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Probe Report

Title: qHTS for Inhibitors of the Interaction of Thyroid Hormone Receptor and Steroid Receptor Coregulator 2

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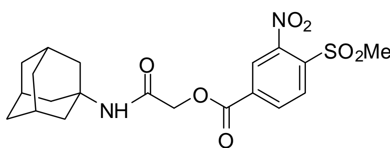
PubChem Summary Bioassay Identifier (AID): 2512

Abstract:

Thyroid hormone receptors (TRs) are members of the nuclear hormone receptor superfamily and regulate many homeostatic processes, including basal metabolism, cardiovascular function, body weight, and lipid trafficking. Upon binding of the ligand triiodothyronine (T3), TR undergoes a conformational change that releases corepressors and recruits coactivators, such as Steroid Receptor Coactivator 2 (SRC2); in turn, these modulate the expression of target genes. In this report, we used a TRβ-SRC2 fluorescence polarization assay to screen the Molecular Libraries Small Molecule Repository (MLSMR) and identify a novel methylsulfonylnitrobenzoate (MSNB)-containing series that blocks the association of TRβ with a SRC2 peptide. This inhibitor probe molecule, ML151 (CID 5184800), blocked TRβ-SRC2 interaction with a potency of 1.8μM. Mechanistic studies revealed that ML151 (CID 5184800) is a covalent inhibitor and binds irreversibly to Cys298 within the AF-2 cleft of TRβ. This series will be useful for *in vitro* mechanistic studies of TR-SRC2 interactions, as well as other nuclear hormone receptor-coactivator interactions.

Probe Structure & Characteristics:



ML151

CID/ ML#	Target Name	IC_{50}/EC_{50} (nM) [SID, AID]	Anti-target Name(s)	IC_{50}/EC_{50} (µM) [SID, AID]	Fold Selective	Secondary Assay(s) Name: IC_{50}/EC_{50} (nM) [SID, AID]
5184800/ ML151	TR-SRC2	1400 [87550851, 2490]	AR-SRC2; VDR-SRC2; PPARg-DRIP	> 130, [87550851, 2448]; 53 [87550851, 2455]; > 130, [87550851, 2449]	> 92; 38; > 92	TR-SRC2 alpha screen: 2700, [87550851, 2444]

Recommendations for scientific use of the probe:

Thyroid receptor (TR) regulates many homeostatic processes including basal metabolism, cardiovascular function, body weight, and lipid trafficking. TR modulators are potential therapeutics for obesity and hyperlipidemias, but current thyroid analogs have undesirable side effects, particularly cardiac stimulation. This probe is a member of a series that inhibits the interaction between the ligand-occupied TR and its coactivator, steroid receptor coregulator 2 (SRC2). This series can be used *in vitro* to study the mechanisms by which coactivators induce nuclear hormone receptor (NHR) activity in general, and how SRC2 activates TR in particular. Because SRC2 interacts with a number of different NHRs, including the androgen receptor (AR) and glucocorticoid receptor, TR selective inhibitors can help elucidate how SRC2 discriminates among different NHRs.

1 Introduction

Thyroid hormone is instrumental in controlling aspects of metabolism, growth, and development (reviewed in [1]). The thyroid receptor (TR) is a member of the family of nuclear hormone receptors (NHR) that induce the expression of transcriptional targets. There are two TR genes, TR alpha and TR beta, which mediate distinct functions. TR alpha regulates key aspects of heart function, while TR beta controls plasma cholesterol levels, as well as feedback regulation between the hypothalamus, pituitary, and thyroid. Like other NHRs, TR is composed of three parts, a transcriptional activation domain, a DNA binding domain, and a ligand binding domain. Ligand binding to TR causes the dissociation of corepressors and the subsequent association of coactivators that activate target gene expression. One coactivator that interacts strongly with TR, as well as other NHRs, is sterol receptor coactivator 2 (SRC2). SRC2 contains a nuclear receptor interaction domain composed of three short leucine-rich motifs that bind to TR. SRC2-2, the second repeat of this domain, binds to TR with an affinity comparable to full length SRC2. The goal of this project is to identify small molecule inhibitors that are TR selective by disrupting the association of SRC2.

2 Materials and Methods

All commercially available reagents and solvents were purchased and used without further purification. All microwave reactions were carried out in a sealed microwave vial equipped with a magnetic stir bar and heated in a Biotage Initiator Microwave Synthesizer. HPLC purification was performed using a Waters semi-preparative HPLC equipped with a Phenomenex Luna® C18 reverse phase (5 micron, 30x 75mm) column having a flow rate of 45 ml/min. The mobile phase was a mixture of acetonitrile and H_2O, each containing 0.1% trifluoroacetic acid. During purification, a gradient of 30% to 80% acetonitrile over 8 minutes was used with fraction collection triggered by UV detection (220nM). ^1H spectra were recorded using an Inova 400 MHz spectrometer (Varian). Two LCMS methods were used to analyze samples' purity.

Method 1: Agilent 1200 series LC/MS equipped with a Zorbax™ Eclipse XDB-C18 reverse phase (5 micron, 4.6x 150mm) column having a flow rate of 1.1 ml/min. The mobile phase was a mixture of acetonitrile and H_2O each containing 0.05% trifluoroacetic acid. A gradient of 5% to 100% acetonitrile over 8 minutes was used during analytical analysis.

Method 2: Acquity HPLC equipped with a Waters BEH C18, 1.7 micron, 2.1 x 50 mm column; Column Temperature: 45 degrees C; Flow: 0.5ml/min; Solvent A: 0.05% TFA in Water; Solvent B: 0.025% TFA in Acetonitrile; Gradient: 2% to 100% Solvent B over 1.3 minutes; Run Time - 3 min.

2.1 Assays

PubChem AID	Type	Target	Conc. Range	Samples Tested
1469	Primary qHTS	Thyroid receptor	92µM – 1.4nM	276,294
1573	Confirmatory	Thyroid receptor	92µM – 9pM	109
2487	Orthogonal	Thyroid receptor	130µM – 7nM	20
2444	Secondary	Thyroid receptor	200µM – 90nM	30
2448	Anti-target	Androgen receptor	130µM – 7nM	25
2449	Anti-target	PPAR gamma	130µM – 7nM	25
2455	Anti-target	Vitamin D receptor	130µM – 7nM	25
2512	Summary			

qHTS for Inhibitors of the Interaction of Thyroid Hormone Receptor and Steroid Receptor Coregulator 2**. [AID: 1469]**

To identify inhibitors that specifically prevent the interaction of TR with the steroid receptor coregulator 2 (SRC2), a fluorescence polarization assay was screened. This assay detects interaction of the ligand-binding domain of human TR beta (TRβ) with a Texas Red labeled SRC2 peptide, corresponding to a 20 amino acid region of the nuclear receptor interaction domain [2]. Small molecule inhibitors that block the interaction of TR and SRC2 are detected by a decrease in fluorescence polarization.

For screening, 5 µl/well 0.6µM TRβ and 20nM SRC2 Texas Red in protein buffer (20mM Tris hydrochloride, 100mM NaCl, 10% glycerol, 1mM EDTA, 0.01% NP-40, 1mM DTT, 1µM T3 and 5% DMSO) were dispensed into black solid 1536-well plates (Grenier) using a solenoid-based dispenser. Following transfer of 23nl compound or DMSO vehicle by a pin tool, the plates were centrifuged 15 s at 1000 RPM and incubated 5 hr at ambient temperature. The plates were read by an Envision (Perkin Elmer) to detect fluorescence polarization of SRC2 Texas Red (555 nm excitation and 632 nm emission). Data were normalized to un-bound (all components except TRβ) and bound SRC2 Texas Red controls.

Table 1: Final 1536-well assay protocol

Step	Parameter	Value	Description
1	Reagent	5µl	0.6µM TRβ, 20nM SRC2
2	Controls	23nl	beta-aminophenylketone inhibitor (CID 3092218)
3	Library compounds	23nl	92µM to 2.9nM dilution series
4	Centrifuge	15 sec	1000 RPM
5	Incubation time	5 hr	Ambient temperature
6	Assay readout	Fluorescence polarization	Envision

Step **Notes**

1 Grenier 1536-well black, medium binding, solid bottom plates; 1 tip dispense to all columns except column 3 of protein buffer (20mM Trizma hydrochloride, 100mM NaCl, 10% Glycerol, 1mM EDTA, 0.01% NP-40, 1mM DTT, 20nM SRC2-2Tex, 600nM TRβ, 1µM T3 and 5% DMSO). Column 3, 1 tip dispense of protein buffer without TRβ.

2 Control compound plate Column 1, beta-aminophenylketone titration starting at 46µM, 16 points in duplicate 1:2 dilutions; Columns 2-4 DMSO.

3 Pin tool transfer. The highest concentration library plates were pinned two times to achieve the highest tested concentrations. Most compounds were screened at 92, 46, 9.2, 1.8, 0.36, and 0.0029µM final assay concentration.

5 Plates covered with stainless steel rubber gasket-lined lids containing pin holes for gas exchange.

6 Envision FP settings - 555 nm excitation, 632 nm emission, 75 flashes.

Confirmatory assay

The confirmation and orthogonal TR assays were performed as follows, with the only difference being the SRC2-2 fluoroprobe; the confirmation assay used a Texas Red label and the orthogonal assay used a fluorescein label. Twenty µl/well 0.6µM TRβ and 20nM SRC2-2 fluoroprobe in protein buffer (20mM Tris hydrochloride, pH 7.4, 100mM NaCl, 10% glycerol, 1mM EDTA, 0.01% NP-40, 1mM DTT, 1µM T3 and 4% DMSO) was dispensed into black solid 384-well plates (Costar 3710) using a Biomek FX (Beckman Coulter) liquid handling system. Following

transfer of 260nl compound (ranging from 7nM to 130µM), the plates were incubated 3 hr at ambient temperature. The plates were read by an Envision (Perkin Elmer) to detect fluorescence polarization of SRC2 Texas Red (555 nm excitation and 632 nm emission) or SRC2 Fluorescein (480 nm excitation and 535 nm emission).

Anti-target assay(s)

The androgen receptor (AR) assay (AID 2448) detects the interaction of the ligand-binding domain of human AR with a Texas Red labeled SRC2-3 peptide, corresponding to a 20 amino acid region of the nuclear receptor interaction domain. Small molecule inhibitors that block the interaction of AR and SRC2-3 are detected by a decrease in fluorescence polarization. Twenty µl/well 1µM liganded AR-LBD and 10nM Tx-SRC2-3 peptide in buffer (50mM HEPES, 150mM Li2SO4, 0.2mM TCEP, 10% glycerol, pH 7.2, and 4% DMSO) were dispensed into black solid 384-well plates (Costar 3710) using a Biomek FX (Beckman Coulter) liquid handling system. Following transfer of 260nl compound (ranging from 7nM to 130µM), the plates were incubated 3 hr at ambient temperature. The plates were read by an Envision (Perkin Elmer) to detect Texas Red polarization of Tx-SRC2-3 (555 nm excitation and 632 nm emission).

The vitamin D receptor (VDR) assay (AID 2455) detects the interaction of the ligand-binding domain of human VDR with an Alexa Fluor 647 labeled SRC2-3 peptide, corresponding to a 20 amino acid region of the nuclear receptor interaction domain. Small molecule inhibitors that block the interaction of VDR and SRC2-3 are detected by a decrease in fluorescence polarization. Twenty µl/well 1µM VDR and 5nM Alexa Fluor 647 SRC2-3 peptide in buffer (25mM PIPES, pH 6.75, 50mM NaCl, 0.01% NP-40, 6µM LG190178, and 4% DMSO) was dispensed into black solid 384-well plates (Costar 3710) using a Biomek FX (Beckman Coulter) liquid handling system. Following transfer of 260nl compound (ranging from 7nM to 130µM), the plates were incubated 3 hr at ambient temperature. The plates were read by an Envision (Perkin Elmer) to detect fluorescence polarization of Alexa Fluor 647 (620 nm excitation and 688 nm emission).

The peroxisome proliferator-activated receptor gamma (PPAR gamma) assay (AID 2449) detects the interaction of the ligand-binding domain of human PPAR gamma with a Texas Red labeled DRIP-2 peptide, corresponding to a 20 amino acid region of the nuclear receptor interaction domain. Small molecule inhibitors that block the interaction of PPAR and DRIP-2 are detected

by a decrease in fluorescence polarization. Twenty µl/well 2µM PPAR gamma and 10nM Tx-DRIP-2 peptide in protein buffer (20mM TRIS, pH 7.5, 100mM NaCl, 0.01% NP-40, 20µM roziglitazone, and 4% DMSO) was dispensed into black solid 384-well plates (Costar 3710) using a Biomek FX (Beckman Coulter) liquid handling system. Following transfer of 260nl compound (ranging from 7nM to 130µM), the plates were incubated 3 hr at ambient temperature. The plates were read by an Envision (Perkin Elmer) to detect Texas Red polarization of Tx-DRIP-2 (555 nm excitation and 632 nm emission).

2.2 Probe Chemical Characterization

Structural verification and purity quantification were performed by ^{1}H NMR analysis using a Varian spectrometer and by LC-MS analysis using an Agilent system in the following conditions:

Column: 3x 75 mm Luna C18, 3 micron

Run time: 4.5 min (short); 8.5 min (long)

Gradient: 4 % to 100 %

Mobile phase: Acetonitrile (0.025 % TFA), water (0.05 % TFA).

Flow rate: 0.8 to 1.0ml

Column temperature: 50°C

Detectors: UV (220 nm, 254 nm) and MS (ESI$^+$).

Both NMR and LC-MS analyses showed purity greater than 98% for ML151 (NCGC00188612, CID: 5184800, SID: 87550851). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 1.65 - 1.74 (m, 6 H), 1.98 - 2.06 (m, 6 H), 2.07 - 2.14 (m, 3 H), 3.46 (s, 3 H), 4.76 (s, 2 H), 5.44 (br. s., 1 H), 8.33 (d, *J*=8.0 Hz, 1 H), 8.44 (dd, *J*=8.1, 1.5 Hz, 1 H), 8.48 (d, *J*=1.6 Hz, 1 H); HPLC: t_1 (short) = 3.77 min and t_2 (long) = 6.36 min, UV_{220} > 98%, UV_{254} > 98%; HRMS (ESI): *m/z* calcd for $C_{20}H_{24}N_2O_7S$ $[M+1]^+$ 437.1394, found 437.1394.

This compound is commercially available from Enamine (ID: T5217128).

Enamine Ltd, USA Office
Princton Corporate Plaza
7 Dear Park Drive, Suite M-3
Monmouth Junction, NJ 08852
Phone: +732 274 9150
Fax: +732 274 9151

The compound, ML151, is soluble at 10mM in DMSO. The compound is not fluorescent with blue excitation wavelengths (~340 nm). Solubility in PBS buffer: 2.9µM.

PubChem CID	5184800
Molecular Weight	436.48
Molecular Formula	C20H24N2O7S
ClogP	1.97
H-Bond Donor	1
H-Bond Acceptor	7
Rotatable Bond Count	6
Tautomer Count	2
Exact Mass	436.130422
Topological Polar Surface Area	133
Heavy Atom Count	30

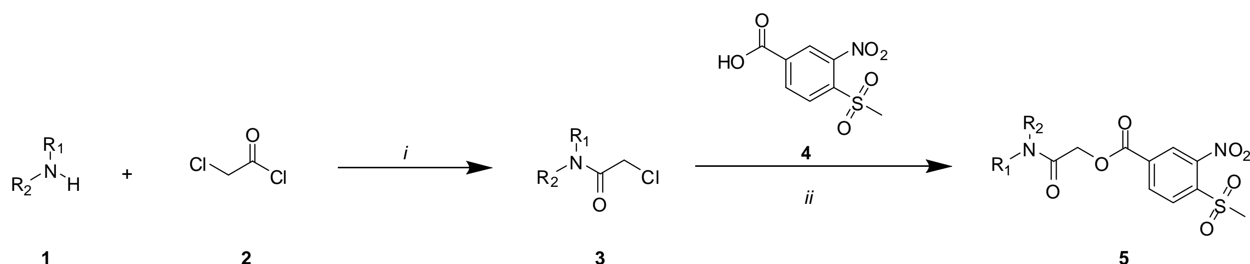
MLS ID	CID	SID	NCGC ID	ML ID	Type
MLS000389544	5184800	87550784	NCGC00188612-01	ML151	Probe
MLS002703101	44623864	87550763	NCGC00188530-01		Analog
MLS002703102	44623849	99495631	NCGC00188531-01		Analog
MLS002703104	44640136	99495633	NCGC00188535-01		Analog
MLS002703105	44640137	99495634	NCGC00188536-01		Analog
MLS002703103	44623872	99495632	NCGC00188537-01		Analog

2.3 Probe Preparation

Schemes 1-3 illustrate the general synthetic strategy for the synthesis of methylsulfonylnitrobenzoate (MSNB). Methylsulfonylnitrobenzoates **5** were prepared by a two-step, one-pot procedure as described in Scheme 1. 2 – Chloroacetic chloride (**2**) was treated with amines **1** to give amides **3**. Following this transformation, the amides reacted with acid **4** to give NSB **5**. Alternatively, methylsulfonylnitrobenzoates **5** were prepared by a reaction sequence described in Scheme 2. Reaction of acid **4** and bromide **6** followed by Boc-deprotection gave

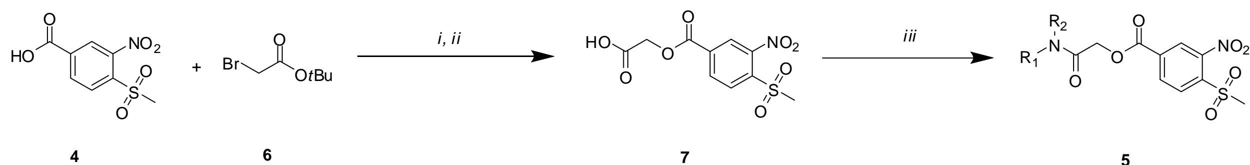
acid **7**, which reacted with amines **1** to generate the desired NSB **5** in high yields. The synthesis of NSB **12a** and **12b** is shown in Scheme 3. Reaction of 2-adamantylamine (**8**) and **9** gave chlorides **10**. The chlorides were converted to alcohols **11** by reacting with sodium acetate followed by a hydrolysis reaction. Mitsunobu reaction of alcohols **11** and acid **4** produced **12a** and **12b**. All NSB analogs were purified by either preparative HPLC or column chromatography on silica gel.

Scheme 1.



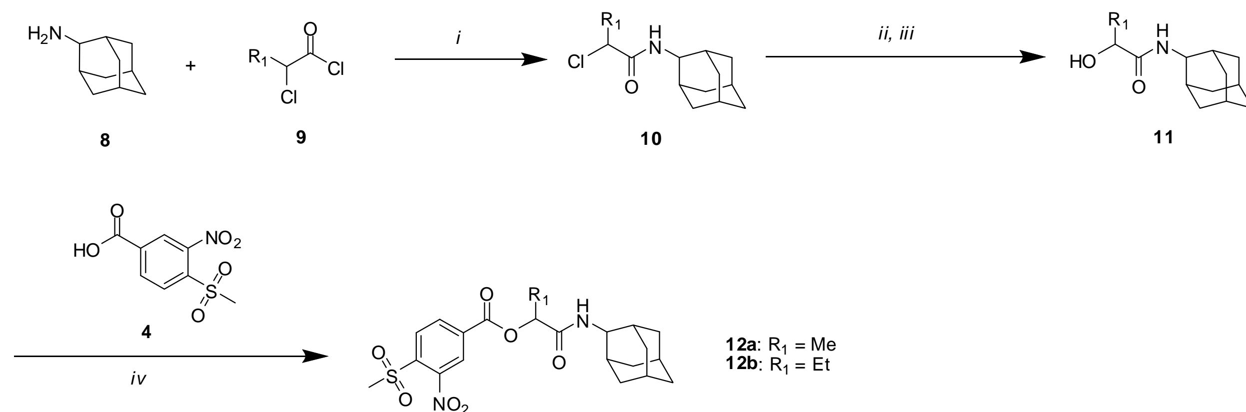
Reagents and conditions: (i) *i*PrNEt$_2$, ACN, r.t. 1 h; (ii) **4**, MW 150 ^{o}C, 10 min

Scheme 2.



Reagents and conditions: (i) *i*PrNEt$_2$, ACN, MW 150 ^{o}C, 10 min; (ii) TFA; (iii) R$_1$R$_2$NH, DMC, *i*PrNEt$_2$

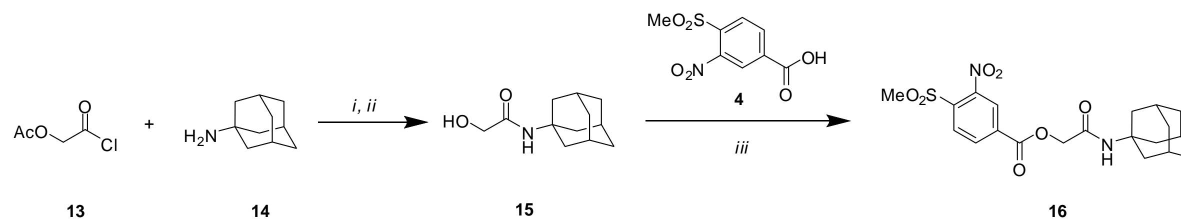
Scheme 3.



Reagents and conditions: (i) *i*$PrNEt_2$, DCM, r.t. 8 h; (ii) NaOAc, DMF, MW 150 °C, 15 min; (iii) LiOH, THF/MeOH/H_2O, MW 100 °C, 15 min; (iv) **4**, DBAD, Ph_3P, THF, 60 °C, 2 h

The probe molecule can be synthesized as depicted in Scheme 4. Reaction of 1-adamantylamine (**14**) with acid chloride **13**, followed by a hydrolysis reaction gave alcohol **15**. Mitsunobu reaction of the alcohol with acid **4** yielded the desired MSNB **16**.

Scheme 4.



Reagents and conditions: (i) *i*$PrNEt_2$, DCM, r.t. o/n; (ii) LiOH, THF/MeOH/H_2O, MW, 100 °C 10 min; (iii) **4**, DBAD, Ph_3P, THF, 60 °C, 2 h

3 Results

The titration-response data was processed by normalizing mP and total fluorescence (TF) values to controls as follows: % Activity = $((V_{compound} – V_{pos})/(V_{pos} – V_{neg}))$ x 100, where $V_{compound}$ denotes the compound well values, V_{pos} denotes the median value of the DMSO-treated control wells containing TRβ, and V_{neg} denotes the median values of the DMSO-treated control well without TRβ (free fluoroprobe). TF was calculated as follows: TF = S + 2P where S and P are fluorescence readings in the parallel and perpendicular channels, respectively. These normalized activity values were then corrected by applying a pattern correction algorithm using DMSO-only plates placed at 24 plate intervals in the screen, as well as at the beginning and end.

Concentration response curves (CRC) were fit and classified as described [5]. Briefly, CRCs were placed into four classes. Class 1 contained complete CRCs showing both upper and lower asymptotes and r2 values > 0.9. Class 2 contained incomplete CRCs having only one asymptote and showed r2 values > 0.9. Class 3 curves were of the lowest confidence because they are poorly fit or based on activity at a single concentration point. Class 1 and 2 curves were divided further into subclasses to indicate efficacies 80% or greater (Class 1.1 and 2.1) or between 30% and 80% (Class 1.2 and 2.2). Class 4 compounds were inactives having either no curve-fit or an efficacy below threshold activity (3 SD of the mean activity). While both activators and inhibitors were recovered from the qHTS, activators were not considered further, as their activity likely arose from compound fluorescence. The qHTS resulted in 910 inhibitors (Class 1-3; Table 2), of which 511 were scored high quality (Class 1.1, 1.2 and 2.1 as well as Class 2.2 with >40% efficacy).

Activity	Distribution	Curve Classification					
		1.1	1.2	2.1	2.2	3	4
Inhibition	Cmpd No.	48	159	65	332	306	291,510
	% library	0.02%	0.05%	0.02%	0.11%	0.1%	99.6%
Activation	Cmpd No.	21	39	23	114	115	
	% library	0.01%	0.01%	0.01%	0.04%	0.04%	

Table 2. Activity profile of the TR qHTS

An in-house program was used to cluster 511 actives, yielding 730 structural series and 128 singletons. Because an active could be part of more than one series, the number of series was larger than the number of actives clustered. After clustering, structurally related compounds with inconclusive or no activity were added to each series. Each series contained at least three compounds, of which at least one was active. Series were flagged for the following potential liabilities (number of series in parentheses): concentration dependent changes in total fluorescence in the FP assay or at 547 nm excitation and 618 emission (206), promiscuous aggregation (17), promiscuous redox activity (55), low potency (> 20μM) or low efficacy (< 50%) (193), significantly lower actives compared to mean actives in all series using a Fisher's exact test, $P < 0.05$ (10), and promiscuous activity in other assays (17). This process identified 8 series and two singletons with no liabilities. To prioritize compounds for follow up studies, the series were examined for previously identified scaffolds, presence of chemically undesirable functional groups, and compounds with 'Rule of Five' violations [6]. Confirmation studies of prioritized series were performed using the primary TR FP assay containing a Texas Red labeled fluoroprobe (AID 1573) and an orthogonal FP assay that utilized a fluorescein labeled probe (AID 2487). These tests identified the nitrosulfonyl benzoate series as the most promising for further optimization.

3.1 Summary of Screening Results

The compound library was tested using quantitative high throughput screening (qHTS), a method where each compound is tested at multiple concentrations to generate a titration-response curve for each sample [5]. The qHTS was conducted over nine days, and 291,510 samples were assayed at six concentrations. The screen performed well; of the 1418 assay plates assayed, 1380 (97%) plates passed quality control, and these showed 0.73 mean Z' scores and 128 mean mP window. The control titration of beta-aminophenylketone inhibitor (CID 3092218) present on each plate performed consistently, showing a mean IC_{50} of 8.2 ± 5.2μM.

3.2 Dose Response Curves for Probe

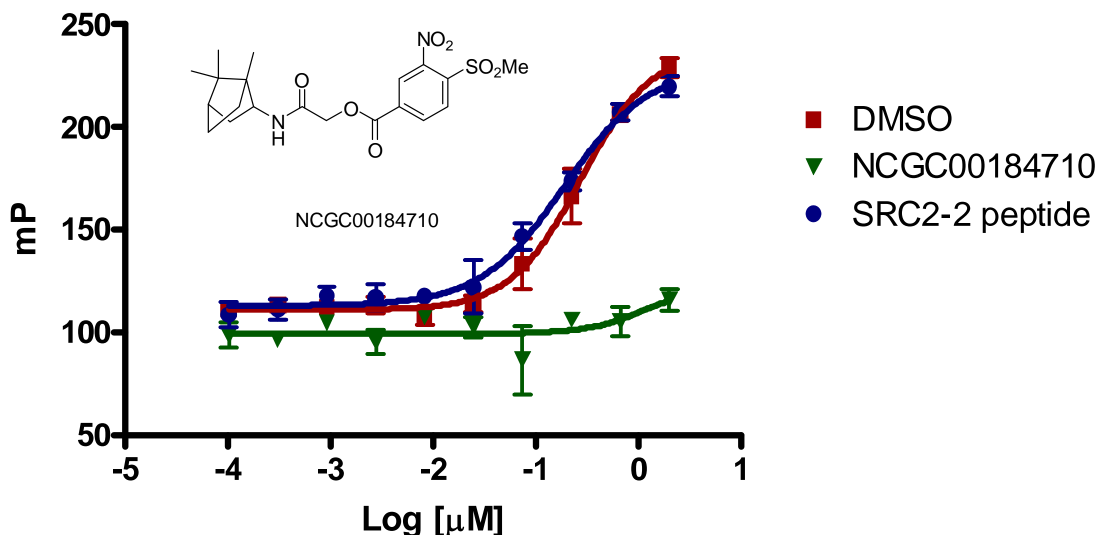


Figure 1. TR inhibition by a methylsulfonylnitrobenzoate series member is irreversible**.** TRβ was incubated with DMSO (red square), NCGC00184710 (CID: 24526448, green triangle) or SRC2-2 peptide (blue circle) for 3hr in assay buffer. Samples were then dialyzed in assay buffer overnight, recovered and tested in the TRβ FP assay at the shown concentrations of TRβ.

3.3 Scaffold/Moiety Chemical Liabilities

The benzoate moiety of the probe molecule could be hydrolyzed by esterase.

3.4 SAR Tables

All analogs were evaluated in the confirmation TR FP assay (AID 1573). The preliminary SAR study of the aromatic (Ar), R1, and R2 positions yielded several important findings (Table 3). At the Ar position, the 3-nitro-4-sulfonyl benzoate moiety is important for inhibitory activity because both 3-nitrobenzoate **19** and 4-methylsulfonylbenzoate **18** were inactive. At the R1 position, methyl and ethyl groups had no effect on potency (compare **17**, **12a** and **12b**). At the R2 position, hydrophilic amines such as morpholine **5e** and N-methylpiperazine **5f** diminished

activity. Among the tested analogs, 1-adamantyl (**12a**, **12b**, and **17-19**) and 2-adamantyl **16** amines were the best groups at this position.

Entry	No	NCGC ID	CID	SID	Ar	R1	R2	TR Tx-SRC2-2 (uM)
1	**17**	NCGC00188514-02	44623856	87550776		H		1.6
2	**18**	NCGC00188499-01	44623871	87550754		H		>130
3	**19**	NCGC00188500-01	7471493	87550755		H		>130
4	**12a**	NCGC00188535-01	44640136	87550751		Me		1.3
5	**12b**	NCGC00188536-01	44640137	87550752		Et		1.2
6	**16**	NCGC00188612-02	5184800	87550851		H		1.4
7	**5a**	NCGC00188538-01	2620272	87550746		H		41.3
8	**5b**	NCGC00188507-01	2620635	87550742		H		9.8
9	**5c**	NCGC00188533-01	44623854	87550747		H		3.3
10	**5d**	NCGC00188541-01	44623874	87550750		H		8.2
11	**5e**	NCGC00188537-01	44623872	87550745		H		>130
12	**5f**	NCGC00188509-01	44623848	87550762		H		>130
13	**5g**	NCGC00188530-01	44623864	87550763		H		42.4
14	**5h**	NCGC00188531-01	44623849	87550765		H		7.6
15	**20**	NCGC00184710-01	24526448	87550722		H		4.3

Table 3. Structure-activity relationships of the methylsulfonylnitrobenzoate series

3.5 Cellular Activity

HEK293 cells were co-transfected with a CMV-TRβ expression vector, a *Photonis* luciferase reporter fused to a thyroid response element and a *Renilla* luciferase reporter, used as a control for transfection efficiency. Cells were treated with 30 nM T3 and different concentrations of ML151 (CID 5184800), incubated for 18 hours, and transcriptional reporter activities measured. ML151 (CID 5184800) showed a concentration-dependent decrease of TRβ-mediated activity over the tested concentration range of 2.5 to 20 μM (Ref 7).

3.6 Profiling Assays

ML151 (CID 5184800) has similar antagonistic potency towards both TRα and TRβ. It has no effects on AR and PPARγ, and only weak effect on VDR (Ref 7).

4 Discussion

Small molecule inhibitors of SRC2 interaction with TR have been identified. One series, containing an aromatic beta-amino ketone core, is an irreversible inhibitor of TR and has an IC_{50} of 2μM [2]. Furthermore, many analogs of this series show off-target activity by blocking hERG channel activity [3]. Our goal was to identify a new chemical entity that reversibly inhibited TR and SRC2 interaction with an IC_{50} value less than 10μM.

Note, because the assay measures a protein-protein interaction and uses near micromolar amounts of target protein, the lower limit of potency detection is about 1μM IC_{50}. Given the historical difficulty in achieving potent inhibitors of protein-protein interactions, the potency goals for this probe were revised to < 10μM.

The one novel series of sufficient potency identified from the screen showed irreversible binding. Thus, the probe goal was modified to identify a new chemical entity that inhibits the TR and SRC2 interaction with an IC_{50} less than 10μM.

4.1 Comparison to existing art and how the new probe is an improvement

This new series has several improvements over the prior art. Analogs of the aromatic beta-amino ketone series reacted to Cys298, and to a lesser degree to Lys211 and Cys388 [4], while the methylsulfonylnitrobenzoate series described here reacts only with Cys298. The presence of an amino functionality in the aromatic beta-amino ketone series has been attributed to the off target inhibition of the hERG channel [3]. The methylsulfonylnitrobenzoate series does not contain an amino group, and hERG inhibition is not expected. The aromatic beta-amino ketone series undergoes β-elimination to form a reactive intermediate that interacts with Cys298 [4]. The methylsulfonylnitrobenzoate series does not from an intermediate and can be tracked more easily than the prior aromatic beta-amino ketone series [3].

4.2 Mechanism of Action Studies

To confirm that inhibitors prevent the interaction of TRβ with the steroid receptor coregulator 2 (SRC2), a TR-SRC2 interaction assay (AID 2444) using Alpha Screen (PerkinElmer) was employed. This assay detects the interaction of the ligand-binding domain of human TRβ with a SRC2 peptide, corresponding to a 20 amino acid region of the nuclear receptor interaction domain. Small molecule inhibitors that block the interaction of TR and SRC2 are detected by a decrease in chemiluminescence. Fifteen µl/well of 100nM TRβ and 100nM SRC2-2-peg2-biotin in buffer (25mM HEPES, 100mM NaCl, 1mM DTT, 0.1% BSA, 0.01% NP-40) were dispensed into white 384 well Optiplates (PerkinElmer). Following transfer of 135nl compound (ranging from 90nM to 200µM), plates were incubated 1 hr, and 5µl acceptor beads (6.3 µg/ml TRβ antibody [Santa Cruz #sc-32754] and 40 µg/ml protein A beads in buffer) were added. After 30 min incubation, 5µl of streptavidin donor beads were added and after 90 min incubation, the plates were read by an Envision (Perkin Elmer) to detect luminescence of the donor and acceptor bead interaction (680 nm excitation and 520-620 nm emission). ML151 A(NCGC00188612, CID: 5184800) showed 1.8µM IC_{50} in the TR Alpha Screen assay, which was similar to 1.4µM IC_{50} determined in the TR FP assay.

The aromatic β-amino ketone inhibitors of TR are irreversible and form a covalent adduct with cysteine residues in the coactivator binding pocket of TR [2]. Members of the nitrosulfonyl benzoate series were tested for irreversible binding as well. TRβ (1µM) was incubated with DMSO, SRC2-2 peptide (100µM) or NCGC00184710 (CID: 24526448, 100µM) for 3 hr in assay buffer. Samples were then dialyzed in assay buffer (4l) overnight using 3000 MW cutoff Slide-A-Lyzer MINI dialysis units (Pierce, IL). The protein samples were concentrated using a 10,000 MW cutoff, spin filter column (Amicon Ultra, Millipore) and then quantified by Bradford analysis. The protein samples were serially diluted from 4 to 0.002µM in assay buffer in 96-well plates. Then, 10µl of diluted protein was added to 10µl of 40nM SRC2 fluorescence probe in 384 well plates. Unlike DMSO or SRC2-2 peptide pretreatment, NCGC00184710 (CID: 24526448) pretreatment continued to inhibit SRC2-2 interaction with TRβ following dialysis (Figure 1).

We wished to identify the nature of the irreversible inhibition of TR by methylsulfonylnitrobenzoate analogs. The TRβ ligand-binding domain (LBD) was incubated in

the absence or presence of NCGC00184816 (CID: 42898880) for 2 hr in assay buffer, and the samples subjected to mass spectrometry. The TR LBD alone had a mass of 30,031 Dalton, but when incubated with NCGC00184816 (CID: 42898880), it had a mass of 30,349 Dalton (Figure 2). The 318 Dalton difference corresponds to the size of NCGC00184816 (CID: 42898880) without the methylsulfonyl group, suggesting a covalent bond was formed *via* an addition-elimination mechanism between one molecule of TRβ LBD and one molecule of NCGC00184816 (CID: 42898880). Mass spectrometry analysis of trypsin digests of the TR-compound pair showed that NCGC00184816 (CID: 42898880) was covalently attached to cysteine 298 of the protein. The proposed mechanism of action for covalent attachment of methylsulfonylnitrobenzoate analogs is shown in Figure 3. Characterization of this series in cell-based assays is ongoing.

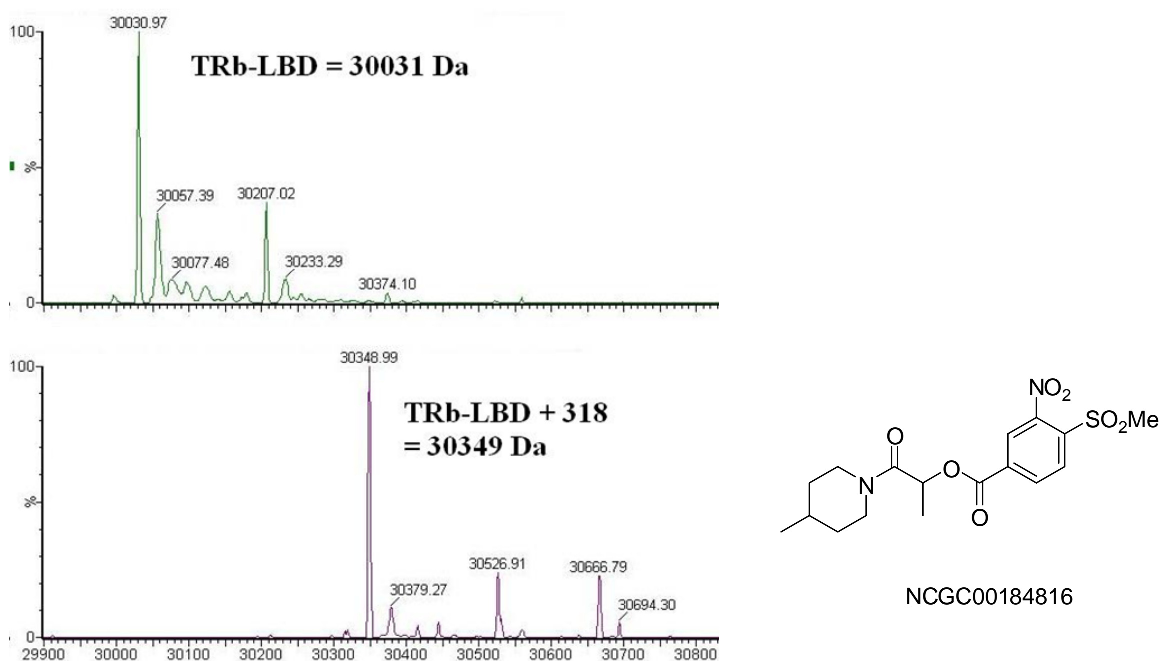


Figure 2. Mass spectrometry analysis of TR and methylsulfonylnitrobenzoate interaction. Five micromolar TRβ was incubated in the presence or absence of 25 μM NCGC00184816 (CID: 42898880) (lower right) for 2 hr in assay buffer, precipitated with cold ethanol for 24 hr air-dried. The samples were desalted and eluted, and the eluent was ionized on a Waters LCT Premier XE mass spectrometer using positive detection mode. The expected error for this mass spectrometer is 1Dalton for every 10,000 Dalton.

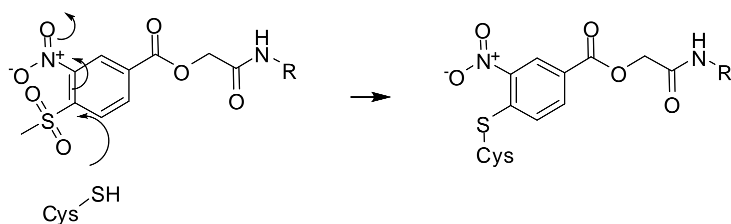


Figure 3. Proposed mechanism of action of methylsulfonylnitrobenzoate analogs. Inside the coactivator binding pocket of TR, the sulfhydryl group of cysteine 298 attacks the 4 position of the phenyl ring of the methylsulfonylnitrobenzoate, aided by the electron withdrawing capacity of the nitro group. Subsequently, the methylsulfonyl group leaves and allows covalent attachment of the nitrobenzoate moiety to the cysteine via a thioether linkage.

4.3 Planned Future Studies

We plan to perform more medicinal chemistry to improve cellular activity.

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