORγ IC_{50} (μM)
15		2-Cl	0.034±0.017	10 ± 1
4		2-Cl	2.4±1.6	n.t.
20		2-Cl	25%	n.t.
21		2-Cl	35%	n.t.
22		2-Cl	0.026±0.027	6.8±3.8
23		2-Cl	25%	n.t.
24		3,4-Cl_2	0.80±0.48	20%
25		2-OMe	0.094±0.009	20%

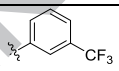
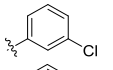
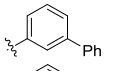
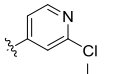
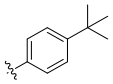
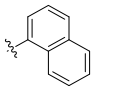
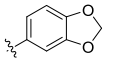
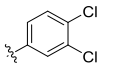
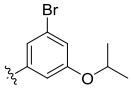
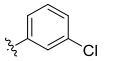
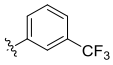
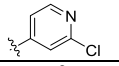
[a] Displacement of [^{3}H]-T09 from human RORβ LBD. Values are the mean ± SEM of at least three replicates. IC_{50} or displacement of [^{3}H]-T09 at 1 μM); [b] Displacement of [^{3}H]-T09 from human RORγ LBD. Values are the mean ± SEM of at least three replicates. IC_{50} or displacement of [^{3}H]-T09 at 1 μM); n.t. = not tested.

The starting point for SAR in the thiophene series was **16** (Table 3). Several substituted aryl rings at the 3-position of the 2-chlorophenyl(thiophen-2-yl)methanones were tolerated. Some showed nice improvements in potency for RORβ (**27**, **29**) though the degree of selectivity for RORγ varied. Compound **27** was the most RORβ-selective analog identified in the thiophene series (~160-fold selectivity for RORβ vs RORγ). The 3-Cl-4-pyridyl motif seemed to be a more selective substitution pattern conferring reasonable RORβ-selectivity in two compounds (**27**, **35**). Increasing the size and hydrophobicity of the aryl substitution had varying effects on RORβ potency, yet this also seemed to increase affinity for RORγ (**29**, **32**-**34**).

Finally, SAR optimization of the 2-aminothiophene core was investigated (Table 4). While mono-3-CF_3 substitution on the 4-phenyl ring was potency enhancing (**3**), bis-CF_3 substitution led to loss of affinity for RORβ (**36**). Bis-substitution with smaller groups (Cl, **37**), however, led to a 10-fold boost in binding affinity for RORβ and one of the most potent analogs identified in this study. Despite an increased affinity for RORγ, there is still a 300-fold window of selectivity for RORβ. 3,4-bis-substitution was not tolerated (**38**). Attempts to bis-chlorinate the benzoyl group at the 5-position of the aminothiophene was less successful leading to mostly impotent compounds (**39**-**43**). A few (2-methoxyphenyl)methanone substituted aminothiophenes were also synthesized. While pairing with the 3-CF_3-phenyl aryl substitution (**44**) reduced RORβ affinity by 2-fold, selectivity against RORγ was also diminished. Additional aryl group substitutions continued to reduce affinity for RORβ (**45**-**47**) as well as selectivity vs RORγ. An interesting comparison is **37** vs **46** wherein the 2-OMe group significantly reduces RORβ potency and selectivity against RORγ.

Table 3. SAR of 4-substitution on the thiophene ring

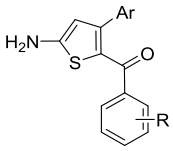


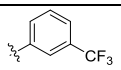
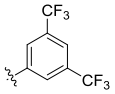
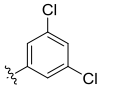
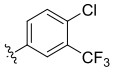
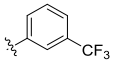
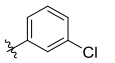
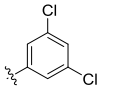
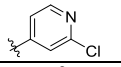
Cmpd	Ar	R	[a]RORβ IC_{50} (μM)	[b]RORγ IC_{50} (μM)
16		2-Cl	0.23 ± 0.04	14 ± 3
5		2-Cl	0.57 ± 0.13	5%
26		2-Cl	0.36	50%
27		2-Cl	0.087±0.02	14 ± 3
28		2-Cl	0.98 ± 0.16	20%
29		2-Cl	0.09±0.02	0.33±0.076
30		2-Cl	0.38±0.06	30%
31		2-Cl	0.47±0.09	30%
32		2-Cl	0.015±0.006	0.46±0.24
33		2-OMe	0.18±0.02	0.78±0.17
34		2-OMe	0.22±0.02	0.79±0.32
35		2-OMe	0.23±0.04	10.7

[a] Displacement of [^{3}H]-T09 from human RORβ LBD. Values are the mean ± SEM of at least three replicates. IC_{50} or displacement of [^{3}H]-T09 at 1 μM); [b] Displacement of [^{3}H]-T09 from human RORγ LBD. Values are the mean ± SEM of at least three replicates. IC_{50} or displacement of [^{3}H]-T09 at 1 μM); n.t. = not tested.

There are clearly some trends in SAR between the different series investigated. Potent and selective RORβ ligands were identified in each series, but it was also possible to lose selectivity in each series, depending on substitution patterns of the groups incorporated.

Table 4. SAR of 4 and 5 substitutions of the amino thiophene ring.



Cmpd	Ar	R	[a]RORβ IC50 (μM)	[b]RORγ IC50 (μM)
3		2-Cl	0.040±0.011	> 40
36		2-Cl	0.24±0.07	25%
37		2-Cl	<0.010±0.03	> 3.0
38		2-Cl	45%	n.t.
39		2,3-Cl	55%	n.t.
40		2,4-Cl	45%	n.t.
41		2,5-Cl	15%	n.t.
42		2,6-Cl	40%	n.t.
43		3,4-Cl	45%	n.t.
44		2-OMe	0.076±0.024	> 5.0
45		2-OMe	0.13±0.02	2.9±0.73
46		2-OMe	0.20±0.02	1.0±0.16
47		2-OMe	0.33±0.04	> 5.0

[a] Displacement of [^{3}H]-T09 from human RORβ LBD. Values are the mean ± SEM of at least three replicates. IC_{50} or displacement of [^{3}H]-T09 at 1 μM); [b] Displacement of [^{3}H]-T09 from human RORγ LBD. Values are the mean ± SEM of at least three replicates. IC_{50} or displacement of [^{3}H]-T09 at 1 μM); n.t. = not tested.

A 3-CF_3 phenyl ring at the 4-position of the thiazoles (**15**) and aminothiophenes (**3**), and the 3-position of the thiophenes (**16**) appears to be optimal for both potency and selectivity. A 2-Cl or 2-OMe benzoyl group appears to be optimal at the 5-position of the thiazoles (**15**, **25**) and aminothiophenes (**3**, **44**), and 2-position of the thiophenes (**16**).

To better understand the structural basis of binding of dual RORβ/RORγ inverse agonist **1** and aminothiophene analog **44** to RORβ, we performed differential hydrogen/deuterium exchange (HDX) mass spectrometry (Figure 2) using purified RORβ ligand binding domain (LBD). HDX data show a clear difference in structural perturbations between the apo receptor and the liganded complexes indicating that both **1** and **44** bind to the LBD in different manners. Furthermore, the HDX signature for **1** is reminiscent of compound binding to the canonical ligand binding pocket (LBP) in RORβ. This profile is similar to 25-hydroxycholesterol (25-HC) binding to RORβ LBD with observed deprotection (increased exchange with solvent D_2O) changes within the LBP indicative of displacement of a fortuitous *E. coli* ligand such as stearic acid that co-purifies with the RORβ LBD.[20]

On the contrary, HDX profiles for the aminothiophene analog **44** and T09 suggests compound binding to the AF2 cleft that harbors the co-regulator binding site in NRs. Allosteric site compounds that bind near the AF2 cleft were recently described for the nuclear receptor RORγ.[21] Differential binding modes observed between **1** and **44** suggests that these molecules are anchored by a variegated set of interactions that help stabilize probe binding and could therefore explain the 2-fold difference in their affinities.

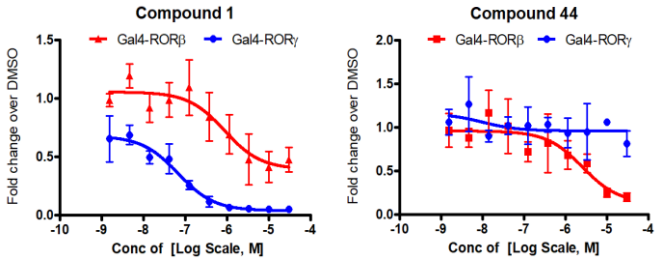


Figure 3: Gal4-LBD assay of compounds **1** and **44**.

To further confirm pharmacology of the compounds *in vitro*, compounds **22**, **33**, **37**, **44** and **45** were screened in HEK293T cells in a Gal4-RORβ::UAS-Luc or Gal4-RORγ::UAS-Luc reporter assay with counter-screening against Gal4-VP16::UAS-Luc (data not shown). While **22** and **33** did not display any significant cellular activity, **37**, **44** and **45** exhibited RORβ inverse agonism (**37**, RORβ IC_{50}= 3.1 μM**,** **44**, RORβ IC_{50}=2.5 μM**,** **45**, RORβ IC_{50}=3.3 μM) with no activity against RORγ or RORα (Figure 3 and data not shown).

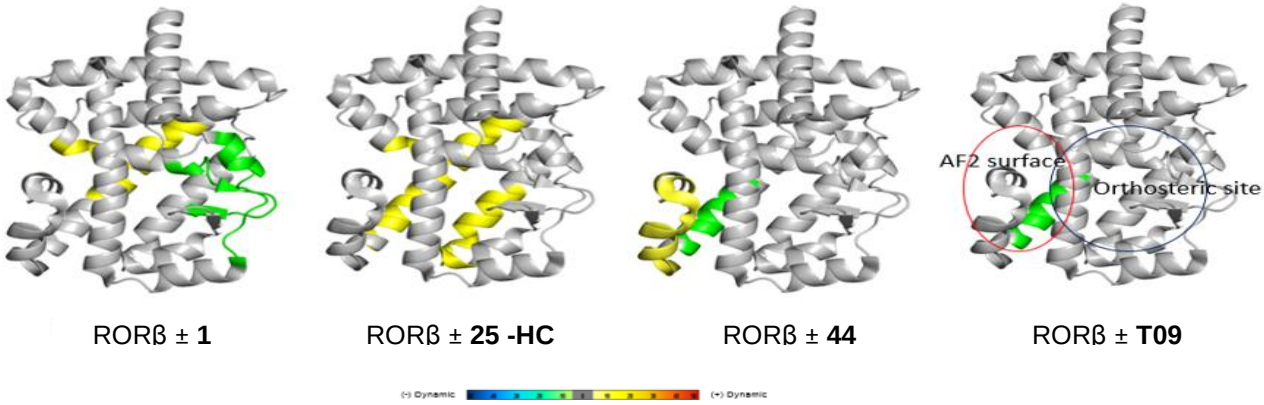
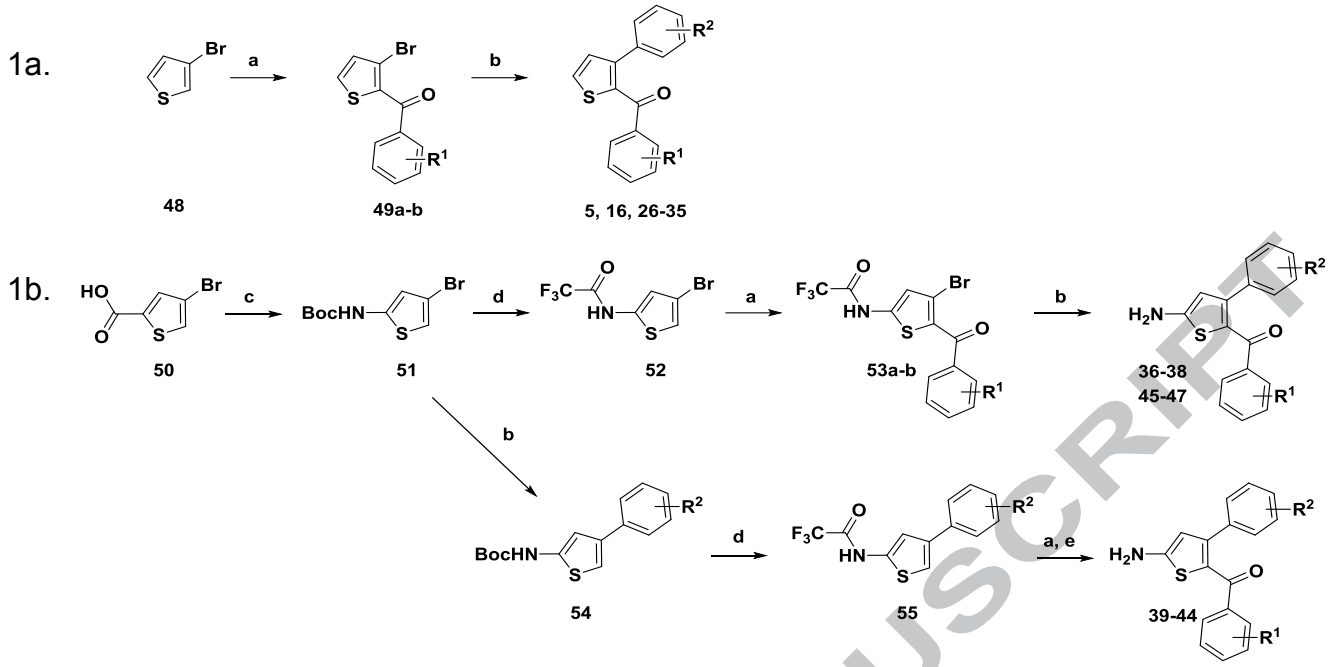


Figure 2: Conformational dynamics probed by HDX of RORβ inverse agonist **1,** 25-Hydroxycholesterol (**25-HC**), **44** and **T09**



Scheme 1. Reagents and Conditions: (a) $R^1PhCOCl$, $AlCl_3$ or $SnCl_4$, DCE, 40 ℃; (b) $R^2PhB(OH)_2$, $Pd(Ph_3P)_4$, Na_2CO_3, toluene/EtOH/H_2O 80 ℃; (c) diphenylphosphorylazide, Et_3N, tBuOH, 90 ℃; (d) TFA/DCM, rt, then trifluoroacetic anhydride, Et_3N, DCM, 0 ℃; (e) K_2CO_3, MeOH, 40 ℃.

It is unlikely that there are cell permeability issues with **22** and **33** given the high hydrophobicity of the compounds. Fortunately, compounds do not appear to be toxic at the doses tested (10 μM and below). However, it is possible that these compounds are neutral antagonists, much like analogs previously described.[19] Despite binding to the receptor and competing out T09, perhaps they are too small or lack specific interactions required to induce a conformational change within the receptor which can disrupt the AF2 surface and alter coregulator interactions. Also, both **22** and **33** lack the 2-amino group substitution on the core heterocycle found in **37** and **44**-**45** which may be a minimal requirement for cellular activity in this series. By comparison, **1**, is a potent dual inverse agonist of RORβ and RORγ, as previously reported (Figure 3).[18]

Different approaches were used in the synthesis of the analogs described herein. For the thiazole series described in Table 2, the corresponding aminothiazoles were fashioned as described in the previous Communication.[19] The 2-amino group was then oxidatively removed using t-BuONO.[22]

For the thiophenes described in Table 3, a simple 2-step approach was used (Scheme 1a). Acylation of the thiophene at the 2-position in presence of Lewis acid ($AlCl_3$) gave 3-bromo-thiophen-2-yl-aryl-methanone derivates (**49a-b)** followed by a Suzuki-Miyaura coupling of substituted arylboronic acids lead to final products (**5**, **16**, **26**-**35**). The aminothiophenes were obtained following methods described in Scheme 1b. A Curtius rearrangement of the 4-bromothiophen-2-carboxylic acid (**50**) in the presence of diphenylphosporylazide (DPPA) and *t*-BuOH afforded the BOC-protected 2-aminothiophene (**51)**. The BOC-protecting group was replaced with an acid stable trifluoroacetate group (**52**). Introduction of the acyl groups was achieved in the presence of a Lewis acid ($SnCl_4$ or $AlCl_3$). $AlCl_3$ was preferred with the 2-methoxybenzoyl chloride to avoid over acylation of the thiophene (**53a-b**). Suzuki-Miyaura coupling with arylboronic acids with in situ trifluoroacetyl cleavage gave final products in good yield (**36-38, 45-47**). To introduce variation in the acetyl portion of the molecule, the Suzuki coupling can be done first (**51→54**), then protecting group exchange (**54→55**). Lewis acid mediated acylation and final deprotection under mild conditions (K_2CO_3/methanol) affords final products (**39**-**44**).

In summary, we have identified a series of disubstituted aminothiophenes as potent RORβ inverse agonists. Starting from a potent dual RORβ/RORγ inverse agonist **1**, removal of the sulfone-containing side chain ablated affinity for RORγ.[19] Optimization of the 4-phenyl and 5-benzoyl groups led to improvements in RORβ potency without compromising selectivity for RORγ. Fine tuning of the core led to cell-active compounds with good potency. Optimization of drug metabolism properties to identify *in vivo* active probes is on-going and will be reported in due course.

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- Truncation of a dual RORβ/RORγ inverse agonist ablated activity at RORγ

- Substituted 2-aminothiophenes were potent RORβ-selective inverse agonists

- Differential HDX-MS analysis of ligands with RORβ highlights receptor perturbations