Title: Campaign to Identify Agonists of Transient Receptor Potential Channels 3 and 2 (TRPML3 & TRPML2)

Authors: Saldanha SA[1], Grimm C[2], Mercer BA[1], Choi JY[3], Allais C[3], Roush WR[3], Heller S[2], Hodder P[1,4].

Affiliations:

1. Lead Identification Division, Translational Research Institute, Scripps Florida, 130 Scripps Way, Jupiter, FL 33458
2. Departments of Otolaryngology-Head and Neck Surgery, Stanford University School of Medicine, Stanford, CA 94305
3. Department of Chemistry, Scripps Florida, 130 Scripps Way, Jupiter, FL 33458
4. To whom correspondence should be addressed, hodderp@scripps.edu

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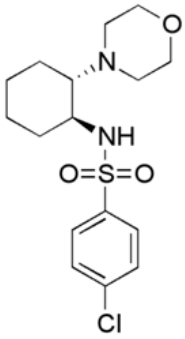
Screening Center Name & PI: Scripps Research Institute Molecular Screening Center (SRIMSC); Hugh Rosen

Chemistry Center Name & PI: SRIMSC; Hugh Rosen

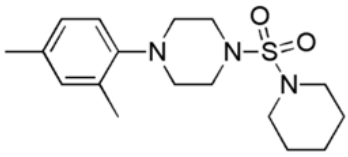
Assay Submitter & Institution: Stefan Heller, Stanford University

PubChem Summary Bioassay Identifier (AID): 1809

Probe 1 (ML123)
Secondary Arylsulfonamide Scaffold
(S, S)-trans isomer, CID1167615
racemate, SR-1984
original (Commercial), CID2911646



Probe 2 (ML122)
Sulfonarylpiperazine Scaffold
CID701237



CID/ ML	Target Name	EC50 (nM) [SID, AID]	Anti-target	EC50 (μM) [SID, AID]	Fold Selective	Secondary Assays: EC50 (nM) [SID, AID]
Probe 1: CID2911646/ ML123	TRPML3	451 nM [powder SID 85786753, AID 1809] 873 nM [SID 24801657, AID 1562]	TRPN1	14.0 μM powder SID85786753, AID 1809] > 29.9 μM SID24801657, AID 1682	>34-fold (TRPN1 Fluo8); 183-fold (YFP-HEK cell-patch clamp)	Inactive: hTRPML1, hTRPM2, mTRPV2, hTRPC3, drTRPN1, and hTRPA1
	TRPML2	54.5% ACT				
Probe 2: CID701237/ ML122	TRPML3	1430 nM [SID24787221, AID 1562] 2000 nM [powder SID85786752, AID 1809]	TRPN1	> 29.9 μM [SID24787221, AID 1682] > 29.9 μM [powder SID85786752, AID 1809]	>20-fold (TRPN1 Fluo8); 60-fold (YFP-HEK cell-patch clamp)	Inactive: hTRPML1, hTRPM2, mTRPV2, hTRPC3, drTRPN1, and hTRPA1
	TRPML2	23.5% ACT				

Abstract

We conducted a high-throughput screen for small molecule activators of the TRPML3 ion channel which, when mutated, causes deafness and pigmentation defects. Cheminformatics analyses of the 53 identified and confirmed compounds revealed nine different chemical scaffolds and 20 singletons. The two probes reported here [SID24801657 (ML123) and SID24787221 ML122)] exhibit potent EC_{50} values against TRPML3 (EC_{50} values of 0.873 µM and 1.43 µM), and activate TRPML2 (54.5%ACT and 23.5% ACT). Importantly, these probes induce a robust increase in intracellular calcium levels, assessed using fluorescence-based Fura-2 calcium assays and patch clamp studies performed by the assay provider, demonstrating their biological value. These probes exhibit no detectable activity against the TRPN1 channel or the YFP-HEK parental cell line, and are inactive against hTRPML1, hTRPM2, mTRPV2, hTRPC3, drTRPN1, and hTRPA1 ion channels. Additionally, these probes are inactive in other ion channel assays including TRPN1, (PubChem AID 1682), the X11L calcium channel (AID 2073), and the ROM K+ channel (AID 1918). We found that agonists strongly potentiated TRPML3 activation with low extracytosolic [Na^+]. This synergism revealed existence of distinct and cooperative activation mechanisms, and a wide dynamic range of TRPML3 activity. Testing compounds on TRPML3-expressing sensory hair cells revealed absence of activator-responsive channels. Epidermal melanocytes showed only weak or no responses to the compounds. These studies validate the selectivity and biological relevance of these probes. Due to the lack of available compounds known to act as TRPML3/TRPML2 agonists (there is no prior art), these compounds are valuable tools for elucidating the functions of these ion channels. These results suggest that TRPML3 in native cells might be absent from the plasma membrane or that the protein is a subunit of heteromeric channels that are non-responsive to the activators identified in this screen.

Recommendations for scientific use of the probe (ML118):

Limitations in state of the art. There are currently no agonists for TRPML subfamily.

Probe Applications. These probes are useful for assays aiming to increase TRPML3 and TRPML2 channel activities, without activating hTRPML1, hTRPM2, mTRPV2, hTRPC3, drTRPN1, and hTRPA1 ion channels. The selectivity and potency of these compounds will enable further investigations into the biological and biochemical roles of TRPML3 and TRPML2. As suggested by the assay provider, compounds which modulate TRPML3 and TRPML2, such as the probes described here, represent potential starting points for the development of therapies for vertigo or tinnitus. Importantly, the probes have already been used to reveal that TRPML3 in native cells is differently configured than when overexpressed.

*Expected end-users of the probe in the research community***.** The probes can be used by academic researchers studying ion channel biology, hair cell, vertigo, and hearing loss. Scientists in diverse fields will be able to apply these chemical probes to elucidate the roles of TRPML3 and TRPML2 in these cellular pathways.

Relevant biology of the probe. The transient receptor potential (TRP) ion channel subunit genes were first defined in the Drosophila visual system. The function of TRP channels is to depolarize cells and raise intracellular Na^+ and Ca^{2+} levels. The goal of this probe development project was to identify small molecules that act as agonists of TRP ion channels.

1 Introduction

TRPML3 (mucolipin 3; MCOLN3) is a TRP channel expressed in inner ear hair cells and stereocilia [1-3], suggesting it may play a role in hearing and mechanotransduction. Reports that mice with mutations in TRPML3 (known as varitint-waddler mutants) exhibit early-onset hearing loss accompanied by head-bobbing and circling behaviors [4-6], provided further support for a role of TRPML3 in hearing and vestibular function.

TRPML2 is a human cation channel, previously known as Mucolipin 2, and is encoded by the MCOLN2 gene [7]. TRPML2 is associated with the Arf6-regulated trafficking pathway and is involved in the intracellular transport of membranes and membrane proteins [8], including TRPML3 [9]. As a result, the identification of probes for TRPML3 and TRPML2 would be useful to investigate the function of TRPML3 in inner ear mechanotransduction and hearing biology, as well as elucidate pathways of intracellular transport of membrane proteins. There are currently no agonists for TRPML2 or TRPML3 [10].

Selective ligands and activators are playing major roles in characterizing the physiological roles of TRP channels. In contrast to many TRP channels, there are no agonists known for members of the TRPML subfamily. This TRPML3 agonist HTS campaign has led to the identification of the first small molecule TRPML3 activators, providing an unparalleled set of novel tools to study the biophysical and physiological features of TRPML3. Maximal activation capabilities for the identified TRPML3 agonists was up to 2-fold higher than the activity observed for a constitutively active TRPML3 *varitint-waddler* mutant channel isoform. Recent research has identified the tight regulation of TRPML3 by dynamin-dependent endocytosis and altered trafficking through heteromerization with TRPML family members. TRPML2 and TRPML3 are known to co-localize in cells and homo- and hetero-multimerization of these channels may modulate channel function. The Heller Lab (Stanford University) is utilizing these newly discovered probes **(SID 24801657 and SID 24787221)** to study possible mechanisms of regulation for TRPML3. In particular the involvement of other TRPML family members such as TRPML2 can be better understood with small molecule modulators with differential selectivity. Towards this end TRPML3/TRPML2 targeted agonists will provide a unique tool for determining the role of these family members in functional hetero-multimerization. Tests with TRPML family-expressing sensory hair cells and epidermal melanocytes are already revealing that activator-responsive TRPML3 channels are tightly controlled in the plasma membrane of cells natively expressing the channel (publication in process).

PubChem BioAssay Table

AID	Assay Name	Assay Type	Target	Powder	Concentration
1448	Primary biochemical HTS assay for agonists of Transient Receptor Potential Channel ML3 (TRPML3) activity.	Primary Assay (1X%ACT)	TRPML3	No	3 µM
1424	Primary biochemical HTS assay for agonists of TRPN1 activity.	Counterscreen (1X%ACT)	TRPN1	No	3 µM
1526	Confirmation biochemical assay for agonists of TRPML3 activity	Confirmation Assay (3X %ACT)	TRPML3	No	3 µM
1525	Fluorescence counterscreen assay for TRPML3 agonists: cell-based HTS assay to identify agonists of TRPN1	Counterscreen (3X%ACT)	TRPN1	No	3 µM
1562	Dose-response biochemical assay for agonists of TRPML3 activity.	Dose Response (3X EC50)	TRPML3	No	10-point, 1:3 dilution starting at 30 µM
1682	Fluorescence counterscreen assay for TRPML3 agonists: dose response cell-based HTS assay to identify agonists of TRPML3 activity.	Dose Response Counterscreen (3X EC50)	TRPN1	No	10-point, 1:3 dilution starting at 30 µM
1809	Summary of probe development efforts to identify agonists of TRPML3.	Summary	TRPML2,3	No	N/A
2116	Late stage results from the probe development efforts to identify inhibitors of TRPML3 and TRPML2.	Late Stage	TRPML2,3	Yes	10 µM

Table of Assay Rationale and Description				
AID	**Assay Rationale**	**Assay Description**	**Z'**	**S:B**
1448	The purpose of this assay is to identify compounds that activate TRPML3.	This assay employs a HEK293 cell line that stably expresses human TRPML3-YFP cation channel. Cells are treated with compounds followed by measurement of intracellular calcium as monitored by a fluorescent, cell permeable calcium indicator dye. Compounds that act as TRPML3 agonists will increase calcium mobilization, resulting in increased relative fluorescence of the indicator dye, and increased well fluorescence.	0.71 +/- 0.09	3.06 +/- 0.17
1424	The purpose of this assay is to identify compounds that activate TRPN1.	Same as AID 1448, except that the assay employs HEK TRPN1-YFP cells.	0.73 +/- 0.15	2.02 +/- 0.13
1526	The purpose of this assay is to confirm activity of compounds active in AID 1448.	Same as AID 1448, except compounds are tested in triplicate.	0.80 +/- 0.02	3.12 +/- 0.02
1525	The purpose of this assay is to determine whether compounds active in AID 1448 were nonselective due to activation of TRPN1.	Same as AID 1424, except compounds are tested in triplicate.	0.82 +/- 0.01	2.13 +/- 0.07
1562	The purpose of this assay is to determine dose response curves for compounds active in AID 1448.	Same as AID 1448, except compounds are tested in triplicate using a dilution series.	0.73 +/- 0.06	3.03 +/- 0.07
1682	The purpose of this assay is to determine whether compounds active in AID 1448 were nonselective due to activation of TRPN1.	Same as AID 1424, except compounds are tested in triplicate using a dilution series.	0.82 +/- 0.03	2.05 +/- 0.13
1809	Summarize probe development efforts.	N/A	N/A	N/A
2116	Describe the assays performed during the probe development effort.	Assays include TRPML3, TRPML2, assays, TRPN1 counterscreens, patch clamp assays, and Fura-2 profiling against various TRP ion channels of different species.	N/A	N/A

2.1 Assays

(Click on the hyperlinks to obtain itemized protocols directly from PubChem; also see Summary AID 1809 and reference [10])

TRPML3 Agonist Assays (PubChem AIDs 1448, 1526, 1562, and 2116)

The purpose of these assays is to identify compounds that act as agonists of the TRPML3 cation channel. These assays employ a HEK293 cell line that stably expresses the human TRPML3-YFP cation channel. The cells are treated with test compounds followed by measurement of intracellular calcium as monitored by the fluorescent, cell permeable calcium indicator dye Fluo-8. As designed, compounds that act as TRPML3 agonists will increase calcium mobilization, resulting in increased relative fluorescence of the indicator dye, and thus increase well fluorescence. Compounds were tested in singlicate (*AID 1448*) and in triplicate (AID *1526*) at a nominal test concentration of 3 µM, and in triplicate using a 10-point, 1:3 dilution series starting at a nominal concentration of 29.9 micromolar (AID *1562* and *2116*).

TRPN1 Agonist Assays (PubChem AIDs 1424, 1525, 1682, and 2116)

The purpose of these assays is to identify test compounds that act as agonists of the TRPN1 cation channel. These assays also serve as counterscreen to identify compounds that are nonselective TRP agonists due to activation of TRPN1. These assays employ a HEK293 cell line that stably expresses the zebrafish TRPN1-YFP cation channel. The cells are treated with test compounds followed by measurement of intracellular calcium as monitored by the fluorescent, cell permeable calcium indicator dye Fluo-8. As designed, compounds that act as TRPN1 agonists will increase calcium mobilization, resulting in increased relative fluorescence of the indicator dye, and thus increase well fluorescence. Compounds were tested in singlicate (*AID 1424*) and in triplicate (AID *1525*) at a nominal test concentration of 3 µM, and in triplicate using a 10-point, 1:3 dilution series starting at a nominal concentration of 29.9 micromolar (AID *1682* and *2116*).

Patch Clamp Assays (PubChem AID 2116)

The purpose of these assays is to determine if test compounds can increase current recordings in TRPML3 ion channels (Assay 3) or the YFP-HEK parental background (Assay 4). Whole-cell currents were recorded with an Alembic Instruments VE-2 amplifier with 100% series resistance compensation, and acquired with JClamp software. The standard bath solution contained (in mM) 138 NaCl, 5.4 KCl, 2 MgCl2, 2 CaCl2, 10 HEPES, and 10 d-glucose, adjusted to pH 7.4 with NaOH. The standard pipette solution contained (in mM) 140 CsCl, 10 HEPES, 3 ATP-Na, 1 BAPTA, and 2 MgCl2, adjusted to pH 7.2. 100 µM 2-Aminoethyl-diphenyl borate was included in the bath solution to block gap junctions and had no effect on the expressed channels. Channel responses were plotted to 10 ms voltage steps (holding potential = +10 mV) between -200 mV and +100 mV in 20 mV incremental steps, normalized by cell capacitance (pF). Compounds were tested at 10 micromolar. These assays were performed by the assay provider.

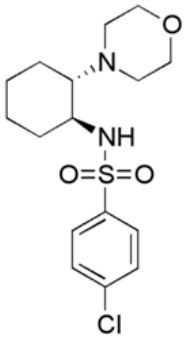
Fura-2 Assays (PubChem AID 2116)

The purpose of these assays is to determine whether compounds identified as probe candidates are able to increase whole cell Ca2+ influx in HEK293 cells transfected with human TRPML3, other human, or murine (m) TRP channels, or zebrafish TRPN1. In this assay cells transiently expressing channels or YFP control plasmid are perfused with test compound, followed by measurement of intracellular [Ca2+] for 2 minutes with the fluorescent indicator fura-2-AM. Compounds are added to cells 20-25 hours after transfection. Values are reported as mean values +/- SEM (n >= 3 independent experiments with 20-30 cells). The % activation values for TRPML2 in the SAR tables were calculated by normalizing the TRPML2 response ratios to TRPML3 response ratios. Compounds are tested at 10 micromolar. These assays were performed by the assay provider.

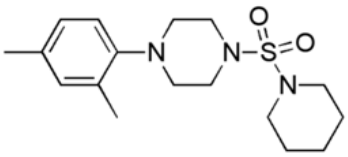
2.2 Probe Chemical Characterization

Probe chemical structure including stereochemistry. *Separation of diastereomers (if necessary)*.
The structures of probes ML122 and ML123 are shown below. Because the stereochemistry of ML123 was not clearly defined in the information provided from commercial suppliers, we synthesized the stereoisomers of ML123 and determined that the most active isomer is the trans-S,S-isomer of ML123, as discussed in this report.

Probe 1 (ML123)
Secondary Arylsulfonamide Scaffold
(S, S)-trans isomer, CID1167615
racemate, SR-1984
original (Commercial), CID2911646



Probe 2 (ML122)
Sulfonarylpiperazine Scaffold
CID701237

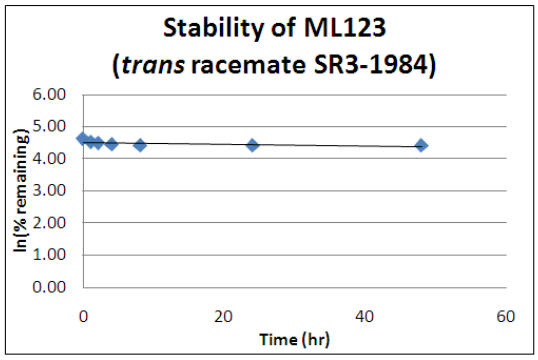


Structure verification with 1H NMR and LCMS results. See next section.

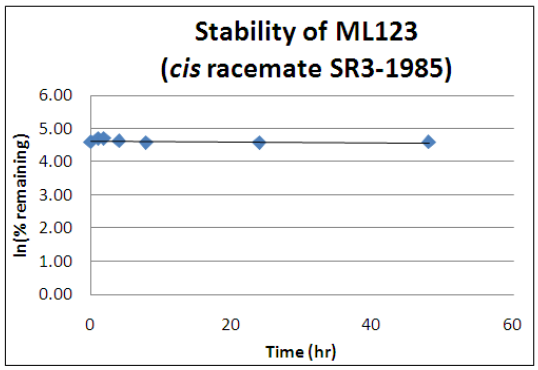
Solubility. The solubility of the probes was measured in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic and a pH of 7.4) at room temperature (23°C). The solubility of probe ML122 and ML123 and the associated stereomers are shown below.

Stability. The stability of the probes was measured at room temperature (23ºC) in PBS (no antioxidants or other protectants; DMSO concentration below 0.1%). The stability, represented by the half-life, was found to be > 48 hours. Below is a graph showing loss of compound with time over a 48 hour period with a minimum of 6 time points. The table indicates the percent of compound remaining at the end of the 48 hours.

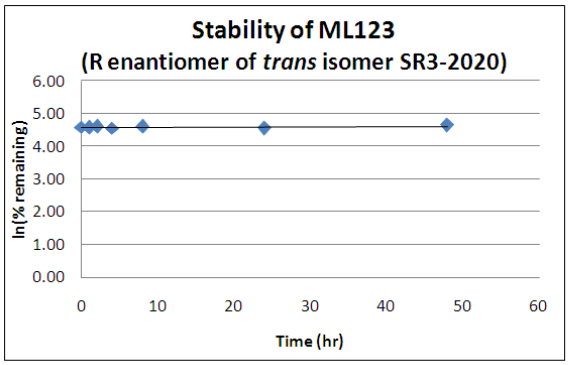
Probe	SR Number (Stereomer)	SID	CID	Solubility in PBS (µM)	Michael Acceptor 100 µM GSH trap	Stability in PBS t1/2 (hr)
ML123	SR3-1984 (*trans* Racemate)	None	None	*49*	No	> 48 hr
ML123	SR3-1985 (*cis* Racemate)	None	None	*121*	No	> 48 hr
ML123	SR3-2020 (*trans* isomer R Enantiomer)	104223082	1167614	*22*	No	> 48 hr
ML123	SR3-2021 (*trans* isomer S Enantiomer)	104223083	1167615	*28*	No	> 48 hr
ML122	SR3-2103 (Resynthesis)	113584823	701237	3.2	No	> 48 hr



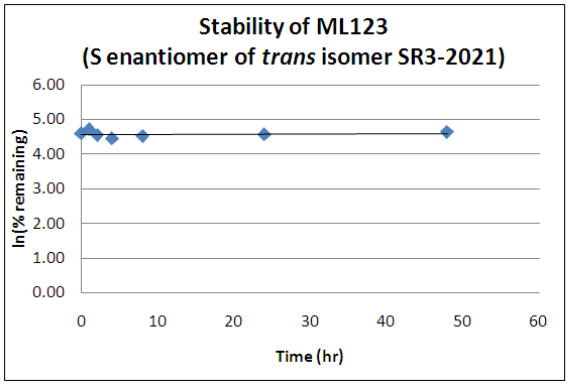
SR3-1984 Stability in PBS Buffer (pH 7.4)		
Sample concentration: 10 µM		
Storage Microcentrifuge Tube on Lab Bench		
Time (hr)	% remaining	ln(%remaining)
0	100	4.61
1	91	4.51
2	88	4.48
4	86	4.46
8	84	4.43
24	83	4.42
48	83	4.42



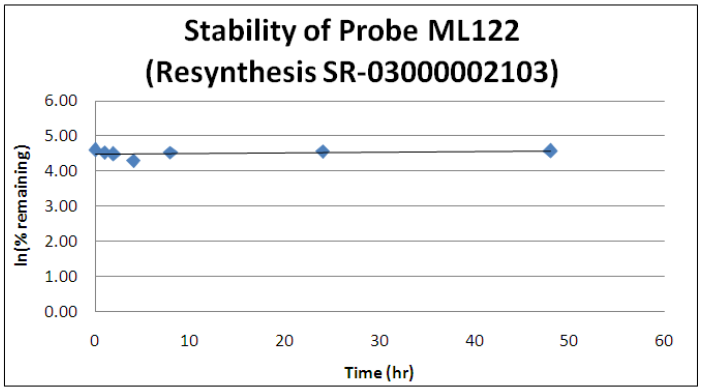
SR3-1985 Stability in PBS Buffer (pH 7.4)		
Sample concentration: 10 µM		
Storage Microcentrifuge Tube on Lab Bench		
Time (hr)	% remaining	ln(%remaining)
0	100	4.61
1	112	4.72
2	111	4.71
4	105	4.65
8	96	4.56
24	98	4.58
48	101	4.62



SR3-2020 Stability in PBS Buffer (pH 7.4)		
Sample concentration: 6 µM		
Storage Microcentrifuge Tube on Lab Bench		
Time (hr)	% remaining	ln(%remaining)
0	100	4.61
1	98	4.58
2	101	4.62
4	97	4.57
8	101	4.62
24	95	4.55
48	105	4.65



SR3-2021 Stability in PBS Buffer (pH 7.4)		
Sample concentration: 10 µM		
Storage Microcentrifuge Tube on Lab Bench		
Time (hr)	% remaining	ln(%remaining)
0	100	4.61
1	112	4.72
2	93	4.53
4	85	4.44
8	91	4.51
24	95	4.55
48	103	4.63



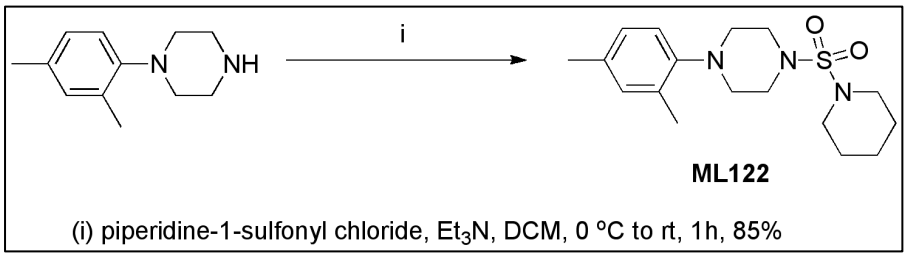
SR-03000002103 Stability in PBS Buffer (pH 7.4)		
Sample concentration: 6 µM		
Storage Microcentrifuge Tube on Lab Bench		
Time (hr)	% remaining	ln(%remaining)
0	100.0	4.61
1	92.3	4.52
2	88.7	4.49
4	73.2	4.29
8	92.9	4.53
24	95.2	4.56
48	97.0	4.57

The probes were measured for their ability to form glutathione adducts. At concentrations of 100 µM reduced GSH, 10 µM of the probes do not appear to react with glutathione. These compounds do not have functionality that would render them to be Michael acceptors [11, 12].

2.3 Probe Preparation

Synthetic route for probes ***ML122 and ML123***. Detailed experimental procedures for the synthesis of probes are below.

Synthesis and Characterization of Probe ML122. Probe **ML122** was synthesized by acylation of N-(2,4-dimethylphenyl)-piperdine as shown in the following equation.



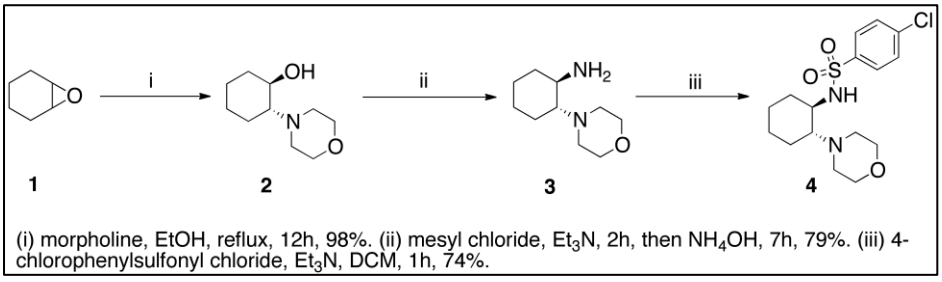
(i) piperidine-1-sulfonyl chloride, Et_3N, DCM, 0 °C to rt, 1h, 85%

1-(2,4-Dimethylphenyl)-4-(piperidin-1-ylsulfonyl)piperazine (ML122, SID113584823, CID701237) To a solution of commercially available N-(2,4-dimethylphenyl)-piperdine (0.22 g, 1.15 mmol) and triethylamine (0.5 mL) in CH_2Cl_2 (5 mL) was added piperidine-1-sulfonyl chloride (ca. 2.0 mmol) at 0 °C. The mixture was stirred for 15 min, then was warmed to room temperature and stirred for an additional hour. The reaction mixture was diluted with dichloromethane (60 mL). The organic layer was separated and was washed with saturated aq. $NaHCO_3$ (20 mL) and brine (20 mL). The combined organic extracts were dried over magnesium sulfate, filtered, and concentrated *in vacuo*. Purification of the crude product by flash chromatography eluting with a step gradient ranging from 6% to 50% EtOAc-hexane provided 0.33 g (0.990 mmol, 85%) of **ML122** as a viscous oil; purity >98% by LCMS; IR (neat) 2939, 2853, 1713, 1500, 1445, 1361, 1336, 1262, 1220, 1161, 1143, 1115,1052, 926, 815, 735 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.00 (s, 1H), 6.97 (d, *J* = 8.0 Hz, 1H), 6.89 (d, *J* = 8.0 Hz, 1H), 3.36 (t, *J* = 4.8 Hz, 4H), 3.25 (t, *J* = 4.8 Hz, 4H), 2.90 (t, *J* = 4.8 Hz, 4H), 2.26 (s, 3H), 2.26 (s, 3H), 1.66-1.54 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 139.11, 138.38, 129.26, 128.67, 67.01, 63.62, 49.71, 48.75, 28.48, 24.59, 24.17, 18.76; MS (ESI) 338 *m/z* $[M+H]^+$; HRMS (ESI) 338.1910 *m/z* $[M+H]^+$, 360.1713 *m/z* $[M+Na]^+$, 697.3569 *m/z* $[2M+Na]^+$; calc. 338.1897 $[M+H]^+$, 360.1716 $[M+Na]^+$, 697.3540 $[2M+Na]^+$.

Synthesis of ML123 and Identification of the Stereochemistry of the Probe

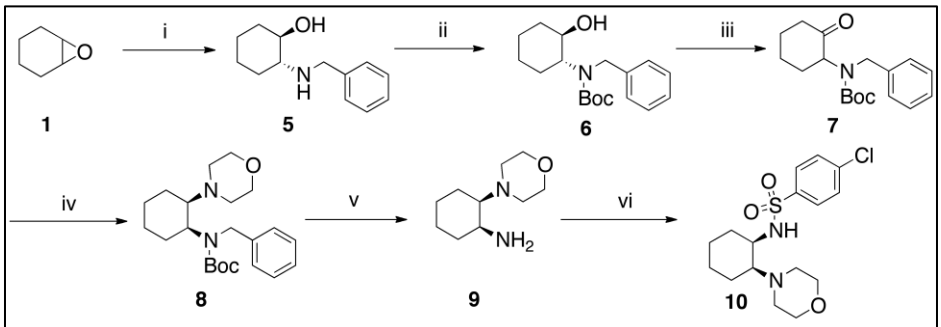
Stereochemistry of the probe, **ML123**, was not specified by the original commercial supplier of samples identified from the HTS. Four possible stereoisomers exist: the racemic trans diastereomer (**4**) and the racemic cis diastereomer (**10**). Samples of the racemic **4** and **10** (both as 1:1 mixtures of enantiomers) were synthesized by the routes summarized below.

Synthesis of Racemic 4 (SR-03000001984)—Racemic Trans isomer. This synthesis involved ring-opening of cyclohexene oxide **1** with morpholine to give **2**. Activation of the hydroxyl group of **2** as a mesylate and then displacement with ammonium hydroxide—via an azidinium ion—provided **3**. Acylation of racemic **3** with p-chlorobenzenesulfonyl chloride then provided racemic **4** (**SR-03000001984**).



(i) morpholine, EtOH, reflux, 12h, 98%. (ii) mesyl chloride, Et_3N, 2h, then NH_4OH, 7h, 79%. (iii) 4-chlorophenylsulfonyl chloride, Et_3N, DCM, 1h, 74%.

Synthesis of Racemic 10 (SR-03000001985)—Racemic Cis isomer. The synthesis of racemic **10** (**SR-03000001985**) was accomplished starting with the reaction of cyclohexene oxide **1** with benzylamine to give **5**. Protection of the secondary amine as the Boc carbamate **6** and then oxidation of the hydroxyl group gave ketone **7**. Reductive amination of **7** with morpholine gave the cis-diamine derivative **8**. Deprotection of the Boc and N-benzyl groups gave the cis diamine derivative **9**, which was then acylated with p-chlorobenzenesulfonyl chloride which provided racemic **10** (**SR-03000001985**).

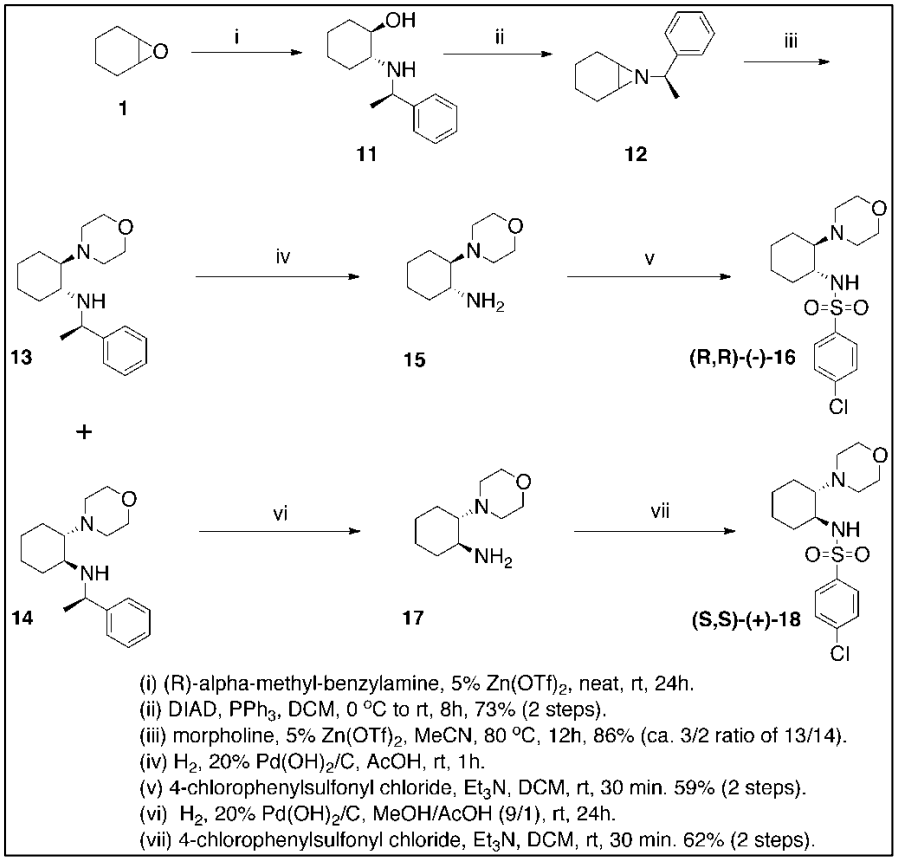


(i) Benzylamine 3% $Zn(OTf)_2$, rt, 24h. (ii) $(Boc)_2O$, DCM, rt, 11h, 85% (2 steps). (iii) PCC, DCM, rt, 24h, 96%. (iv) morpholine, 6% p-toluenesulfonic acid, benzene, 110 ^oC, 36h, then H_2, Pd/C, THF, rt, 48 h, 53%. (v) TFA, 1h, then H_2, Pd/C MeOH, 3h. (vi) 4-chlorophenylsulfonyl chloride, Et_3N, DCM, 1h, 11% over 2 steps.

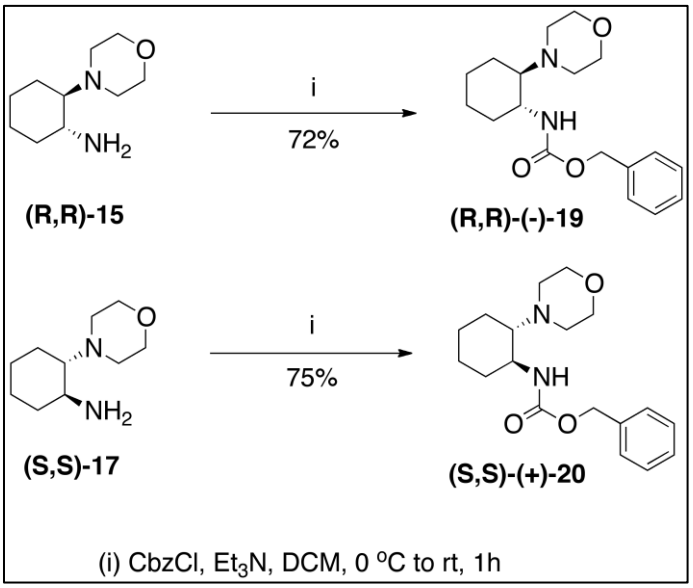
Because several of the TRPML3/2 agonist probe analogs—such as analogs 1-5—are ortho-substituted aniline sulfonamide derivatives, and because the racemic trans diastereomer **4** of **ML123** (but not the cis diastereomer **10**) maps best onto the structures of analogs 1-5, we surmised that the ML123 probe would have the trans stereochemistry. This assumption was verified by the results of testing of **4** and **10**, which demonstrated that **4** is about 10-fold more active than **10** in the TRPML3/2 assay. Therefore, we proceeded to determine the structure of the most active enantiomer by the synthesis summarized below.

Synthesis of Single Trans Enantiomers 16 (SR-03000002020) and 18 (SR-03000002021). This synthesis was accomplished by treatment of cyclohexene oxide **1** with (R)-a-methyl-benzylamine. Conversion of **11** to the aziridine **12** set the stage for ring opening of the aziridine with morpholine. This reaction gave a

ca. 3:2 mixture of the diastereomeric diamines **13** and **14** which were separated chromatographically. Debenzylation of **13** gave the (R,R)-**15**, whereas debenzylation of **14** gave the enantiomeric diamine, (S,S**)-17**. Acylation of **15** and **17** with p-chlorobenzenesulfonyl chloride then provided (R,R)-(-)-**16 (SR-03000002020)** and (S,S)-(+)-**18 (SR-03000002021)**, respectively.



Finally, the absolute stereochemistry of the primary amine intermediates **15** and **17** was verified by conversion of each to the corresponding Cbz derivatives (R,R)-(-)-**19** and (S,S)-(+)-**20**, respectively. The optical rotation obtained for (S,S)-(+)-**20** ($[\alpha]_D^{28}$ = +37.9 (*c* = 1.0 in $CHCl_3$)), but not for (R,R)-(-)-**20** ($[\alpha]_D^{28}$ = -35.7 (*c* = 1.0 in $CHCl_3$)), was in close agreement with the value reported in the literature for this compound ($[\alpha]_D^{28}$ = +45.7 (*c* = 1.0 in $CHCl_3$); [13]



Detailed experimental procedures for synthesis of Probe ML123 and Stereoisomers

***trans*-2-Morpholinocyclohexanol (2)**. To a solution of cyclohexene oxide (5.0 mL, 49.5 mmol) in anhydrous ethanol (25 mL) was added morpholine (7.0 mL, 79.3 mmol) at room temperature. The reaction mixture was stirred at 100 ^{o}C for 12 hours. After being cooled to ambient temperature, the solvent was removed under reduced pressure. Purification of the residue by flash chromatography eluting with a step gradient ranging from 0% to 20% MeOH-EtOAc provided 9.0 g (48.6 mmol, 98%) of **2** as a viscous oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.02 (d, *J* = 2.4 Hz, 1H), 3.60-3.52 (m, 4H), 3.38-3.31 (m, 1H), 2.63-2.40 (m, 4H), 2.13-2.07 (m, 1H), 1.91-1.87 (m, 1H), 1.74-1.66 (m, 2H), 1.60-1.58 (m, 1H), 1.19-1.07 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 69.29, 68.20, 66.77, 48.72, 33.91, 24.89, 23.74, 23.22; MS (ESI) 186 *m/z* $[M+H]^+$.

***trans*-2-Morpholinocyclohexanamine (3)**. To a solution of **2** (2.3 g, 12.5 mmol) and triethylamine (2.6 mL, 18.6 mmol) in dry ether (50 mL) was added methanesulfonyl chloride (1.2 mL, 15.4 mmol) dropwise at 0 ^{o}C. A white precipitate formed, and the reaction mixture was stirred at room temperature for 2 hours. After completion of the mesylation step (monitored by LCMS for the absence of 186 *m/z* $[M+H]^+$), NH_4OH (50 mL) was added. The reaction mixture was vigorously stirred at ambient temperature for additional 7 hours. The reaction mixture was then diluted with ether (50 mL) and washed with saturated aq. $NaHCO_3$ (30 mL) and brine (30 mL). The organic layer was dried over magnesium sulfate, filtered, and concentrated *in vacuo*. Purification of the crude product by flash chromatography using a SNAP KP-NH Cartridge by eluting with a step gradient ranging from 12% to 100% EtOAc-hexane provided 1.8 g (9.92 mmol, 79%) of **3** as a viscous oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.70-3.60 (m, 4H), 2.67-2.55 (m, 3H), 2.39-2.34 (m, 2H), 2.01-1.91 (m, 2H), 1.79-1.74 (m, 2H), 1.65-1.60 (m, 3H), 1.22-1.02 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 70.98, 67.90, 50.65, 49.01, 35.30, 25.98, 25.12, 22.85; MS (ESI) 185 *m/z* $[M+H]^+$.

***trans*-4-Chloro-N-(trans-2-morpholinocyclohexyl)benzenesulfonamide (racemic-4, ML123, SR-0300001984-1)**. To a solution of **3** (0.11g, 0.60 mmol) and triethylamine (0.12 mL, 0.90 mmol) in dry CH_2Cl_2 (10 mL) was added 4-chlorophenylsulfonyl chloride (0.15 g, 0.72 mmol) at room temperature. The reaction mixture was stirred for 1 hour at ambient temperature. The solvent was removed under reduced pressure, and the crude product was diluted with ethyl acetate (50 mL). The organic layer was washed with saturated aq. $NaHCO_3$ (10 mL) and brine (10 mL). The organic layer was dried over magnesium sulfate, filtered, and concentrated *in vacuo*. Purification of the crude product by flash chromatography eluting with a step gradient ranging from 12% to 100% EtOAc-hexane provided a viscous oil. The oil crystallized at 0°C to yield 0.16 g (0.449 mmol, 74%) of **4**; purity >98% by LCMS; m. p. = 110 ^{o}C; IR (neat) 3236, 2942, 2861, 2817, 1588, 1479, 1451, 1333, 1166, 1070, 1013, 910, 827, 754 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (d, *J* = 8.4 Hz, 2H), 7.44 (d, *J* = 8.4 Hz, 2H), 5.97 (s, 1H), 3.54-3.45 (m, 4H), 2.72 (td, *J* = 10.4, 4.0 Hz, 1H), 2.35-2.31 (m, 1H), 2.19-2.09 (m, 5H), 1.80-1.71 (m, 2H), 1.62-1.60 (m, 1H), 1.24-0.98 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 139.15, 138.80, 129.39, 128.72, 67.28, 66.92, 53.33, 48.13, 32.95, 25.24, 24.19, 22.93; MS (ESI) 359 *m/z* $[M+H]^+$; HRMS (ESI) 359.1203 *m/z* $[M+H]^+$, 381.1027 *m/z* $[M+Na]^+$; calc. 359.1191 $[M+H]^+$, 381.1010 $[M+Na]^+$.

***trans*-2-(Benzylamino)cyclohexanol (5)**. To cyclohexene oxide (3 mL, 29.6 mmol) were added zinc trifluoromethanesulfonate (0.33 g, 3 mol%) and benzylamine (3 mL, 27.5 mmol) at room temperature. The reaction mixture was stirred neat at ambient temperature for 24 hour. The reaction mixture solidified, and the un-reacted reagents were removed under reduced pressure overnight. The crude solid product **5** (6.3 g, 30. 6 mmol) was directly used in the next step without further purification; MS (ESI) 206 *m/z* $[M+H]^+$.

***trans*-tert-Butyl benzyl(trans-2-hydroxycyclohexyl)carbamate (6)**. To a solution of crude **5** (6.3 g, 30.6 mmol) in dry CH_2Cl_2 (50 mL) was added di-tert-butyl dicarbonate (8.8 g, 40.2 mmol) at room temperature. The reaction mixture was stirred for 11 hours at ambient temperature. The mixture was diluted with CH_2Cl_2

(100 mL) and washed with water (30 mL) and brine (30 mL). The organic layer was dried over magnesium sulfate, filtered, and concentrated *in vacuo*. Purification of the crude product by flash chromatography eluting with a step gradient ranging from 12% to 100% EtOAc-hexane provided 7.1 g (23.4 mmol, 85% over 2 steps) of **6** as a viscous oil; ^{1}H NMR (400 MHz, DMSO-d_6, Temp = 60 ^{o}C) δ 7.30-7.18 (m, 5H), 4.44-4.35 (m, 2H), 4.23 (d, *J* = 5.2 Hz, 1H), 3.60 (septet, *J* = 4.8 Hz, 1H), 3.48 (br, 1H), 1.94-1.91 (m, 1H), 1.57-1.53 (m, 3H), 1.36 (s, 10H), 1.24-1.09 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 157.09, 140.00, 128.41, 126.86, 80.17, 71.15, 62.07, 47.43, 35.14, 29.76, 28.36, 25.39, 24.38; MS (ESI) 206 (Boc decomposition) *m/z* $[M+H]^+$.

tert-butyl Benzyl(2-oxocyclohexyl)carbamate (7). To a solution of **6** (7.1 g, 23.4 mmol) in dry CH_2Cl_2 (100 mL) was added pyridinium chlorochromate (PCC) (8.1 g, 37.6 mmol), and the reaction mixture was stirred at room temperature for 24 hours. After completion of the reaction (monitored by TLC), the mixture was diluted with ether and filtered through a pad of Celite. The solvent was removed under reduced pressure. Purification of the crude product by flash chromatography eluting with a step gradient ranging from 6% to 50% EtOAc-hexane provided 6.8 g (22.4 mmol, 96%) of **7** as a viscous oil; ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.32-7.19 (m, 5H), 4.57-4.47 (m, 1H), 4.36 (q, *J* = 1.6 Hz, 1H), 4.12-4.02 (m, 1H), 2.41-2.29 (m, 2H), 1.99-1.53 (m, 6H), 1.31 (d, *J* = 41.2 Hz, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 207.37, 156.30, 140.29, 128.38, 126.75, 126.59, 80.49, 77.43, 64.73, 49.67, 41.69, 32.49, 31.09, 28.43, 26.58, 25.07; MS (ESI) 204 (Boc decomposition) *m/z* $[M+H]^+$.

tert-butyl Benzyl(*cis*-2-morpholinocyclohexyl)carbamate (8). To a solution of **7** (0.41 g, 1.34 mmol) in dry benzene (10 mL) was added p-toluenesulfonic acid (17 mg, 0.089 mmol) at room temperature. The reaction mixture was stirred at 50 ^{o}C until all of acid had dissolved. Morpholine (0.15 mL, 1.73 mmol) was added to the reaction mixture, and the reaction mixture was refluxed at 100 ^{o}C for 3 hours. The solvent was removed by distillation. Additional morpholine (0.15 mL) and dry benzene (10 mL) was added to the reaction mixture, which was refluxed at 110 ^{o}C for 14 hours. The solvent was distilled at atmospheric pressure, and excess morpholine was removed under reduced pressure with a vacuum pump to give the crude enamine intermediate; MS (ESI) 373 *m/z* $[M+H]^+$. The crude enamine was dissolved in dry THF (10 mL), and 10% Pd/C (78.5 mg) was added. After removing air in the flask using a vacuum pump, hydrogen gas was introduced using a balloon. The reaction mixture was stirred for 48 hours at ambient temperature. Palladium on carbon was removed by filtration. The filtrate was evaporated to give a crude viscous oil, which was purified by flash chromatography using SNAP KP-NH Cartridge by eluting with a step gradient ranging from 6% to 50% EtOAc-hexane to yield **8** (0.27 g, 0.712 mmol, 53%) as a viscous oil; MS (ESI) 375 *m/z* $[M+H]^+$.

***cis*-2-Morpholinocyclohexanamine (9)**: To a solution of **8** (0.27 g, 0.712 mmol) in CH_2Cl_2 (4 mL) was added trifluoroacetic acid (4 mL) at room temperature. The reaction mixture was stirred at ambient temperature and monitored by mass spectrometer. The Boc-deprotection was complete after one hour, and the solvent and trifluoroacetic acid were removed under reduced pressure; MS (ESI) 275 *m/z* $[M+H]^+$. To the crude intermediate were added MeOH (10 mL) and 10% Pd/C (ca. 0.1 g), and the reaction mixture was stirred at ambient temperature. Air in the flask was removed using a vacuum pump, and hydrogen gas was introduced using a balloon. The reaction was monitored by mass spectrometer and after 3 hours, benzyl deprotection was complete. Palladium on carbon was removed by filtration. The filtrate evaporated to give a crude viscous oil **9**, which was directly used in the next step without further purification; MS (ESI) 185 *m/z* $[M+H]^+$.

***cis*-4-Chloro-N-(cis-2-morpholinocyclohexyl)benzenesulfonamide (10, SR-0300001985-1)**. Racemic cis-isomer **10** (29 mg, 80.7 µmol, 11% over 2 steps) was obtained as a white solid by following the procedure described for the synthesis of **4**; m. p. = 140 ^{o}C; IR (neat) 3171, 2928, 2867, 2806, 1584, 1449, 1392, 1370, 1329, 1170, 1113, 1093, 995, 754 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (dt, *J* = 8.8, 2.0 Hz, 2H), 7.46 (dt, *J* = 8.8, 2.0 Hz, 2H), 5.34 (s, 1H), 3.49 (t, *J* = 4.8, 4H), 3.35 (d, *J* = 3.2, 1H), 2.27-2.23 (m, 3H), 2.09-2.02 (m, 3H), 1.74 (d, *J* = 10.8, 2H), 1.46 (qd, *J* = 13.2, 3.2 Hz, 1H), 1.34-1.30 (m, 1H), 1.24-1.09 (m, 3H);

^{13}C NMR (100 MHz, $CDCl_3$) δ 148.56, 133.38, 132.76, 132.03, 127.26, 119.31, 51.80, 47.44, 47.01, 25.73, 24.00, 20.84, 17.75; MS (ESI) 359 *m/z* $[M+H]^+$; HRMS (ESI) 359.1199 *m/z* $[M+H]^+$, 381.1019 *m/z* $[M+Na]^+$; calc. 359.1191 $[M+H]^+$, 381.1010 $[M+Na]^+$.

***trans*-2-(((R)-1-Phenylethyl)amino)cyclohexanol (11)**. Compound **11** (7.3 g, 33.5 mmol) was obtained by following the procedure described for the synthesis of **5** using **1** (3 mL, 29.7 mmol), (R)-alpha-methylbenzylamine (4 mL, 31.4 mmol), and zinc trifluoromethanesulfonate (0.32 g, 3 mol%); MS (ESI) 220 *m/z* $[M+H]^+$.

7-((R)-1-Phenylethyl)-7-azabicyclo[4.1.0]heptanes (12). To a solution of **11** (ca. 30 mmol) in dry CH_2Cl_2 (30 mL) was added PPh_3 (9.5 g, 36.0 mmol) at room temperature. The reaction mixture was stirred until PPh_3 completely dissolved, then was cooled to 0 ^oC. Diisopropyl azodicarboxylate (DIAD, 7 mL, 36.0 mmol) was added dropwise to the reaction mixture. The mixture was warmed to ambient temperature and stirred for 8 hours. The solvent was removed under reduced pressure. The crude product was diluted with ethyl acetate (90 mL) and was washed with water (20 mL) and brine (20 mL). The organic layer was dried over magnesium sulfate, filtered, and concentrated *in vacuo*. Purification of the crude product by flash chromatography eluting with a step gradient ranging from 0% to 50% EtOAc-hexane provided 4.4 g (22.0 mmol, 73% over 2 steps) of **12** as a viscous oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 (d, *J* = 7.2 Hz, 2H), 7.30 (t, *J* = 7.2 Hz, 2H), 7.21 (t, *J* = 7.2 Hz, 1H), 2.43 (q, *J* = 6.8, 1H), 1.90-1.68 (m, 3H), 1.65-1.57 (m, 2H), 1.48-1.38 (m, 3H), 1.36 (d, *J* = 6.8, 3H), 1.22-1.10 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 145.93, 128.29, 126.88, 126.67, 70.04, 38.34, 38.05, 25.12, 24.82, 23.81, 20.94, 20.80; MS (ESI) 202 *m/z* $[M+H]^+$.

(1R,2R)-2-Morpholino-N-((R)-1-phenylethyl)cyclohexanamine (13) and **(1S,2S)-2-Morpholino-N-((R)-1-phenylethyl)cyclohexanamine (14)**. To a solution of **12** (1.5 g, 7.64 mmol) in dry CH_3CN were added zinc trifluoromethanesulfonate (0.13g, 5 mol%) and morpholine (0.8 mL, 9.25 mmol) at room temperature. The reaction mixture was stirred at 80 ^oC for 12 hours. The mixture was cooled to ambient temperature, then the solvent was removed by evaporation. The reaction mixture was diluted with ethyl acetate (80 mL) and washed with water (20 mL) and brine (20 mL). The organic layer was dried over magnesium sulfate, filtered, and concentrated *in vacuo*. Purification of the crude product by flash chromatography using a SNAP KP-NH Cartridge by eluting with a step gradient ranging from 0% to 50% EtOAc-hexane provided 1.2 g (4.29 mmol, 56%) of **13** as a white solid (R_f = 0.51 at 25% EtOAc/Hexane on KP-NH TLC plate) and 0.66 g (2.29 mmol, 30%) of **14** as a viscous oil (R_f = 0.39 at 25% EtOAc/Hexane on KP-NH TLC plate)

Data for **13**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.36 (d, *J* = 7.2 Hz, 2H), 7.29 (t, *J* = 7.2 Hz, 2H), 7.22-7.17 (m, 1H), 3.77-3.65 (m, 5H), 2.90 (s, 1H), 2.69-2.64 (m, 2H), 2.52-2.40 (m, 3H), 2.27-2.20 (m, 1H), 1.86-1.82 (m, 1H), 1.74-1.66 (m, 2H), 1.53-1.49 (m, 1H), 1.36 (d, *J* = 6.8 Hz, 3H), 1.23-1.00 (m, 3H), 0.93-0.87 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 147.73, 128.23, 126.54, 126.50, 67.82, 67.77, 57.62, 57.49, 48.60, 33.58, 25.60, 24.64, 24.41, 22.96; MS (ESI) 289 *m/z* $[M+H]^+$.

Data for **14**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32-7.28 (m, 2H), 7.24-7.20 (m, 3H), 3.77 (q, *J* = 6.8 Hz, 1H), 3.70-3.58 (m, 4H), 3.09 (br, 1H), 2.24-2.13 (m, 6H), 2.03 (td, J = 10.4, 4.4 Hz, 1H), 1.77-1.70 (m, 2H), 1.66-1.62 (m ,1H), 1.43 (d, *J* = 6.8 Hz, 3H), 1.19-0.93 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 146.33, 128.44, 126.96, 126.47, 67.87, 67.81, 53.95, 53.26, 48.67, 31.88, 25.73, 24.56, 24.53, 22.68; MS (ESI) 289 *m/z* $[M+H]^+$.

(1R,2R)-2-Morpholinocyclohexanamine (15). To a solution of **13** (16 mg, 56.5 μmol) in AcOH (10 mL) was added 20% $Pd(OH)_2/C$ (9.6 mg) at room temperature. After removing air in the flask using a vacuum pump, hydrogen gas was introduced using a balloon. The mixture was stirred for 5 minutes, the balloon was removed, and the reaction mixture was stirred for 1 hour at ambient temperature. Palladium on carbon was

removed by filtration through a Celite pad. The filtrate was evaporated to give a crude viscous oil **15**, which was directly used in the next step without further purification; MS (ESI) 185 *m/z* $[M+H]^+$.

4-chloro-N-((1R,2R)-2-Morpholinocyclohexyl)benzenesulfonamide (16, CID1167614, SID104223082, SR-0300002020). Compound **16** (12 mg, 33.4 µmol, 59% over 2 steps) was obtained as a white solid by following the procedure described for synthesis of **4**. The spectroscopic data (^1H, ^{13}C, and MS) of **16** are same to those of **4**; m.p. = 168 ^oC; $[\alpha]_D^{28}$ = -72.2 (*c* = 1.0 in $CHCl_3$). The purity of **16** was >98% based on LCMS.

(1S,2S)-2-Morpholinocyclohexanamine (17). To a solution of **14** (0.20 g, 0.695 mmol) in MeOH/AcOH (50 uL/5 mL) was added 20% $Pd(OH)_2/C$ (38 mg) at room temperature. After removing air in the flask using a vacuum pump, hydrogen gas was introduced using a balloon. The reaction mixture was stirred for 24 hour at ambient temperature. Palladium on carbon was removed by filtration through a Celite pad. The filtrate was collected and evaporated to give a crude viscous oil, which was directly used for the next step without further purification. The spectra data of **17** (^1H and MS) are same to those of **3**.

4-Chloro-N-((1S,2S)-2-morpholinocyclohexyl)benzenesulfonamide (18, CID1167615, SID104223083, SR-0300002021). Compound **18** (0.15 g, 0.428 mmol, 62% over 2 steps) was obtained as a white solid by following the procedure described for synthesis of **4**. The spectroscopic data (^1H, ^{13}C, and MS) for **18** are same to those of **4**; m.p. = 173 ^oC; $[\alpha]_D^{28}$ = +79.9 (*c* = 1.0 in $CHCl_3$). The purity of **18** was >98% based on LCMS.

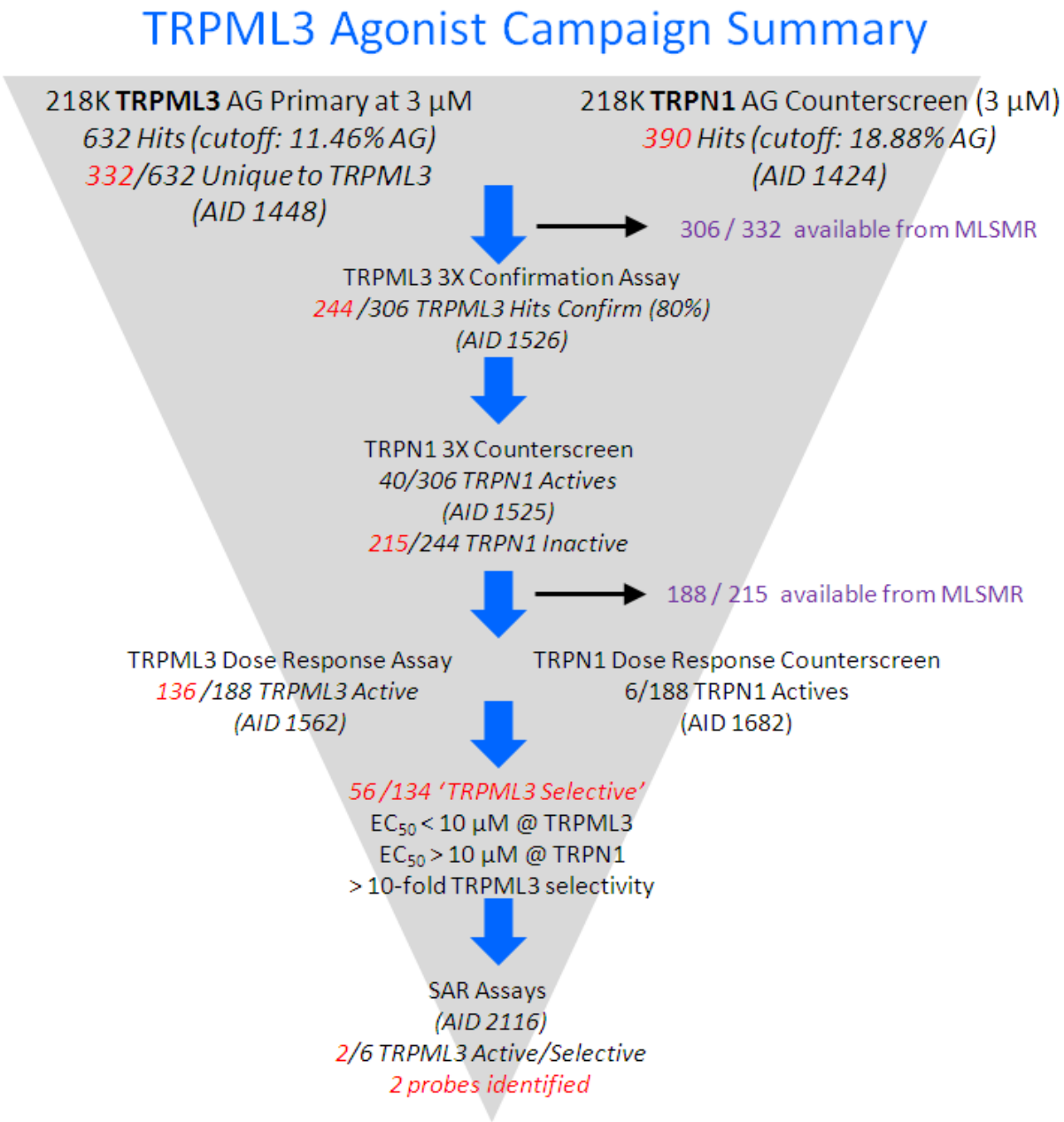
Benzyl ((1R, 2R)-2-Morpholinocyclohexyl)carbamate (19). To a solution of **15** (20 mg, 0.107 mmol) and triethylamine (0.1 mL) in dry CH_2Cl_2 (5 ml) was added benzyl chloroformate (0.02 mL) dropwise at 0 ^oC. After being stirred 15 minutes, the reaction mixture was warmed to room temperature and stirred for additional 1 hour. The reaction mixture was diluted with dichloromethane (40 mL) and washed with saturated aq. $NaHCO_3$ (10 mL) and brine (10 mL). The organic layer was dried over magnesium sulfate, filtered, and concentrated *in vacuo*. Purification of the crude product by flash chromatography eluting with a step gradient ranging from 12% to 100% EtOAc-hexane provided 24 mg (77 µmol, 72%) of **19** as a viscous oil: $[\alpha]_D^{28}$ = -35.7 (*c* = 1.0 in $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 7.35-7.28 (m, 5H), 5.36 (br, 1H), 5.08 (s, 2H), 3.66-3.55 (m, 4H), 3.31 (septet, *J* = 4.4 Hz, 1H), 2.65-2.60 (m, 2H), 2.43-2.41 (m, 1H), 2.35-2.30 (m, 2H), 2.15 (td, *J* = 10.8, 3.2 Hz, 1H), 1.88-1.77 (m, 2H), 1.68-1.62 (m, 1H), 1.28-1.03 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 156.77, 137.08, 128.71, 128.23, 128.22, 67.88, 67.69, 66.56, 51.47, 48.58, 33.28, 25.61, 24.79, 23.34; MS (ESI) 319 *m/z* $[M+H]^+$.

Benzyl ((1S, 2S)-2-Morpholinocyclohexyl)carbamate (20). Compound **20** (23 mg, 73 µmol, 75%) was obtained as a viscous oil by following the procedure described for synthesis of **19**: $[\alpha]_D^{28}$ = +37.9 (*c* = 1.0 in $CHCl_3$), (ref. $[\alpha]_D^{28}$ = +45.7 (*c* = 1.0 in $CHCl_3$) [13]; MS (ESI) 319 *m/z* $[M+H]^+$.

3 Results

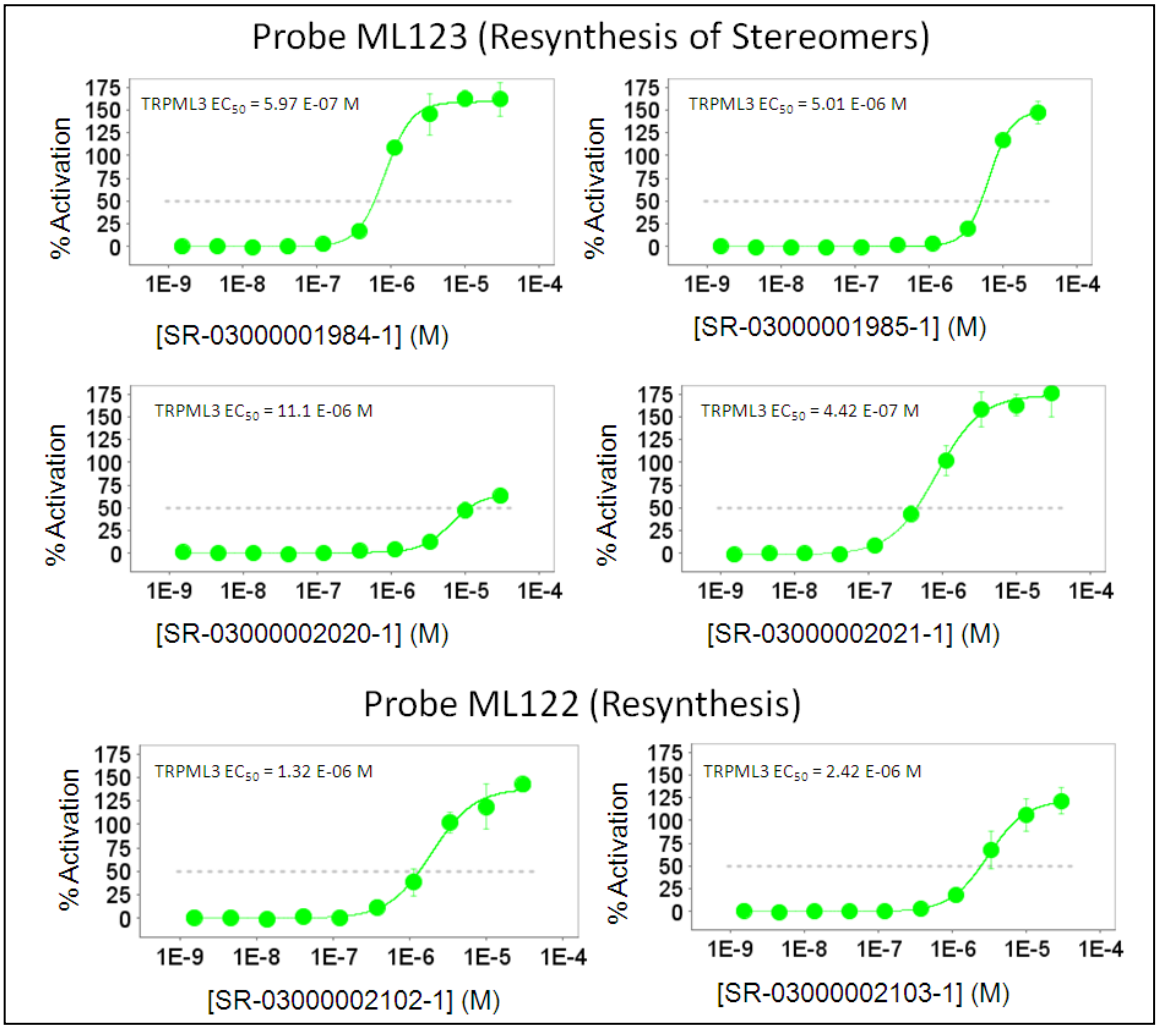
3.1 Summary of Screening Results

Following primary HTS in singlicate to identify TRPML3 agonists (AID 1448), counterscreening against TRPN1 to identify nonselective agonists in singlicate (AID 1424) and triplicate (1525), confirmation of TRPML3 activity in triplicate (AID 1526), titration assays in triplicate to determine potency (AID 1562) and selectivity (AID 1682), certain compounds were identified as possible candidates for probe development. Two HTS lead compounds belonging to the secondary arylsulfonamide and sulfonarylpiperazine scaffolds (SID 24801657 and SID 24787221), exhibited EC50 values of 0.873 µM and 1.43 µM, respectively. These compounds and related analogs were ordered as powders (SID 85786753 and SID 85786752) for retesting by the SRIMSC in TRPML3 dose response assays. The powder samples exhibited EC50 values consistent with the liquid samples: 0.451 µM and 2.00 µM, respectively. Next, the assay provider tested these two probe candidates and select analogs in TRPML3 patch clamp, TRPN1 patch clamp counterscreening, and Fura-2 imaging assays. Results of the assay provider Fura-2 assays revealed that the two probes have activity against both TRPML3 and TRPML2, and are inactive against hTRPML1, hTRPM2, mTRPV2, hTRPC3, drTRPN1, and hTRPA1 ion channels. As a result, two probes were identified. Probe compounds **SID 24801657** and **SID 24787221** were tested in more than 190 PubChem Bioassays and active only in 3 TRPML3 assays.



3.2 Dose Response Curves for Probes

The dose response curves shown below demonstrate that the racemic trans-isomer, SR-1984, is about 10-fold more potent than the racemic cis-isomer, SR-1985, and that the (S,S)-trans isomer of ML123 (e.g., SR-2021) is >10-fold more potent than the (R,R)-trans isomer SR-2020. Therefore, the most potent isomer of ML123 is SR-2021 (442 nM EC_{50}).

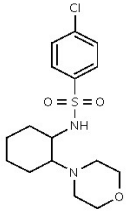
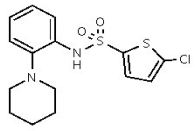
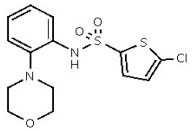
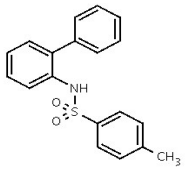
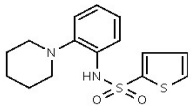
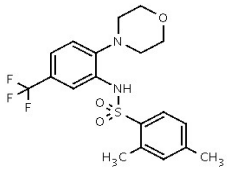


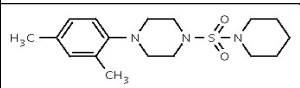
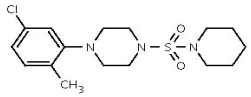
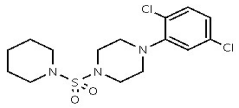
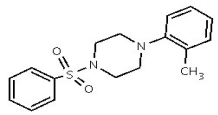
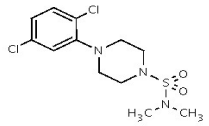
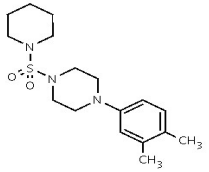
TRPML3 % Activation vs Agonist Concentration. TRPML3 Activation is shown in percentage, normalized to the maximum Carbachol response. EC_{50} values are reported as mean values (n = 3).

3.3 Scaffold/Moiety Chemical Liabilities

There are no known instability or chemical liabilities associated with probes ML122 or ML123.

3.4 SAR Tables

TRPML3/2 Agonist SAR Table 1: Secondary Arylsulfonamides Scaffold															
Compound Information							HTS Information		Probe Development Information						
Compound	**Structure**	**CID**	**SID**	**MLS**	**Vendor**	**Vendor Catalog ID**	**TRPML3 Dose Response (AID 1562) EC50, µM**	**TRPN1 Dose Response Counterscreen (AID 1682) EC50, µM**	**SID**	**TRPML3 Dose Response EC50, µM (AID 2116)**	**TRPN1 Dose Response EC50, µM (AID 2116)**	**TRPML3 Patch Clamp Assay (AID 2116)**	**TRPN1 Patch Clamp Assay (AID 2116)**	**TRPML3, TRPML2 Fura-2 Imaging (AID 2116)**	**Fura-2 Profiling Assays (AID 2116)**
Probe		2911646	24801657	MLS 000713 637	Asinex	BAS 03787124	Active (0.873)	Inactive (>29.9)	85786753	Active (0.451)	Inactive (14.0)	Active	Inactive	Active	Inactive in hTRPML1, hTRPM2, mTRPV2, hTRPC3, drTRPN1, and hTRPA1.
Analog 1		2111037	14746905	MLS 000570 381	Enamine	T5241132	Active (0.872)	Inactive (>29.9)	85786755	Active (0.624)	Inactive (>29.9)	Active	Inactive	Inactive	Inactive in hTRPML1, hTRPM2, mTRPV2, hTRPC3, drTRPN1, and hTRPA1.
Analog 2		2124908	3716245	MLS 000054 019	Enamine	T5252809	Active (1.36)	Inactive (>29.9)	85786754	Active (1.09)	Inactive (>29.9)	Active	Inactive	Inactive	Inactive in hTRPML1, hTRPM2, mTRPV2, hTRPC3, drTRPN1, and hTRPA1.
Analog 3		818647	17414375	MLS 000575 428	Chem Bridge	5182458	Active (1.59)	Inactive (>29.9)	85786756	Active (2.14)	Inactive (>29.9)	Active	Inactive	Inactive	Inactive in hTRPML1, hTRPM2, mTRPV2, hTRPC3, drTRPN1, and hTRPA1.
Analog 4		2814040	26731263	MLS 000850 372	Maybridge	HTS 08158	Active (2.3)	Inactive (>29.9)	Not tested due to lower target potency in AID 1562, compared to the probe.						
Analog 5		2078255	22405272	MLS 000394 056	Enamine	T5268454	Active (2.48)	Inactive (>29.9)							

TRPML3/2 Agonist SAR Table 2: Sulfonylaryl piperazines Scaffold															
Compound Information							**HTS Information**		**Probe Development Information**						
Compound	**Structure**	**CID**	**SID**	**MLS**	**Vendor**	**Vendor Catalog ID**	**TRPML3 Dose Response (AID 1562) EC50, µM**	**TRPN1 Dose Response Counterscreen (AID 1682) EC50, µM**	**SID**	**TRPML3 Dose Response EC50, µM (AID 2116)**	**TRPN1 Dose Response EC50, µM (AID 2116)**	**TRPML3 Patch Clamp Assay (AID 2116)**	**TRPN1 Patch Clamp Assay (AID 2116)**	**TRPML3, TRPML2 Fura-2 Imaging (AID 2116)**	**Fura-2 Profiling Assays (AID 2116)**
Probe		701237	24787221	MLS 000715 887	Asinex	BAS 08196462	Active (1.43)	Inactive (>29.9)	85786752	Active (2.00)	Inactive (>29.9)	Active	Inactive	Active	Inactive in hTRPML1, hTRPM2, mTRPV2, hTRPC3, drTRPN1, and hTRPA1.
Analog 1		991660	24792359	MLS 000690 533	Chem Bridge	7797638	Active (1.84)	Inactive (>29.9)	85786751	Active (3.46)	Inactive (>29.9)	Not tested due to lower target potency in AID 1562, compared to the probe.			
Analog 2		991661	24787220	MLS 000715 888	Asinex	BAS 08196464	Active (2.07)	Inactive (>29.9)	Not tested due to lower target potency in AID 1562, compared to the probe.						
Analog 3		740952	14720556	MLS 000035 471	Asinex	BAS 00898171	Active (5.11)	Inactive (>29.9)							
Analog 4		849896	4255423	MLS 000063 673	Chem Bridge	7727036	Inactive (26.5)	Inactive (>29.9)							
Analog 5		849900	24784768	MLS 000715 886	Asinex	BAS 08196460	Not tested because it was inactive in the TRPML3 Primary Assay (AID 1448).								

3.5 Cellular Activity

With regard to the cytotoxicity of the probes, **ML123** (SID 24801657CID 2911646) was found inactive in several cytotoxicity assays in PubChem, including AIDs 1486, 1554, 1825, 449728. Similarly, because this compound was inactive in numerous assays examining inhibition of diverse cellular pathways such as STAT3, STAT1, luciferase reporters, PERK, P97 ATPase, Rml C and D reductase, TNAP, alkaline phosphatase, Mcl/Bid, aldehyde dehydrogenase, and bacterial streptokinase, it is likely not a general inhibitor of cell-based transcription or metabolism at the doses and timepoints tested.

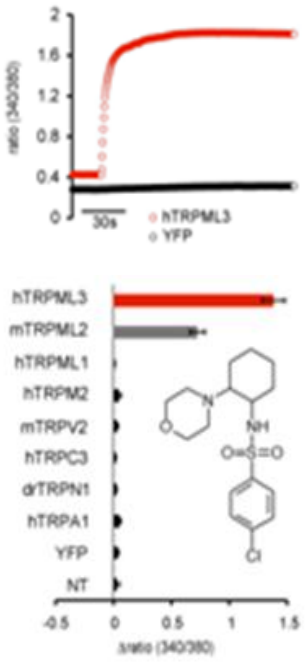
Probe ML122 (SID 24787221/CID 701237) was found inactive in several cytotoxicity/viability assays in PubChem, including AIDs 1554, and 504466. ML122 was also found inactive in assays examining cell-based pathways such as DNA repair, beta arrestin, NFkB, ERK Map Kinase, aldehyde dehydrogenase, histone methyltransferase, cysteine protease, and alpha glucosidase.

Probes were also tested in a variety of cell-based assays performed by the SRIMSC and assay provider, as shown below. These assays were performed to determine the probes' selectivity, cellular activity, and mechanism of action.

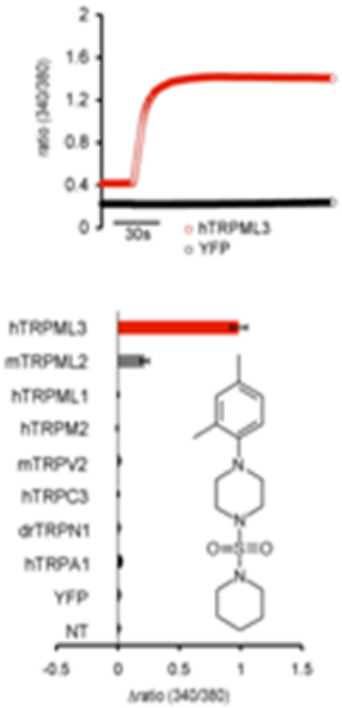
Fura-2 Assays

These assays employ the calcium indicator dye fura-2-AM to detect increases in intracellular calcium in TRPML3-transfected HEK cells. The results of these assays for the two probes are shown below.

Probe 1 (ML123):
Secondary Arylsulfonamide Scaffold
SID 24801657/CID2911646



Probe 2 (ML122):
Sulfonarylpiperazine Scaffold
SID 24787221/ CID701237



Patch Clamp Assays

The purpose of these assays is to determine if test compounds can increase current recordings in TRPML3 and TRPN1 ion channels. Whole-cell currents were recorded with an Alembic Instruments VE-2 amplifier with 100% series resistance compensation, and acquired with JClamp software. The standard bath solution

contained (in mM) 138 NaCl, 5.4 KCl, 2 MgCl2, 2 CaCl2, 10 HEPES, and 10 d-glucose, adjusted to pH 7.4 with NaOH. The standard pipette solution contained (in mM) 140 CsCl, 10 HEPES, 3 ATP-Na, 1 BAPTA, and 2 MgCl2, adjusted to pH 7.2. 100 μM 2-Aminoethyl-diphenyl borate was included in the bath solution to block gap junctions and had no effect on the expressed channels. Channel responses were plotted to 10 ms voltage steps (holding potential = +10 mV) between –200 mV and +100 mV in 20 mV incremental steps, normalized by cell capacitance (pF). Compounds were tested at 10 micromolar.

3.6 Profiling Assays

The MLSMR sample of probe **ML123** (SID 24801657) has been tested in a total of 371 PubChem Bioassays and was identified as active in only the 3 TRPML3 assays (AIDs 1562, 1526, and 1448). This gives a 0.8% promiscuity ratio. These findings demonstrate the high level of selectivity of this novel probe.

In addition, the MLSMR sample of probe **ML122** (SID24787221) has been tested in a total of 363 PubChem Bioassays and was identified as active in only 4 assays. This result gives a 1.1% promiscuity ratio. Three of these assays are the SRIMSC's TRPML3 agonist assays (AIDs 1562, 1526, and 1448). The fourth assay (AID 504444) is a fluorescence-based cell-based reporter assay which attempted to identify inhibitors of the Nrf2 transcription factor out of the entire MLP collection. The compound did not confirm activity in the subsequent validation study, thereby confirming the selectivity of this probe.

4 Discussion

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

There is no prior art for agonists of TRPML3 and TRPML2.

4.2 Mechanism of Action Studies

These novel probes activate the TRPML3 channel to increase intracellular Ca2+ influx (see Section 3.5 for Fura-2 based Calcium assay results). In addition, these probes act synergistically in the presence of endolymph-like extracellular solution (ELS) containing high K^+ (150 mM) and low Na+ (2 mM) concentrations, leading to a 10-fold increase in current response [10]. These findings indicate that these probes act cooperatively with extracellular Na^+ on TRPML3.

4.3 Planned Future Studies

The probes generated in this report are first in class, and thus best in class, agonists for TRPML3. The leads have excellent selectivity against other TRP receptors and ion channels, with moderate activity against TRPML2. The SAR studies suggest that additionally synthesized compounds may exhibit greater potency and may be superior probes than the leads selected. We encourage members of the community to use the (S,S)-trans isomer of ML123 as the starting point for further optimization, as it is the most potent of the TRPML3 agonists identified in this work, and are chemically amenable to further structural optimization. Such analogs are likely to be increasingly useful compounds for elucidating the biology of TRPML3. The SRIMSC is pursuing the development of additional TRPML-3–selective probes, based on other chemical scaffolds. These studies will be presented in a future probe report.

5 References

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