Probe Report

Title: Identification of Selective Agonists of the Transient Receptor Potential Channels 3 (TRPML3)

Authors: S Adrian Saldanha[1], Christian Grimm[2], Christophe Allais[3], Emery Smith[1], Souad Ouizem[3], Becky A. Mercer[1], William R. Roush[3], Stefan Heller[2], Peter Hodder[1,4].

[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]Corresponding author, hodderp@scripps.edu

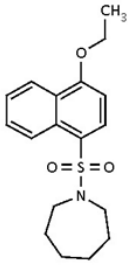
Assigned Assay Grant #: 1 R03 MH083077-01
Screening Center Name & PI: Scripps Research Institute Molecular Screening Center (SRIMSC); Hugh Rosen
Chemistry Center Name & PI: SRIMSC, Hugh Rosen
Assay Submitter & Institution: Stefan Heller, Stanford University
PubChem Summary Bioassay Identifier (AID): 1809

Abstract:

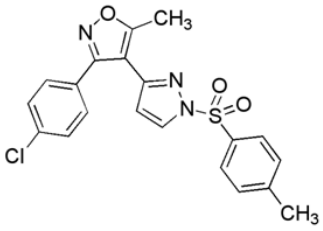
When mutated, the Transient Receptor Potential Channels 3 (TRPML3) ion channel causes deafness and pigmentation defects. Due to the lack of available compounds known to act as selective TRPML3 agonists, the identification of selective probes for TRPML3 are useful to investigate the function of TRPML3 in inner ear mechanotransduction and hearing biology. The two probes reported here, CID 776924 (ML268) and CID 53239838 (ML269), emerged from an HTS-based effort to identify small molecule activators of TRPML3. They exhibit submicromolar EC_{50} values against TRPML3 in intracellular calcium functional assays. Further, their selectivity & mechanism-of-action has been confirmed in various in patch clamp and functional assays. Interestingly, testing these probes and other TRPML3 activators on TRPML3-expressing sensory hair cells revealed the absence of activator-responsive channels. Similarly, epidermal melanocytes showed only weak or no responses when exposed to the compounds. These studies validate the biological relevance of these probes, as they have now been used to demonstrate that TRPML3 might be absent from the plasma membrane or that the protein is a subunit of heteromeric channels in native cells. A comprehensive summary of their activity has been published (reference [1]).

ML268 and ML269 are first-in-class tools for elucidating the functions of the TRPML3 ion channel. The significance of their impact was the subject of a special review article (reference [2])

Probe Structures & Characteristics:



Probe 1
ML268 / CID 776924
Liquid SID 14722627
Powder SID 87692368



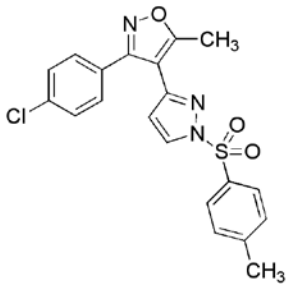
Probe 2
ML269 / CID 53239838
Liquid SID 26731169/ Powder SID 124349367

Figure 1. Selective TRPML3 Agonist Probes

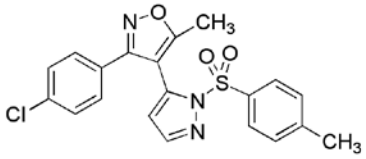
Table 1. Selective TRPML3 Agonist Results Summary

CID/ ML#	Target Name	EC50 (nM) [SID, AID]	Anti-Target Name	EC50* (μM) [SID, AID]	Fold Selective[a]	Secondary Assay EC50 (nM) [SID, AID]
Probe 1 CID 776924/ ML268	TRPML3	**LIQUID:** 1030 nM [SID 14722627, AID 1562] **POWDER:** 950 nM [SID 87692368, AID 2510]	TRPN1	**LIQUID:** >29.9 [SID 14722627, AID 1682] **POWDER:** >29.9 SID 87692368, AID 2583] >32-fold selectivity over TPRN1 in Fluo-8 screening assays.	**LIQUID** >29-fold **POWDER** >31-fold	Both probes were inactive against all TRP channels tested as determined using whole cell patch clamp assays. (AID 2116): hTRPML1 hTRPM2 mTRPV2 hTRPC3 drTRPN1 and hTRPA1
			TRPML2	**POWDER:** Inactive [SID 87692368, AID 2770]		
Probe 2 CID 53239838/ ML269	TRPML3	**LIQUID:** 325nM [SID 26731169, AID 1562 DRUN] **POWDER:** 253 nM SID 87692372, AID 2510] 290 nM [SID 124349367, AID 602128]	TRPN1	**LIQUID:** >29.9 [SID 26731169, AID 1682] **POWDER:** >29.9 [SID 124349367, AID 2583]	**LIQUID** >92-fold **POWDER** >118-fold	
			TRPML2	**POWDER:** Inactive [SID 87692372, AID 2770]		
			TRPML2	23.5% ACT		

[a]Fold-selectivity was calculated as IC_{50} for anti-target/IC_{50} for target, using an approximate value for the anti-target due to its flat agonist curve (low maximal activation at the highest dose tested, 30μM).



6a, Probe 2 (CID53239838)
(correct structure)



6b (CID2745583)
(original structure--INCORRECT)

Recommendations for the scientific use of this probe:

These novel *selective* probes are useful for assays aiming to increase TRPML3 channel activities, without activating hTRPML1, hTRPML2, hTRPML1 NC (plasma membrane variant), 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. The Heller Lab at Stanford University is utilizing these probe molecules to study possible mechanisms of regulation for TRPML3. In particular, the involvement of other TRPML family members can be better understood with small molecule modulators with differential selectivity. Towards this end TRPML3-targeted agonists such as the probes reported here 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 [1]. As suggested by the Assay Provider, selective TRPML3 modulators represent potential starting points for the development of therapies for vertigo or tinnitus. The HTS assays, chemistry effort, and biological experiments detailing the identification and characterization of these selective probes, related analogs, and our previously reported non-selective probes have been described together in [1] (see Appendix).

1 Introduction

Cell signaling pathways that mediate osmosensation, photosensation, and thermosensation depend on a family of diverse transient receptor potential (TRP) cation channels, which are activated by agonist-receptor coupling [3-9]. A role for these channels in inner ear hair cell mechanotransduction was gleaned from TRP channel mutations identified in flies, worms, and lower vertebrates with defective balance and impaired sensitivity to touch [3-7]. TRPML3 (mucolipin 3; MCOLN3) is a TRP channel expressed in inner ear hair cells and stereocilia [7-9], suggesting it may play a role in hearing and mechanotransduction. Reports that mice with mutation in TRPML3 (known as varitint-waddler mutants) exhibit early-onset hearing loss accompanied by head-bobbing and circling behaviors [10-12], provided further support for a role of TRPML3 in hearing and vestibular function. The varitant-waddler defect is the result of a A419P substitution mutation in TRPML3, which causes the channel to be constitutively active, resulting in calcium overload and apoptosis in cells that express the channel, particularly melanocytes and sensory hair cells[13]. Unfortunately, although efforts into the roles of the wildtype and mutant TRPMLS channels have characterized the response of TRPML3 to luminal pH changes and extracellular ions such as H^+ and Na^{2+}, little is known about its physiological role, cellular localization, and heteromer formation under normal and diseased states. As a result, the identification of selective probes for TRPML3 would be useful to investigate the function of TRPML3 in inner ear mechanotransduction and hearing biology.

2 Materials and Methods

Chemistry: All chemical reagents and solvents were acquired from commercial vendors.
Biology: All protocols are reported in the relevant PubChem AIDs provided in **Table 2** and in Grimm et al [1].
Compound Properties: Solubility, stability, and glutathione reactivity analyses were conducted in accordance with NIH guidelines. CYP inhibition and microsome stability analyses were performed as previously described [14].

2.1 Assays

Table 2 lists all of the PubChem AIDs for the TRPML3 agonist project. Descriptions of the assay protocols follow the tables.

Table 2. PubChem BioAssay Table (Summary can be found at AID 1809)

Project Stage	AID	Assay Name	Samples Tested	Samples Active	Compound Concentration
HTS (Liquids)	1448	TRPML3 Primary Agonist Assay (Fluo-8; 1X%ACT)	218,117	632	3 µM
	1424	Full-deck Counterscreen to identify agonists of TRPN1 (Fluo-8; 1X%ACT)	218,117	390	
	1526	TRPML3 Confirmation Assay (Fluo-8; 3X %ACT)	306	244	
	1525	TRPN1 Counterscreen (Fluo-8; 3X%ACT)	306	40	
	1562	TRPML3 Dose Response Assay (Fluo-8; 3X EC50)	188	136	10-point, 1:3 dilution starting at 30 µM
	1682	TRPN1 Dose Response Counterscreen (Fluo-8; 3X EC50)	188	6	
SAR (Powders) Run by Scripps	2116	TRPML3 Profiling and Counterscreening Assays; TRPML3 Patch clamp; TRPML3 Fura-2 Imaging; TRPML2 Fura-2 Counterscreening; Ion Channel Profiling (Fura-2)	6	6	10 µM
	2510	TRPML3 Dose Response Assay (Fluo-8: 3X EC50)	6	6	10-point, 1:3 dilution starting at 30 µM
	2583	TRPN1 Dose Response Counterscreen (Fluo-8; 3X EC50)	6	0	
	602128	TRPML3 Dose Response Assay (3X EC50): Round 2	3	1	
	602129	TRPML3 Dose Response Assay (3X EC50): Round 3	34	12	
SAR (Powders) Run by Assay Provider	2692	TRPN1 Patch Clamp Counterscreen (Whole-cell currents recorded at 10 µM)	3	0	10 µM
	2694	TRPML3 Patch Clamp Assay (Whole-cell currents recorded at 10 µM)	3	3	10 µM
	2719	TRPML3 Fura-2 Profiling Assay	3	3	10 µM
	2770	Ion Channel Fura-2 Counterscreen (HEK cells, mTRPML2, hTRPML1, hTRPML1 (NC), hTRPM2, mTRPV2, hTRPC3, drTRPN1, hTRPA1).	3	0	10 µM

Assay Descriptions

TRPML3 Agonist Assays (AID 1448, AID 1526, AID 1562, AID 2510, AID 602128, and AID 602129)
The purpose of these assays is to identify test compounds that act as agonists of the TRPML3 cation channel. This assay employs 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 a fluorescent, cell permeable calcium indicator dye. 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) or triplicate (AID 1526) at a nominal dose of 3 µM, and in a 10-point, 1:3 dilution series starting at a nominal concentration of 30 µM (AID 1562, AID 2510, AID 602128, and AID 602129).

The TRPML3 HEK293 cell line was routinely cultured in T-175 sq cm flasks at 37 degrees C and 95% relative humidity (RH). The growth media consisted of Minimum Essential Medium with GlutaMAX and supplemented with 10% v/v heat-inactivated qualified fetal bovine serum, 800 micrograms/mL Geneticin, and 1X antibiotic mix (penicillin, streptomycin, and neomycin). The day before the assay 1500 cells in 3 µl of growth media were seeded into each well of 1536 well microtiter plates and allowed to incubate at 37 degrees C, 5% CO2, and 95 % RH for 23 hours. Next, 2 µl of the fluorogenic Fluo-8 intracellular calcium indicator mixture with 1 mM trypan red plus (prepared according to the manufacturer's protocol) was added to each well. After a 1 hour incubation at 37 degrees C, 5% CO2, and 95 % RH followed by a 30 minute incubation at room temperature, the assay was started by performing a basal read of plate fluorescence (470-495 nm excitation and 515-575 nm emission) for 5 seconds on the FLIPR Tetra (Molecular Devices). Next, 15 nL of test compound (3 µM final nominal concentration) in DMSO, DMSO alone (0.3% final concentration), or the cholinergic agonist carbachol (87 µM final concentration) in DMSO were dispensed to the appropriate wells. Then a real time fluorescence measurement was immediately performed for the remaining 120 seconds of the assay. A ratio for each well was calculated to normalize assay data, according to the following mathematical expression: Ratio = I_Max / I_Min. Where I_Max represents the maximum measured fluorescence emission intensity over the 125 second read and I_Min represents the minimum (basal) measured fluorescence emission intensity before compound was added. The percent activation was calculated from the median ratio as follows:
*%Activation = ((Ratio_Test_Compound- Median_Ratio_Low_Control) / (Median_Ratio_High_Control - Median_Ratio_ Low_Control)) *100*

Where:
Test_Compound is defined as wells containing test compound.
High_Control is defined as wells with carbachol.
Low_Control is defined as wells with DMSO.

TRPN1 Counterscreen Assays (AID 1424, AID 1525, AID 1682, and AID 2583)
The purpose of this assay is to identify test compounds that act as agonists of the TRPN1 cation channel. This assay serves as a counterscreen to determine whether compounds are non-selective TRP agonists. This assay employs 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 a fluorescent, cell permeable calcium indicator dye. 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) or triplicate (AID 1525) at a nominal dose of 3 µM, and in a 10-point, 1:3 dilution series starting at a nominal concentration of 30 µM (AID 1682, and AID 2583).

The TRPN1 HEK293 cell line was routinely cultured in T-175 sq cm flasks at 37 degrees C and 95% relative humidity (RH). The growth media consisted of Minimum Essential Medium with GlutaMAX and supplemented with 10% v/v heat-inactivated qualified fetal bovine serum, 800 micrograms/mL Geneticin, and 1X antibiotic mix (penicillin, streptomycin, and neomycin). The day before the assay 1500 cells in 3 µl of growth media were seeded into each well of 1536 well microtiter plates and allowed to incubate at 37 degrees C, 5% CO2, and 95 % RH for 23 hours. Next, 2 µl of the fluorogenic Fluo-8 intracellular calcium indicator mixture with 1 mM trypan red plus (prepared according to the manufacturer's protocol) was added to each well. After a 1 hour incubation at 37 degrees C, 5% CO2, and 95 % RH followed by a 30 minute incubation at room temperature, the assay was started by performing a basal read of plate fluorescence (470-495 nm excitation and 515-575 nm emission) for 5 seconds on the FLIPR Tetra (Molecular Devices). Next, 15 nL of test compound (3 µM final nominal concentration) in DMSO, DMSO alone (0.3% final concentration), or the cholinergic agonist carbachol (87 µM final concentration) in DMSO were dispensed to the appropriate wells. Then a real time fluorescence measurement was immediately performed for the remaining 120 seconds of the assay. A ratio for each well was calculated to normalize assay data, according to the following mathematical expression: *Ratio = I_Max / I_Min.* Where *I_Max* represents the maximum measured fluorescence emission intensity over the 125 second read and *I_Min* represents the minimum (basal) measured fluorescence emission intensity before compound was added. The percent activation was calculated from the median ratio as follows:
*%Activation = ((Ratio_Test_Compound- Median_Ratio_Low_Control) / (Median_Ratio_High_Control - Median_Ratio_ Low_Control)) *100*

Where:
Test_Compound is defined as wells containing test compound.
High_Control is defined as wells with carbachol.
Low_Control is defined as wells with DMSO.

Patch Clamp Assays (AID 2116, AID 2694, and AID 2692)
The purpose of these assays is to determine if test compounds can increase current recordings in TRPML3 ion channels (AID 2116 and AID 2694) or TRPN1 (AID 2692), along with controls employing the YFP-HEK parental background. 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.

Fura-2 Calcium Influx Assays (AID 2116, AID 2719, and AID 2770)
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 were tested at 10 micromolar.

2.2 Probe Chemical Characterization

Solubility

As shown in **Table 3** the solubility of probes 1 and 2 in PBS was determined to be 0.28 μM and 0.02μM, respectively (solution used: 137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, pH 7.4, room temperature). Owing to these poor solubilities, ML269 was tested in PBS supplemented with 6% fetal bovine serum (FBS), under conditions that are more relevant to the conditions used in the TRPML3 assay. Under these conditions, ML269 (specifically the re-assigned compound identified here as **6a**) has a solubility of 12 μM. Therefore, probe solubilities are fully adequate to provide the high potency seen in multiple cell-based assays (< 1 μM) and are also adequate for broad use as a biological probe to be used in a variety of media.

Table 3. Probe Solubility and Stability

Compound	SR Number	MLS	CID	SID	Solubility in PBS (μM)[a]	Solubility in PBS-6%FBS (μM)	Michael Acceptor 100 μM GSH trap	Stability in PBS (t1/2 (hr)
Probe #1: ML268	SR-03000002102-1	MLS00387 5009	776924	113584822	0.28	ND	No	> 48 hr
Probe #2 (**6a**): ML269	SR-03000002360-1	MLS00367 5345	53239838	124349367	0.02	12.0	No	> 48 hr (57hr)
Original Probe #2 (**6b**)	SR-01000768170-3	MLS00111 1122	2745583	26731169	0.10	5.9	No	> 48 hr

[a]Solubility measurements demonstrated that Probe 2 has very low kinetic solubility in PBS buffer (20 nM). However, solubility for this compound is 12 μM in PBS buffer with 5% fetal bovine serum (FBS)—which is used in the TRPML3 assays. Similarly, solubility for the structure of the originally assumed probe 2 structure (**6b**) similarly was markedly enhanced in PBS-6% FBS compared to PBS alone (5.9 μM vs. 100 nM, respectively). ND, Not determined.

Stability and Reactivity

The probes have a half-life of >48 hours in PBS at room temperature (**Figures 2 and 3**), when tested at 10 μM concentration. No erosion of LCMS peak intensity was seen in the 2 day duration of the study. The probes were found to be unreactive with excess glutathione, indicating that they are not a Michael acceptor under physiologically-relevant conditions. The compounds were tested at 10 μM concentration in the presence of 100 μM GSH (10-fold excess) and no erosion of LCMS peak intensity for the probe was seen for duration of the study.

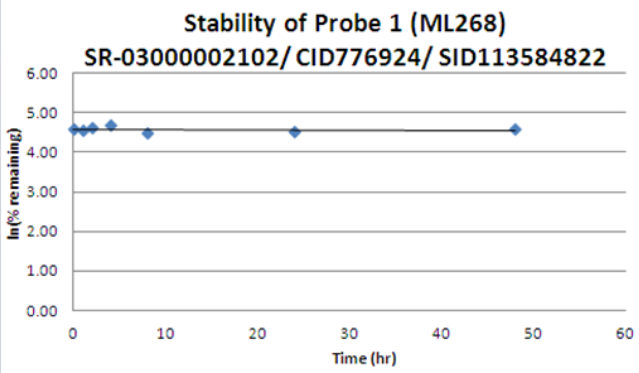
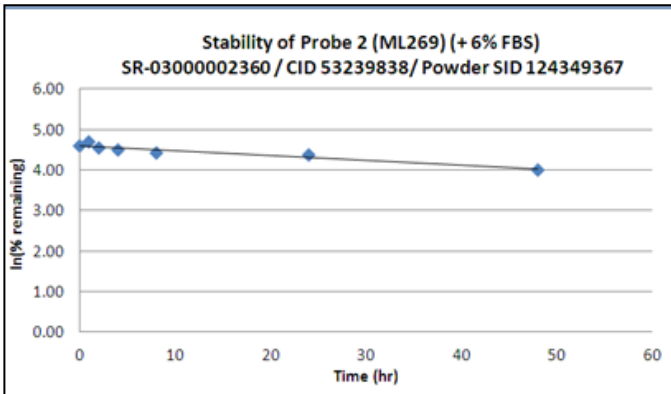
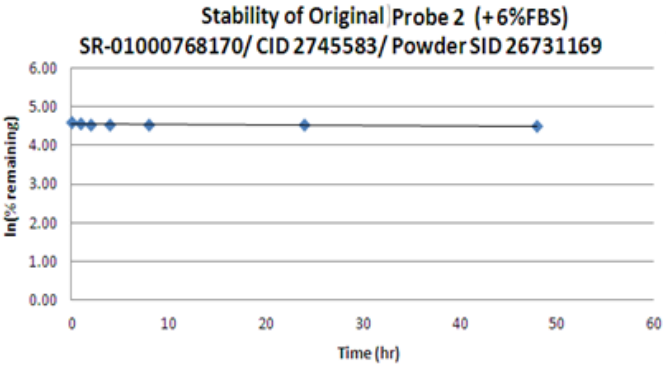


Figure 2. Probe 1 Stability.

Probe 1 (ML268) SR-03000002102 / CID 776924/ Powder SID 113584822		
Stability in PBS Buffer (pH 7.4)		
Sample concentration: 5 µM		
Storage: microcentrifuge tube on lab benchtop		
Time (hr)	% remaining	ln(%remaining)
0	100	4.61
1	95	4.55
2	101	4.62
4	108	4.68
8	89	4.49
24	92	4.53
48	98	4.58
slope =		-0.0007
half-life =	-ln2/slope	> 48 hr





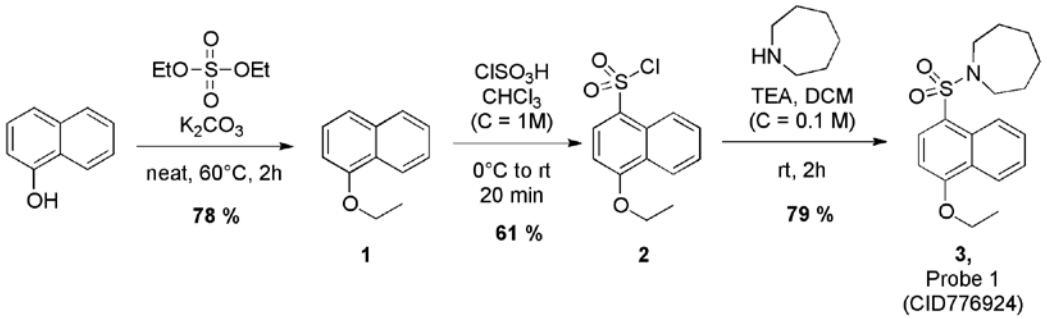
Probe 2 (ML269) SR-03000002360/ CID 53239838/ Powder SID 124349367		
Stability in PBS Buffer (pH 7.4) + 6% FBS (heat inactivated)		
Sample concentration: 1 µM		
Storage: microcentrifuge tube on lab benchtop		
Time (hr)	% remaining	ln(%remaining)
0	100	4.61
1	109	4.69
2	95	4.55
4	89	4.49
8	83	4.42
24	79	4.37
48	55	4.00
slope =		-0.0121
half-life =	-ln2/slope	57.2

Original Probe 2 SR-01000768170/ CID 2745583/ Powder SID 26731169		
Stability in PBS Buffer (pH 7.4) + 6% FBS (heat inactivated)		
Sample concentration: 1 µM		
Storage: microcentrifuge tube on lab benchtop		
Time (hr)	% remaining	ln(%remaining)
0	100.0	4.61
1	97.2	4.58
2	94.6	4.55
4	94.1	4.54
8	94.9	4.55
24	92.9	4.53
48	89.8	4.50
slope =		-0.0016
half-life =	-ln2/slope	> 48 hr

Figure 3. Probe 2 Stability

2.3 Probe Preparation

(1) Synthesis of Probe 1 (CID 776924, compound 3)



Synthesis of **1**.

1-Naphthol (577 mg, 4 mmol, 1 equiv.) and potassium carbonate (1 g, 2 mmol) were ground together in a in a 10-mL round-bottomed flask until a fine powder was obtained. This mixture was then heated at 60 °C, and then diethyl sulfate (524 µL, 1 equiv.) was added under vigorous stirring. The mixture was heated at 60 °C until the reaction was complete as judged by TLC analysis. Water (10 mL) was added to the mixture and the aqueous layer was extracted three times with diethyl ether (10 mL). The combined organic layers were washed with 1N HCl, water and brine, and then dried over sodium sulfate. Solvent was removed under reduce pressure and compound **1** was obtained as a colorless oil (535 mg, 78 %) after purification by flash chromatography (silica gel, 100 % hexanes, $R_f = 0.32$).

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.40-8.36 (1H, m), 7.87-7.84 (1H, m), 7.57-7.51 (2H, m), 7.49-7.40 (2H, m), 6.84 (1H, dd, *J* = 0.8 Hz, *J* = 7.6 Hz), 4.25 (2H, q, *J* = 7.1 Hz), 1.60 (3H, t, *J* = 7.0 Hz). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 154.7, 134.5, 127.4, 126.3, 125.9, 125.7, 125.0, 122.1, 120.0, 104.6, 63.6, 14.8.

Synthesis of **2**.

A solution of **1** (535 mg, 3.11 mmol, 1 equiv.) in chloroform (3.1 mL) in a 10-mL round-bottomed flask was cooled to -5 °C under an inert atmosphere. Chlorosulfonic acid (410 µL, 2 equiv.) was added dropwise to this solution. The stirred mixture was allowed to warm to room temperature and stirred for an additional 20 min. The mixture was then poured onto ice (13 g); chloroform (20 mL) was added along with a small amount of brine (5 mL). The heterogeneous solution was filtered through a short pad of Celite, the layers were separated and the organic layer was washed twice with water and once with brine. The organic phase was dried over sodium sulfate, filtered, and solvent was removed under reduce pressure to yield compound **2** as an off-white solid (511 mg, 61 %). This material was used in the next step without purification.

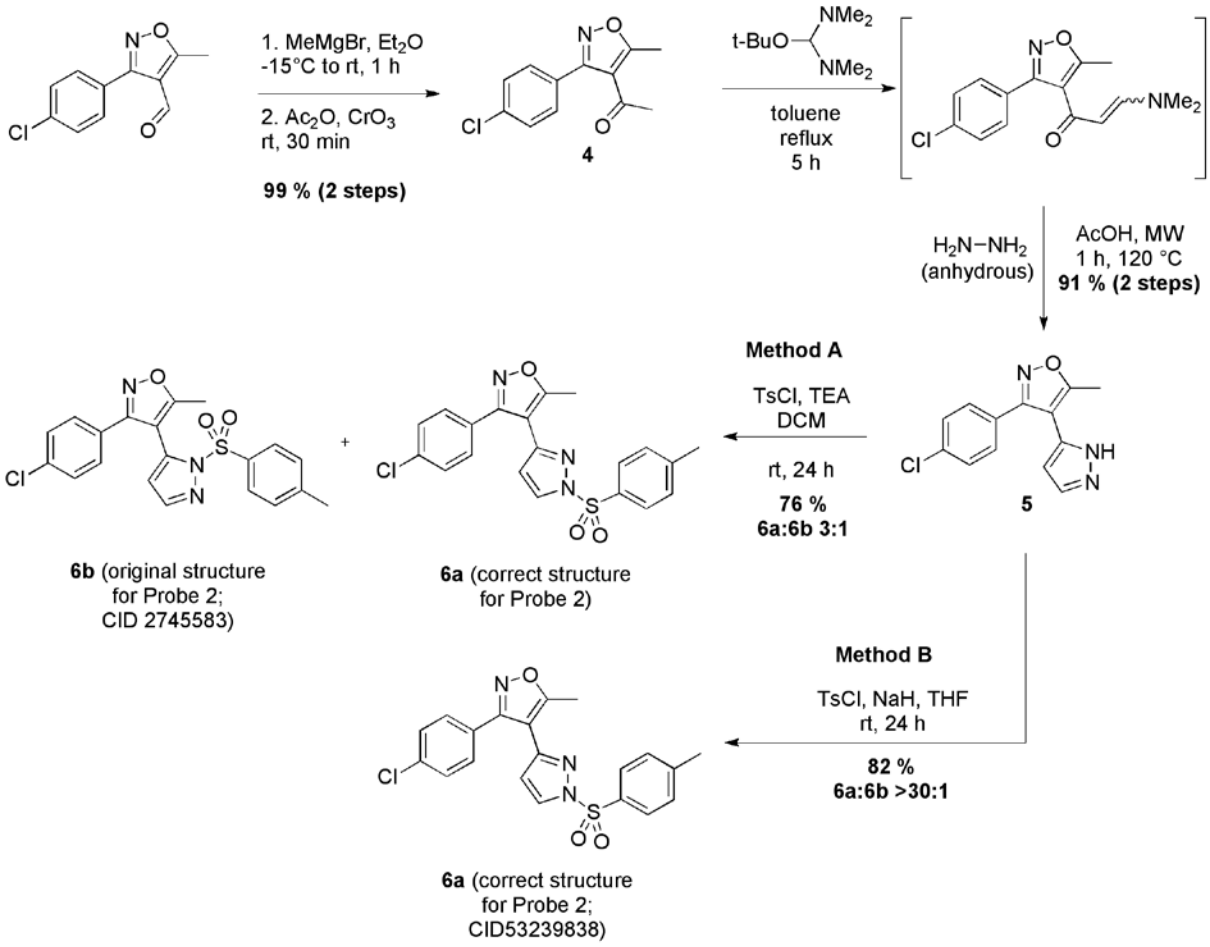
^{1}H NMR (400 MHz, $CDCl_3$) δ 8.71 (1H, ddd, *J* = 0.8 Hz, *J* = 0.8 Hz, *J* = 8.6 Hz), 8.41 (1H, ddd, *J* = 0.6 Hz, *J* = 1.2 Hz, *J* = 8.6 Hz), 8.28 (1H, d, *J* = 8.6 Hz), 7.78 (1H, ddd, *J* = 1.3 Hz, *J* = 6.9 Hz, *J* = 8.4 Hz), 7.62 (1H, ddd, *J* = 1.1 Hz, *J* = 6.9 Hz, *J* = 8.3 Hz), 6.77 (1H, d, *J* = 8.6 Hz), 4.28 (2H, q, *J* = 7.0 Hz), 1.59 (3H, t, *J* = 7.0 Hz). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 161.3, 132.0, 130.8, 129.7, 128.7, 126.7, 126.0, 123.7, 123.3, 102.2, 64.9, 14.4.

Synthesis of **3** (Probe 1, CID776924)

To a room temperature solution of sulfonyl chloride **2** (510 mg, 1.88 mmol, 1 equiv.) in dichloromethane (20 mL) were added triethylamine (525 µL, 2 equiv.) and azepane (318 µL, 1.5 equiv.). This mixture was stirred for 2 h. The mixture was poured into water (20 mL) and aqueous layer was extracted three times with dichloromethane. The combined organic layers were washed with 1N HCl, water and brine, and then dried over sodium sulfate. The mixture was filtered, solvent was removed under reduce pressure and compound **3** (493 mg, 79 %, white solid— mp = 103-104 °C) was obtained after purification by flash chromatography (silica gel, ethyl acetate-hexanes 20/80, R_f = 0.66).

Compound 3 (Probe 1, CID 776924): ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.60 (1H, ddd, *J* = 0.9 Hz, *J* = 0.9 Hz, *J* = 8.5 Hz), 8.37 (1H, ddd, *J* = 0.8 Hz, *J* = 1.2 Hz, *J* = 8.4 Hz), 8.12 (1H, d, *J* = 8.3 Hz), 7.63 (1H, ddd, *J* = 1.4 Hz, *J* = 6.9 Hz, *J* = 8.4 Hz), 7.54 (1H, ddd, *J* = 1.1 Hz, *J* = 6.9 Hz, *J* = 8.3 Hz), 6.77 (1H, d, *J* = 8.4 Hz), 4.25 (2H, q, *J* = 7.0 Hz), 3.36 (4H, dd, *J* = 5.8 Hz, *J* = 6.0 Hz), 1.68-1.64 (4H, m), 1.59-1.55 (7H, m). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 158.7, 131.5, 129.9, 128.1, 126.2, 126.1, 125.8, 124.9, 122.8, 102.3, 64.2, 47.7, 29.1, 26.9, 14.6. **HRMS** ($[M+Na]^+$) expected: 356.1291, observed: 356.1307. **IR** (cm^{-1}): 2932, 2859, 1590, 1574, 1509, 1376, 1314, 1148, 1229, 1086, 1041, 961, 775, 709.

(2) Synthesis of Probe 2 (CID 532239838, compound 6a)



Synthesis of **4**

3-(4-Chlorophenyl)-5-methylisoxazole-4-carboxaldehyde (1 g, 4.52 mmol, 1 equiv, purchased from Sigma-Aldrich, # 703281-1G) was dissolved in freshly distilled tetrahydrofuran (45 mL) under inert atmosphere in a flame-dried flask. The solution was cooled to -15 °C and then methylmagnesium bromide (3 M in diethyl ether, 2.26 mL, 1.5 equiv.) was slowly added. The mixture was allowed to warm up room temperature and was stirred for 1 h. The mixture was then poured into ice-water and 1N HCl was slowly added until the pH = 1. The aqueous layer was extracted with diethyl ether (3 x 100 mL) and the combined organic layers were washed with water (100 mL), brine (100 mL) and then dried over sodium sulfate. The solution was filtered, solvent was removed under reduced pressure to yield the corresponding secondary alcohol as a colorless oil (1.07 g, quant.) which was used in the following step without purification.

Chromic acid (904 mg, 9.04 mmol, 2 equiv.) and acetic anhydride (50 mL) were mixed with stirring in a 100-mL round-bottomed flask until homogeneous. A solution of secondary alcohol from the previous step (1.07 g, 4.52 mmol, 1 equiv.) in acetic anhydride (20 mL) was then added dropwise via syringe pump (10 min). The resulting mixture was stirred for 30 min, then was extracted with diethyl ether (4×50 mL). The combined organic layers were washed several times with saturated aqueous ammonium chloride solution. The organic layer was dried over sodium sulfate, filtered and then solvent was removed under reduced pressure. The crude

methyl ketone was purified by flash chromatography over silica gel (ethyl acetate-hexanes 20/80, R_f = 0.28) to yield **4** (1.07 g, 99 % over two steps) as a colorless oil.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45 (4H, s), 2.70 (3H, s), 2.13 (3H, s). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 192.7, 174.7, 161.0, 136.3, 130.4, 128.9, 127.3, 117.2, 30.6, 13.7.

Synthesis of **5**

A solution of **4** (530 mg, 2.25 mmol, 1 equiv.) in dry toluene (7.5 mL) in a flame-dried sealed tube was treated with tert-butoxy bis(dimethylamino)methane (511 µL, 1.1 equiv.) under an inert atmosphere. The tube was sealed and the mixture was heated at 110 °C overnight. Toluene was then removed under reduce pressure to give the crude enamino-ketone intermediate as a pale yellow oil (641 mg, 98%) which was used in the next step without further purification.

The enamino-ketone from the preceding experiment (360 mg, 1.24 mmol, 1 equiv.) was dissolved in acetic acid (2 mL) in a 5-mL microwave-vial, and anhydrous hydrazine (43 µL, 1.1 equiv.) was added. The mixture was submitted to microwave irradiations for 1 h at 120 °C. Water (5 mL) and dichloromethane (5 mL) were added and then the resulting mixture was transferred to a separatory funnel. Saturated aqueous sodium hydrogenocarbonate solution was added until pH = 4-5, then the aqueous layer was extracted with dichloromethane (3 x 15 mL). The combined organic layers were washed with saturated aqueous ammonium chloride solution (30 mL), brine (30 mL) and dried over sodium sulfate. The solution was filtered and solvent was removed under reduce pressure. Pyrazole **5** was obtained as a pale yellow solid (300 mg, 91 %, 2 steps) after purification by flash chromatography (silica gel, ethyl acetate-hexanes 40/60, R_f = 0.65).

^{1}H NMR (400 MHz, $CDCl_3$) δ 10.31 (1H, br s), 7.51 (1H, d, *J* = 2.2 Hz), 7.46 (2H, d, *J* = 8.5 Hz), 7.30 (2H, d, *J* = 8.5 Hz), 6.16 (1H, d, *J* = 2.1 Hz), 2.50 (3H, s). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 168.4, 160.3, 139.7, 135.8, 131.5, 129.7, 128.8, 127.5, 108.1, 106.0, 12.0.

Synthesis of Probe 2 (**6a**, CID 53239838**)** and Its Regioisomer **6b**

Method A: Pyrazole **5** (115 mg, 0.44 mmol, 1 equiv.) and *p*-tosyl chloride (84 mg, 1 equiv.) were dissolved in dry dichloromethane (4.4 mL) under inert atmosphere. Freshly distilled triethylamine (124 µL, 2 equiv.) was added and the mixture was stirred at room temperature for 24 h. At this time water (10 mL) and aqueous 1N HCl solution (10 mL) were added. The aqueous layer was extracted with dichloromethane (3 x 15 mL) The combined organic layers were washed with water (15 mL) and brine (15 mL) and dried over sodium sulfate. The solution was filtered, then solvent was removed under reduce pressure to give a 3:1 mixture of regioisomers **6a** and **6b**. The major regioisomer **6a** (104 mg, white solid—mp = 133-134 °C) and the minor isomer **6b** (34 mg, pale yellow solid) were separated by flash chromatography over silica gel (ethyl acetate/hexanes 20/80, R_{f6a} = 0.26, $R_{f\,6b}$= 0.14) with an overall yield of 76 %. *The minor regioisomer **6b** has the structure originally listed for Probe 2 the commercial supplier.*

Method B: Pyrazole **5** (20 mg, 0.08 mmol, 1 equiv.), *p*-tosyl chloride (14.7 mg, 1 equiv.) and 97 % sodium hydride (3.8 mg, 2 equiv.) were weighed into a flame-dried 10-mL round-bottomed flask inside an inert atmosphere glovebox. The flask was sealed under inert atmosphere and the solids were dissolved in freshly distilled tetrahydrofuran (0.8 mL). The mixture was stirred at room temperature for 24 h. At this time water (5 mL) and aqueous 1N HCl solution (5 mL) were added with precaution. The aqueous layer was separated and extracted with dichloromethane (3 x 10 mL) The combined organic layers were washed with water (10 mL) and brine (10 mL) and dried over sodium sulfate. The solution was filtered, and solvent was removed under reduce pressure yielding regioisomers **6a** (minor regioisomer **6b** was not observed by 1H NMR analysis of the crude product). The major regioisomer **6a** (26 mg, 82 %, white solid— mp = 133-134 °C) was purified by flash chromatography over silica gel (ethyl acetate/hexanes 20/80, $R_{f\,6a}$= 0.26).

Major regioisomer 6a (Probe 2, CID 53239838): ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08 (1H, d, *J* = 2.8 Hz), 7.87 (2H, d, *J* = 8.4 Hz), 7.37-7.33 (4H, m), 7.24 (2H, d, *J* = 8.7 Hz), 6.12 (1H, d, *J* = 2.7 Hz), 2.50 (3H, s), 2.46 (3H, s). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 169.3, 160.3, 147.7, 146.1, 135.6, 133.7, 131.8, 130.0, 129.9, 128.6, 128.2, 127.2, 108.8, 107.7, 21.7, 12.2. **MS** ([M+H]+): 414.1. **IR** (cm^{-1}): 3142, 3126, 2921, 1641, 1475, 1370, 1178, 1157, 1093, 1048, 929, 778, 669.

Minor regioisomer 6b (with the structure listed for CID 2745583): ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (1H, d, *J* = 1.6 Hz), 7.48 (2H, d, *J* = 8.4 Hz), 7.15-7.14 (4H, m), 7.10 (2H, d, *J* = 8.1 Hz), 6. 04 (1H, d, *J* = 1.6 Hz), 2.34 (3H, s), 2.29 (3H, s). **^{13}C NMR** (100 MHz, $CDCl_3$) δ 170.3, 159.6, 146.0, 143.5, 135.8, 134.8, 133.9, 129.7, 128.7, 128.4, 128.0, 127.1, 113.2, 103.9, 21.6, 11.7. **MS** ([M+H]+): 414.1. **IR** (cm^{-1}): 3128, 2924, 2853, 1640, 1596, 1452, 1418, 1389, 1269, 1194, 1172, 1124, 1089, 914, 844, 809, 678, 661.

Determination of the Structures of Regioisomers **6a** and **6b**

It is expected that sulfonylation of pyrazole 5 should lead selectively to regioisomer **6a**. Indeed, isomer **6a** was obtained exclusively when the sulfonylation was performed according to Method B. Fortunately, regioisomer **6b** was obtained as the minor component of a 3 : 1 mixture from the sulfonylation performed as described in method A. Therefore, samples of both compounds were subjected to testing in the TRPML3 assay, from which it was determined that regioisomer **6a** was the active probe compound.

Further evidence that regioisomer **6a** is the correct structure of Probe 2 was provided as follows. TLC analysis demonstrated conclusively that the synthetic compound **6a** is the same as a sample of Probe 2 obtained from a commercial supplier. However, compound **6b**—which is inactive in the TRPML3 assay and which is different from commercial samples of Probe 2 by TLC analysis—has the structure as originally listed for Probe 2 in PubChem (CID 2745583) and in the commercial vendor's catalogs. Therefore, we suspected that the structure of the compound is incorrect in the vendor catalogs, and that this error has been duplicated in the PubChem database.

We have performed NMR analysis of a commercial sample of the probe compound, obtained from Maybridge Ltd (the supplier ID is SPB04991), and have verified that synthetic probe **6a** is **IDENTICAL** to the material—written with the wrong structure—in the vendor catalog.

Therefore, the following NMR experiments were performed in order to clarify the structures of regioisomers **6a** and **6b**. The 1H nOe summarized in the following figures are highly instructive. The nOe data in **Figure 1**, obtained by irradiation of the ortho hydrogen atoms of the toluenesulfonyl group, resulted in a strong enhancement of the indicated ortho pyrazoles ring hydrogen in structure **6a**, but only a weak enhancement in **6b**. Conversely, this nOe study (**Figure 4)** led to a stronger enhancement of the oxazoles methyl group in **6b** than in **6a**. These data are fully consistent with the assigned structures. This conclusion is enhanced by the 1H nOe study summarized in **Figure 5**, in which the ortho pyrazole hydrogen was irradiated in the two regioisomers. This resulted in a strong enhancement of the ortho hydrogen atoms of the toluenesulfonyl group in regioisomer **6a** but only a weak enhancement in **6b**.

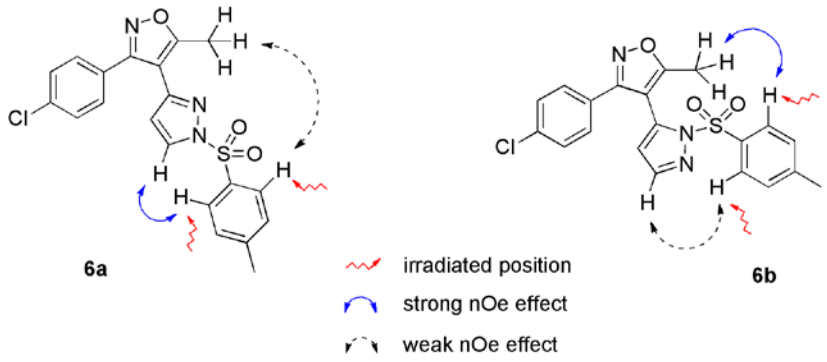


Figure 4: 1H nOe enhancements from irradiation of **6a** at 7.87 ppm and **6b** at 7.48 ppm.

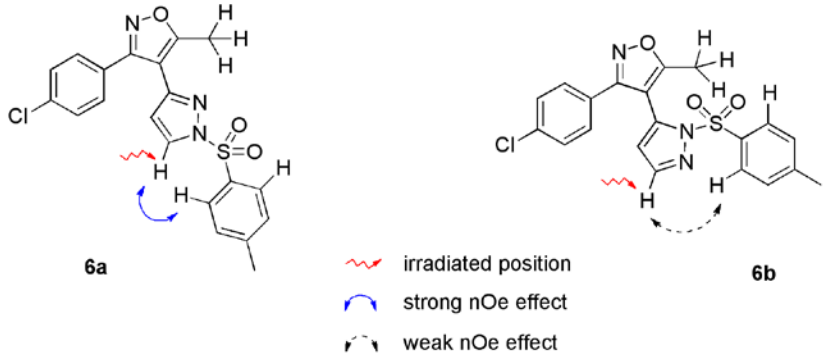
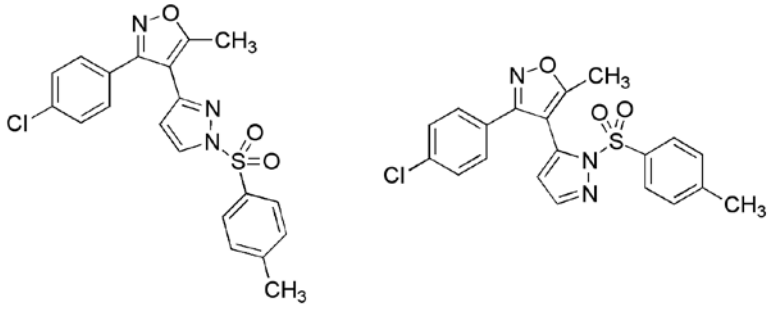


Figure 5: 1H nOe enhancements from irradiation of **6a** at 8.08 ppm and **6b** at 7.83 ppm.

Therefore, based on these studies, the structure of Probe 2 must be reassigned from the original structure (listed in PubChem as CID 2745583, as in isomer **6b**) to that of compound **6a** (CID 53239838).



6a, Probe 2 (CID53239838)
(correct structure)

6b (CID2745583)
(original structure--INCORRECT)

3 Results

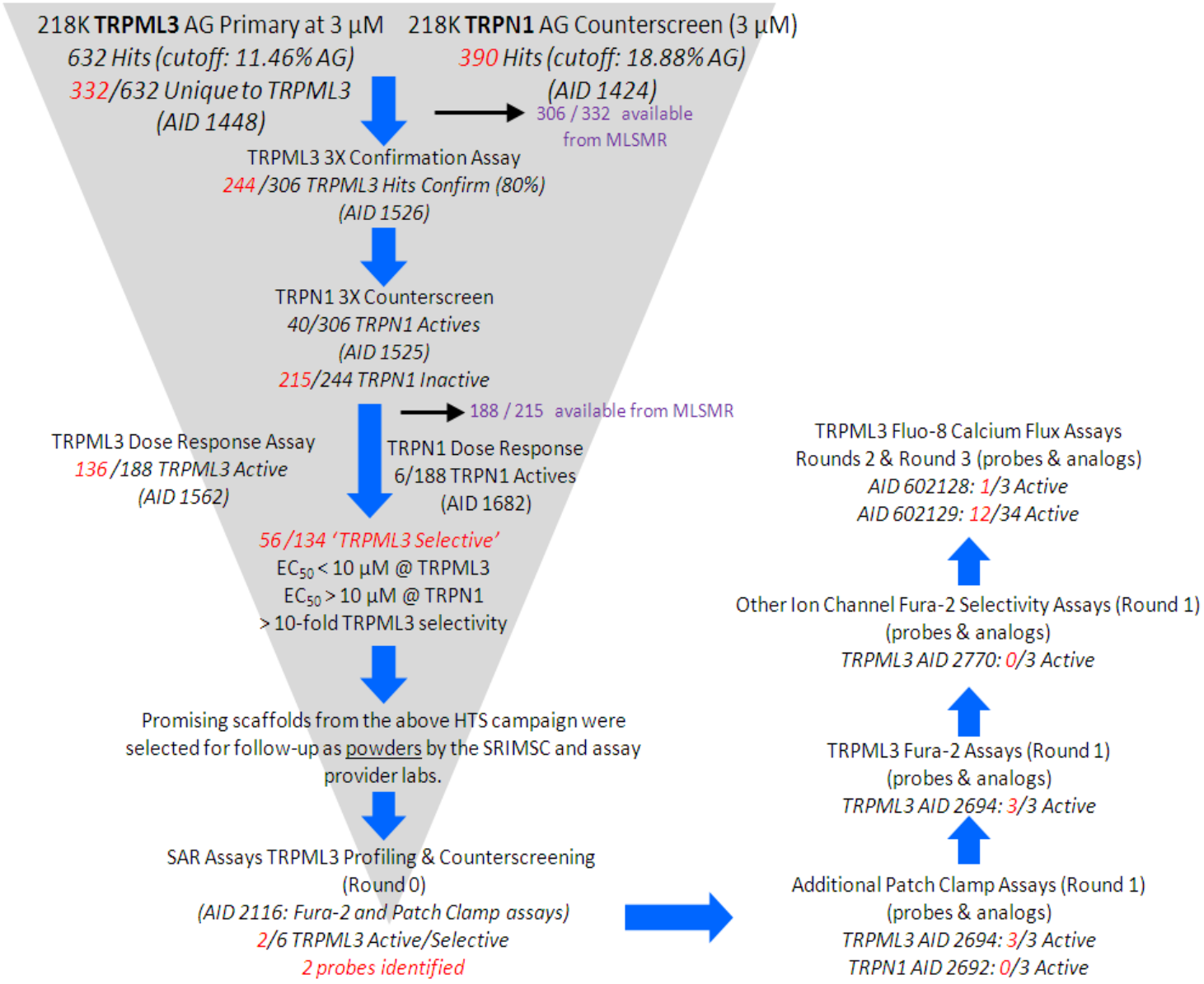
Summary. Following primary HTS in singlicate to identify TRPML3 agonists (AID 1448), counterscreening against TRPN1 to identify nonselective agonists in singlicate (AID 1424) and triplicate (1525), confirmation of TRPML3 activity in triplicate (AID 1526), titration assays in triplicate to determine potency (AID 1562) and selectivity (AID 1682), certain compounds were identified as possible candidates for probe development. Compounds were ordered as powders samples for testing by the SRIMSC and assay provider labs. The results of powder dose response assays (Rounds 0 and 1: AID 2510; Round 2: 602128; Round 3: AID 602129), and selectivity dose response assays against TRPN1 (AID 2583), patch clamp assays against TRPML3 (AID 2694) and TRPN1 (AID 2692), and Fura-2 ion channel profiling (AID 2719 and AID 2770), resulted in the identification of two *selective* TRPML3 agonist probes ML268 and ML269.

3.1 Summary of Screening Results

The purpose of the HTS assays was to identify compounds that can increase calcium flux selectively through the TRPML3 channel, as measured using the Fluo-8 calcium reporter dye. These assays are cell-based, and employ HEK293 cells that stably express the human TRPML3-YFP cation or TRPN1 (anti-target) channel. The HTS assays were run in 1536-well plate format and were conducted at the SRISMC facility in Jupiter, Florida. Two HTS lead compounds belonging to the tertiary arylsulfonamides and Pyrazol-5-yl-isoxazole scaffolds (SID 14722627 and SID 26731169), exhibited EC50 values in HTS assays of 1.03 µM and 0.33 µM, respectively. These compounds and related analogs were ordered as powders (SID 87692368 and SID 87692372) for retesting by the SRIMSC in TRPML3 dose response assays. The powder samples exhibited EC50 values consistent with the liquid samples: 0.95 µM and 0.25 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 selective activity against TRPML3, being inactive against all other ion channels tested, including the prior anti-targetsTRPN1 and TRPML2. See the SAR tables for additional data.

The flow chart (**Figure 6**) outlines the results of the ultra high throughput screening (uHTS) campaign. Briefly, 306 available hits were initially identified, 215 were confirmed as TRPML3-specific, and dose response potency and selectivity screens reduced the number of actives under consideration to 56.

Figure 6. TRPML3 Selective Agonist Screening Campaign Funnel



3.2 Dose Response Curves for Probe

The dose response curves for the TRPML3 selective agonist probes as determined using Fluo-8 calcium dye cell-based fluorescence assays are shown in **Figure 7.** Please refer to the indicated PubChem AIDs for details.

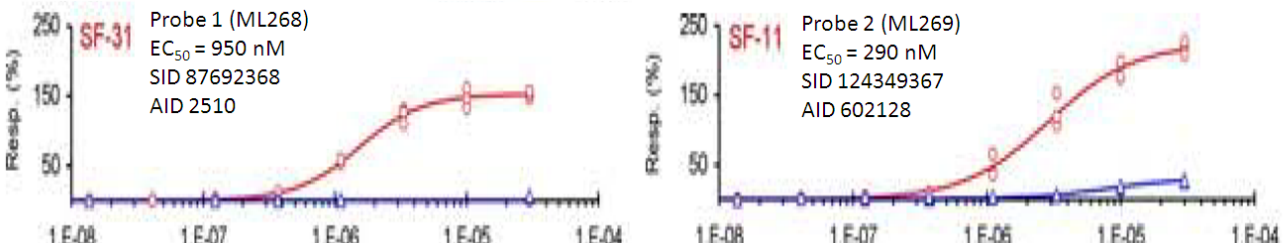


Figure 7. Dose Response Curves for TRPML3 selective probes. Left panel, probe 1 (ML268). Right panel. Probe 2(ML269). TRPML3 curves are shown in red. TRPN1 curves are shown in blue. Note lack of activity for probes on the TRPN1 channel (AID 1682). See indicated PubChem AIDs for details.

the TRPN1 channel (AID 1682). See indicated PubChem AIDs for details.

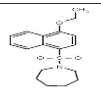
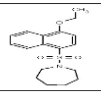
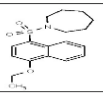
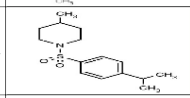
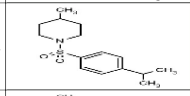
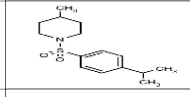
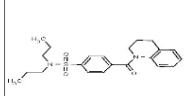
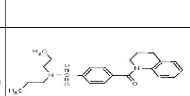
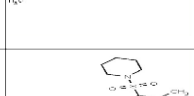
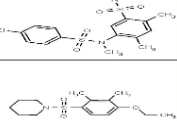
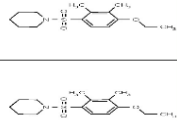
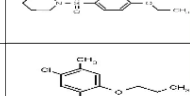
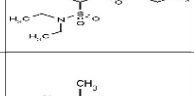
3.3 Scaffold/Moiety Chemical Liabilities

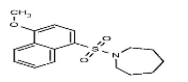
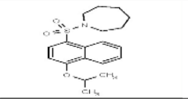
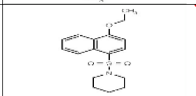
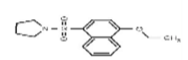
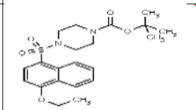
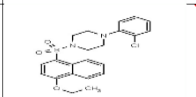
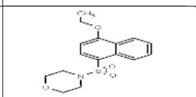
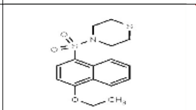
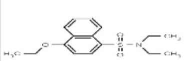
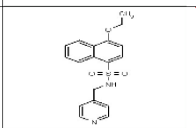
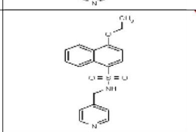
There are no known chemical liabilities with the two probes described in this probe report.

3.4 SAR Tables

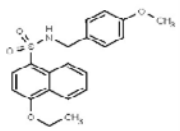
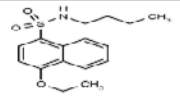
Please see next page for SAR tables.

SAR Table 1. Tertiary arylsulfonamides Scaffold

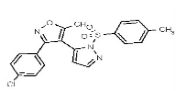
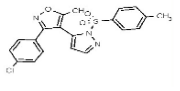
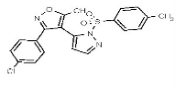
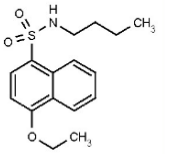
Compound Information								Screening Assays						Probe Development Data							
Compound	**SR-Number**	**Structure**	**CID**	**SID**	**MLS**	**Vendor**	**Vendor Catalog ID**	**AID 1448: TRPML3 Primary 1X%ACT**	**AID 1424: TRPN1 Primary 1X%ACT**	**AID 1526: TRPML3 CRUN 3X%ACT**	**AID 1525: TRPN1 3X%ACT**	**AID 1562: TRPML3 EC50, µM**	**TRPN1 CS DRUN (AID 1682) EC50, µM**	**AID 2510: TRPML3 EC50, µM**	**AID 2583: TRPN1 EC50,**	**AID 2694: TRPML3 Patch Clamp: nA/pF, -80 mV**	**AID 2692: TRPN1 Patch Clamp: nA/pF, -80 mV**	**AID 2719: TRPML3 Fura-2 Imaging: AF, 340 nm/ 380 nM**	**AID 2770: Fura-2 Assay: Other channels DF340/ F380**	**AID 602128: TRPML3 EC50, µM**	**AID 602129: TRPML3 EC50, µM**
Probe 1 (liquid SID)	SR-01000430563-2		776924	14722627	MLS000123160	Asinex	BAS 00667918	Active (101.27)	Inactive (-1.58)	Active (128.69)	Inactive (-0.72)	Active (1.03)	Inactive (>29.9)	Not tested: this SID is an MLSMR liquid and was not tested during MedChem							
Probe 1 (**ML268**) (powder SID, purchased)	SR-01000430563-3		776924	87692368	None	ChemBridge	5852491	Not tested: this SID is a powder and was not tested during HTS						0.95 (Active) CID776924 SID	>29.9 (Inactive)	-0.19 ±0.03 (Active)	- 0.003 ±0.001 (Inactive)	1.0 ±0.1 (Active)	-0.011 ± 0.001 (Inactive)	SID Not Tested	SID Not Tested
Probe 1 (powder SID, synthesized)	SR-03000002102-1		776924	113584822	MLS003875009	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Compound was synthesized iin order to submit to MLSMR							
Analog1a	SR-01000724392-1		801435	24801969	MLS000706525	Asinex	BAS 02912185	67.73 (Active)	Inactive	118.18 (Active)	Inactive	1.33 (Active)	Inactive (>29.9)	Not tested: this SID is an MLSMR liquid and was not tested during MedChem							
Analog 1b (purchased)	SR-01000724392-2		801435	87692370	MLS000706525:	Asinex	BAS 02912185	Not tested: this SID is a powder and was not tested during HTS						1.124 (Active)	>29.9 (Inactive)	- 0.02 ±0.01 (Active)	- 0.003 ±0.001 (Inactive)	0.52 ±0.05 (Active)	−0.008 ± 0.005 (Inactive)	Not Tested	Not Tested
Analog 1c (synthesized)	SR-01000724392-3		801435	TBD	MLS003875013	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: Compound was synthesized for submission to MLSMR							
Analog 2a (liquid)	SR-01000529615-2		569100	14740076	MLS000530984	Chem Bridge	6930808	Active (133.27)	Inactive (6.36)	Active (104)	Inactive (6.27)	Active (1.33)	Inactive (>29.9)	Not tested: this SID is an MLSMR liquid and was not tested during MedChem							
Analog 2b (purchased)	SR-01000529615-3		569100	126723256	MLS003875014	Chemical Block Ltd	A2255/ 0095000	Not tested: this SID is a powder and was not tested during HTS						Not tested: Compound was purchased for submission to MLSMR							
Analog 3	SR-01000503984-2		2920855	17412681	MLS000575809	Chem Bridge	6963034	Active (68.49)	Inactive (-0.16)	Active (96.95)	Inactive (-1.04)	Active (1.37)	Inactive (>29.9)	Not tested: this SID is an MLSMR liquid and was not tested during MedChem							
Analog 4 (MLSMR liquid)	SR-01000271732-2		976573	24786634	MLS000693818	Chem Bridge	7726423	Active (84.45)	Inactive (-1.46)	Active (77.37)	Inactive (-1.72)	Active (1.43)	Inactive (>29.9)	Not tested: this SID is an MLSMR liquid and was not tested during MedChem							
Analog 4 (purchased)	SR-01000271732-3		976573	87692367	None	Enamine	T5579947	Not tested: this SID is a powder and was not tested during HTS						1.798 (Active)	>29.9 (Inactive)	Not tested due to lower TRPML3 activity, compared to probe.					
Analog 5 (MLSMR liquid)	SR-01000683090-1		1818631	14727674	MLS000548862	Specs	AP-263/ 42611152	Active (25.88)	Inactive (-2.06)	Active (68.51)	Inactive (-2.49)	Active (1.6)	Inactive (>29.9)	Not tested: this SID is an MLSMR liquid and was not tested during MedChem							
Analog 5 (purchased)	SR-01000683090-2		1818631	87692369	None	Enamine	T5580266	Not tested: this SID is a powder and was not tested during HTS						4.724 (Active)	>29.9 (Inactive)	Not tested due to lower TRPML3 activity, compared to probe.					

SAR Table 1. Tertiary arylsulfonamides Scaffold(Continued)																					
Compound Information								**Screening Assays**						**Probe Development Data**							
Compound	**SR-Number**	**Structure**	**CID**	**SID**	**MLS**	**Vendor**	**Vendor Catalog ID**	**AID 1448: TRPML3 Primary 1X%ACT**	**AID 1424: TRPN1 Primary 1X%ACT**	**AID 1526: TRPML3 CRUN 3X%ACT**	**AID 1525: TRPN1 3X%ACT**	**AID 1562: TRPML3 EC50, µM**	**TRPN1 CS DRUN (AID 1682) EC50, µM**	**AID 2510: TRPML3 EC50, µM**	**AID 2583: TRPN1 EC50,**	**AID 2694: TRPML3 Patch Clamp: nA/pF, -80 mV**	**AID 2692: TRPN1 Patch Clamp: nA/pF, -80 mV**	**AID 2719: TRPML3 Fura-2 Imaging: ΔF, 340 nm/ 380 nM**	**AID 2770: Fura-2 Assay: Other channels DF340/ F380**	**AID 602128: TRPML3 EC50, µM**	**AID 602129: TRPML3 EC50, µM**
Analog 6	SR-01000316809-2		846974	125311233	MLS003875010	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: compound was synthesized after these assays were completed.							0.476 (Active)
Analog 7	SR-03000002558-1		53384704	125311234	MLS003875012	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: compound was synthesized after these assays were completed.							17.99 (Inactive)
Analog 8	SR-01000407579-3		772123	125311235	MLS003875011	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Was synthesized to complete SAR after Probe ML289 was fully characterized. While analog 1 is more active than probe ML289 in the primary assays, it has not been subjected to all final characterization assays (e.g., Patch Clamp assay, etc)							3.457 (Active)
Analog 9	SR-01000320627-3		1085524	125311236	None	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: compound was synthesized after these assays were completed.							>29.9 (Inactive)
Analog 10	SR-03000002559-1		53384709	125311237	None	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: compound was synthesized after these assays were completed.							>29.9 (Inactive)
Analog 11	SR-03000002560-1		53384719	125311238	None	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: compound was synthesized after these assays were completed.							>29.9 (Inactive)
Analog 12	SR-01000803473-2		818895	125311239	None	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: compound was synthesized after these assays were completed.							>29.9 (Inactive)
Analog 13	SR-03000002561-1		52418320	125311240	None	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: compound was synthesized after these assays were completed.							>29.9 (Inactive)
Analog 14	SR-01000321000-2		801442	125311241	None	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: compound was synthesized after these assays were completed.							>29.9 (Inactive)
Analog 15 (purchased)	SR-01000216251-1		794388	131269030	None	ChemBridge	5661396	Not tested: this SID is a powder and was not tested during HTS						Not tested: compound was purchased after these assays were completed.							>29.9 (Inactive)
Analog 15 (synthesized)	SR-01000216251-2		794388	125311242	None	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: compound was synthesized after these assays were completed.							

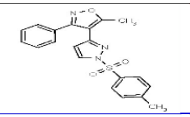
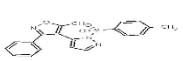
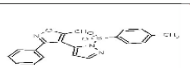
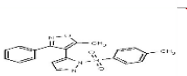
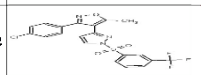
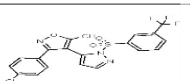
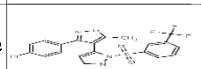
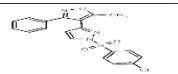
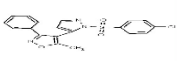
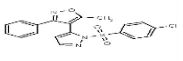
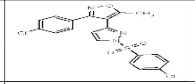
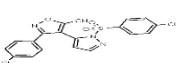
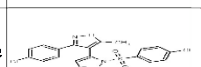
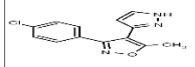
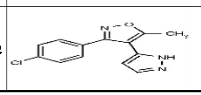
SAR Table 1. Tertiary arylsulfonamides Scaffold(Continued)

Compound Information								Screening Assays						Probe Development Data							
Compound	SR-Number	Structure	CID	SID	MLS	Vendor	Vendor Catalog ID	AID 1448: TRPML3 Primary 1X%ACT	AID 1424: TRPN1 Primary 1X%ACT	AID 1526: TRPML3 CRUN 3X%ACT	AID 1525: TRPN1 3X%ACT	AID 1562: TRPML3 EC50, µM	TRPN1 CS DRUN (AID 1682) EC50, µM	AID 2510: TRPML3 EC50, µM	AID 2583: TRPN1 EC50,	AID 2694: TRPML3 Patch Clamp: nA/pF, -80 mV	AID 2692: TRPN1 Patch Clamp: nA/pF, -80 mV	AID 2719: TRPML3 Fura-2 Imaging: ΔF, 340 nm/ 380 nM	AID 2770: Fura-2 Assay: Other channels DF340/ F380	AID 602128: TRPML3 EC50, µM	AID 602129: TRPML3 EC50, µM
Analog 16	SR-03000002562-1		53384693	125311243	None	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: compound was synthesized after these assays were completed.							>29.9 (Inactive)
Analog 17	SR-03000002563-1		16645312	125311244	None	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: compound was synthesized after these assays were completed.							>29.9 (Inactive)

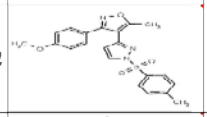
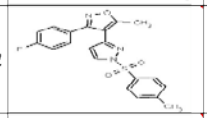
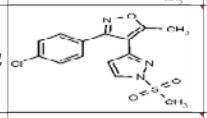
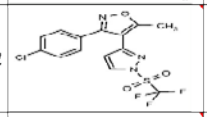
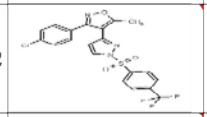
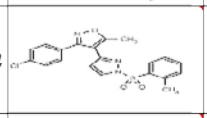
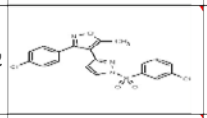
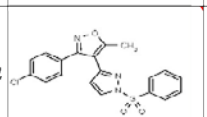
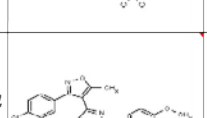
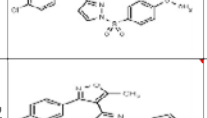
SAR Table 2. Pyrazol-5-yl-isoxazole Scaffold

Compound Information								HTS Assays						Probe Development Assays									
Compound	SR-Number	Structure	Solubility in PBS (µM)	Solubility in PBS-6% FBS (µM)	CID	SID	MLS	Vendor	Vendor Catalog ID	AID 1448: TRPML3 Primary 1X%ACT	AID 1424: TRPN1 Primary 1X%ACT	AID 1526: TRPML3 CRUN 3X%ACT	AID 1525: TRPN1 3X%ACT	TRPN1 CS DRUN (AID 1682) EC50, µM	AID 2510: TRPML3 EC50, µM	AID 2583: TRPN1 EC50,	AID 2694: TRPML3 Patch Clamp: nA/pF, -80 mV	AID 2692: TRPN1 Patch Clamp: nA/pF, -80 mV	AID 2719: TRPML3 Fura-2 Imaging: ΔF, 340 nm/ 380 nM	AID 2770: Fura-2 Assay: Other channels DF340/ F380	AID 602128: TRPML3 EC50, µM	AID 602129: TRPML3 EC50, µM
Probe 2 Compound (6a) (this liquid SID is associated with an incorrect CID structure in PubChem).	SR-01000768170-1		0.1	5.9	2745583	26731169	MLS 001111122	Maybridge	SPB 04991	Active (81.8)	Inactive (1.01)	Active (106.23)	Inactive (0.49	Inactive (>29.9)	Not tested: this SID is an MLSMR liquid and was not tested during MedChem								
Probe 2 (this powder SID was purchased to confirm activity; SID is associated with an incorrect CID structure in PubChem)	SR-01000768170-2				2745583	87692372	None	Maybridge	SPB 04991	Not tested: this SID is a powder and was not tested during HTS					0.2526 (Active)	>29.9 (Inactive)	- 0.14 ±0.02 (Active)	- 0.004 ±0.001 (Inactive)	0.72 ±0.05 (Active)	−0.005 ± 0.002 (Inactive)	SID Not Tested	SID Not Tested
Probe 2 (this powder SID was synthesized to prove that the CID structure in PubChem is incorrect; compound is inactive)	SR-01000768170-3				2745583	124349366	None	TSRI	None	Not tested: this SID is a powder and was not tested during HTS					Not tested: Compound was synthesized after these assays were completed.						>29.9 (Inactive)	SID Not Tested
Probe 2 (ML269) Compound (6a) (this powder was synthesized to prove SAR; compound associated with this structure is active. Note new CID).	SR-03000002360-1		0.02	12	53239838	124349367	MLS00367 5345	TSRI	None	Not tested: this SID is a powder and was not tested during HTS					Not tested: Compound synthesized after these assays were completed.						0.291 (Active)	SID Not Tested

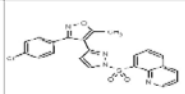
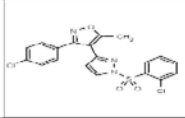
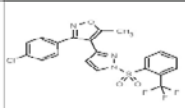
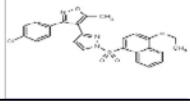
SAR Table 2. Pyrazol-5-yl-isoxazole Scaffold (Continued)

Compound Information								HTS Assays						Probe Development Assays							
Compound	SR-Number	Structure	CID	SID	MLS	Vendor	Vendor Catalog ID	AID 1448: TRPML3 Primary 1X%ACT	AID 1424: TRPN1 Primary 1X%ACT	AID 1526: TRPML3 CRUN 3X%ACT	AID 1525: TRPN1 3X%ACT	AID 1562: TRPML3 EC50, µM	TRPN1 CS DRUN (AID 1682) EC50, µM	AID 2510: TRPML3 EC50, µM	AID 2583: TRPN1 EC50,	AID 2694: TRPML3 Patch Clamp: mA/pF, -80 mV	AID 2692: TRPN1 Patch Clamp: mA/pF, -80 mV	AID 2719: TRPML3 Fura-2 Imaging: ΔF, 340 nm/ 380 nM	AID 2770: Fura-2 Assay: Other channels DF340/ F380	AID 602128: TRPML3 EC50, µM	AID 602129: TRPML3 EC50, µM
Analog 1a	SR-03000002573-1		53384691	125311254	MLS003875016	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: Compound synthesized after these assays were completed.							1.459 (Active)
Analog 1b (MLSMR liquid SID is associated with incorrect structure in PubChem)	SR-01000767070-1		2745600	26731235	MLS001111124	Maybridge	SPB 05016	119.6	Inactive (1.19)	Active (94.33)	Inactive (-1.09	Active (0.819)	Inactive (>29.9)	Not tested: this SID is an MLSMR liquid and was not tested during MedChem.							
Analog 1b (this sample was purchased to confirm HTS activity)	SR-01000767070-2		2745600	87692371	None	Maybridge	SPB 05016	Not tested: this SID is a powder and was not tested during HTS						1.005 (Active)	>29.9 (Inactive)	This SID not tested in these assays					
Analog 1b (this sample was synthesized to obtain correct structure shown)	SR-03000002582-1		2745600	125311263	None	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Compound was synthesized in order to confirm the structure was incorrect in PubChem.							>29.9 (Inactive)
Analog 2a	SR-03000002574-1		53384705	125311255	None	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: Compound synthesized after these assays were completed.							3.336 (Active)
Analog 2b (MLSMR liquid SID is associated with an incorrect structure in PubChem)	SR-01000767049-1		2745598	26731234	MLS001111123	Maybridge	SPB 05013	33.0	Inactive (1.49)	Active (51.03)	Inactive (-0.27	Active (4.26)	Inactive (>29.9)	Not tested: this SID is an MLSMR liquid and was not tested during MedChem.							
Analog 2b (this sample was synthesized to obtain correct structure shown)	SR-03000002583-1		2745598	125311264	None	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: Compound synthesized after these assays were completed.							>29.9 (Inactive)
Analog 3a	SR-03000002576-1		53384724	125311257	None	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: Compound synthesized after these assays were completed.							>29.9 (Inactive)
Analog 3b (MLSMR SID is associated with an incorrect structure in PubChem)	SR-01000631911-2		2745601	26731237	MLS001111125	Maybridge	SPB 05017	Inactive (8.45)	Inactive (2.08)	Not tested due to lack of TRPML3 activity in HTS Primary assay.				Not tested: this SID is an MLSMR liquid and was not tested during MedChem.							
Analog 3b (this sample was synthesized to obtain correct structure shown)	SR-03000002584-1		2745601	125311265	None	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: Compound synthesized after these assays were completed.							>29.9 (Inactive)
Analog 4a	SR-03000002575-1		53384697	125311256	None	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: Compound synthesized after these assays were completed.							15.82 (Inactive)
Analog 4b (the MLSMR liquid SID is associated with an incorrect structure in PubChem)	SR-01000773206-1		2745582	26731233	MLS001111121	Maybridge	SPB 04990	Inactive (3.88)	Inactive (0.14)	Not tested due to lack of TRPML3 activity in HTS Primary assay.				Not tested: this SID is an MLSMR liquid and was not tested during MedChem.							
Analog 4b (this sample was synthesized to obtain correct structure shown)	SR-03000002585-1		2745582	125311266	None	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: Compound synthesized after these assays were completed.							>29.9 (Inactive)
Analog 5 (MLSMR Liquid)	SR-01000761039-1		2745567	26729029	MLS000834032	Maybridge	SPB 04969	Inactive (-1.12)	Inactive (-1.49)	Not tested due to lack of TRPML3 activity in HTS Primary assay.				Not tested: this SID is an MLSMR liquid and was not tested during MedChem.							
Analog 5 (synthesized)	SR-03000002361-1		2745567	124349368	None	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: Compound synthesized after these assays were completed.					>29.9 (Inactive)		Not tested

SAR Table 2. Pyrazol-5-yl-isoxazole Scaffold (Continued)

Compound Information								HTS Assays						Probe Development Assays							
Compound	SR-Number	Structure	CID	SID	MLS	Vendor	Vendor Catalog ID	AID 1448: TRPML3 Primary 1X% ACT	AID 1424: TRPN1 Primary 1X% ACT	AID 1526: TRPML3 CRUN 3X% ACT	AID 1525: TRPN1 3X% ACT	AID 1562: TRPML3 EC50, µM	TRPN1 CS DRUN (AID 1682) EC50, µM	AID 2510: TRPML3 EC50, µM	AID 2583: TRPN1 EC50,	AID 2694: TRPML3 Patch Clamp: nA/pF, -80 mV	AID 2692: TRPN1 Patch Clamp: nA/pF, -80 mV	AID 2719: TRPML3 Fura-2 Imaging: ΔF, 340 nm/ 380 nM	AID 2770: Fura-2 Assay: Other channels DF340/ F380	AID 602128: TRPML3 EC50, µM	AID 602129: TRPML3 EC50, µM
Analog 6	SR-03000002577-1		53384721	125311258	None	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: Compound synthesized after these assays were completed.							2.988 (Active)
Analog 7	SR-03000002578-1		53384692	125311259	MLS003875015	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: Compound synthesized after these assays were completed.							0.68 (Active)
Analog 8	SR-03000002580-1		53384707	125311261	None	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: Compound synthesized after these assays were completed.							>29.9 (Inactive)
Analog 9	SR-03000002581-1		53384683	125311262	None	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: Compound synthesized after these assays were completed.							>29.9 (Inactive)
Analog 10	SR-03000002579-1		53384703	125311260	None	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: Compound synthesized after these assays were completed.							>29.9 (Inactive)
Analog 11	SR-03000002564-1		53384708	125311245	None	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: Compound synthesized after these assays were completed.							4.89 (Active)
Analog 12	SR-03000002565-1		53384678	125311246	MLS003875018	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: Compound synthesized after these assays were completed.							1.945 (Active)
Analog 13	SR-03000002566-1		53384730	125311247	MLS003875019	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: Compound synthesized after these assays were completed.							2.386 (Active)
Analog 14	SR-03000002567-1		53384702	125311248	None	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: Compound synthesized after these assays were completed.							3.78 (Active)
Analog 15	SR-03000002568-1		53384723	125311249	None	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: Compound synthesized after these assays were completed.							>29.9 (Inactive)

SAR Table 2. Pyrazol-5-yl-isoxazole Scaffold (Continued)

Compound Information								HTS Assays						Probe Development Assays							
Compound	SR-Number	Structure	CID	SID	MLS	Vendor	Vendor Catalog ID	AID 1448: TRPML3 Primary 1X% ACT	AID 1424: TRPN1 Primary 1X% ACT	AID 1526: TRPML3 CRUN 3X% ACT	AID 1525: TRPN1 3X% ACT	AID 1562: TRPML3 EC50, µM	TRPN1 CS DRUN (AID 1682) EC50, µM	AID 2510: TRPML3 EC50, µM	AID 2583: TRPN1 EC50,	AID 2694: TRPML3 Patch Clamp: nA/pF, -80 mV	AID 2692: TRPN1 Patch Clamp: nA/pF, -80 mV	AID 2719: TRPML3 Fura 2 Imaging: ΔF, 340 nm/ 380 nM	AID 2770: Fura 2 Assay: Other channels DF340/ F380	AID 602128: TRPML3 EC50, µM	AID 602129: TRPML3 EC50, µM
Analog 16	SR-03000002569-1		53384713	125311250	None	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: Compound synthesized after these assays were completed.							>29.9 (Inactive)
Analog 17	SR-03000002570-1		53384729	125311251	MLS003875017	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: Compound synthesized after these assays were completed.							1.533 (Active)
Analog 18	SR-03000002571-1		53384687	125311252	None	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: Compound synthesized after these assays were completed.							3.576 (Active)
Analog 19	SR-03000002572-1		53384682	125311253	None	TSRI	None	Not tested: this SID is a powder and was not tested during HTS						Not tested: Compound synthesized after these assays were completed.							>29.9 (Inactive)

3.5 Cellular Activity

Fura-2-AM Assays (AID 2719 and AID 2770) Reveal Cellular Activity of Probes. The SRIMSC next pursued cell-based assays to determine whether the powders samples of probes could increase whole cell Ca^{2+} influx in HEK293 cells transfected with human TRPML3**.** These assays were run in a manner similar to the HTS assays (**Figure 8**). Cells transiently expressing human TRPML3 channel or the YFP control plasmid were perfused with test compound (10 µM), followed by measurement of intracellular Ca^{2+} for 2 minutes with the fluorescent indicator fura-2-AM (the membrane-permeable derivative of Fura-2). Compounds were added to cells 20-25 hours after transfection. Values are reported as mean values +/- SEM (n ≥ 3 independent experiments with 20-30 cells). Note that these probes do not increase Ca^{2+} influx in cells expressing the YFP control plasmid (black bar).

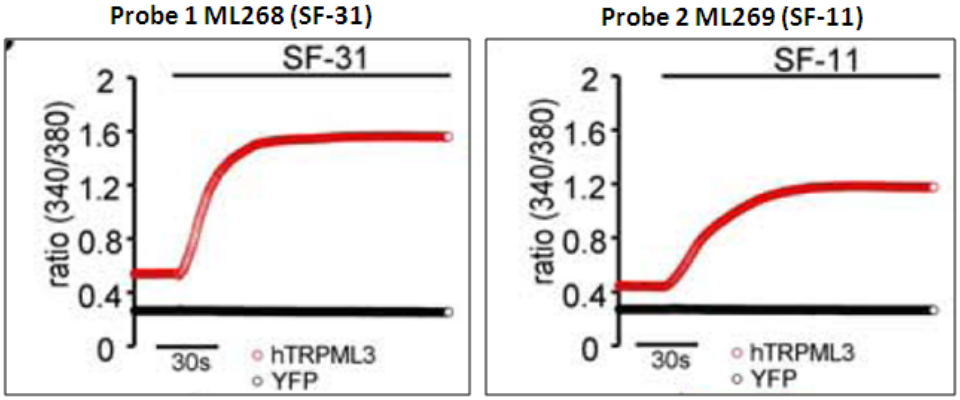


Figure 8. Probes ML268 and ML269 increase TRPML3-mediated intracellular Ca^{2+} flux. Cell-based Fura-2-AM assays using HEK293 cells transfected with human TRPML3 or YFP control plasmid.

Lack of Effect of Probes on Native Cells provides insight into TRPML3 function. Because varitant-waddler epidermal melanocytes express mutant TRPML3 in the plasma membrane, leading to calcium loading, cell death, and variegated coat color [10], we next explored the effect of these TRPML3 probes in a relevant cell type: primary human epidermal skin melanocytes (HEMs). Melanocytes also express all three TRPML channels. Measurements of [Ca2+]i with the fluorescent indicators fura-2-AM (Invitrogen) were performed using a monochromator-based imaging system (iMIC platform and Polychrome V monochromator, TILL Photonics). HEK293 cells, plated onto glass coverslips, were loaded with 4 mM fura-2-AM (Invitrogen) in a standard bath solution (SBS) containing 138 mM NaCl, 6 mM KCl, 2 mM MgCl2, 2 mM CaCl2, 10 mM HEPES, and 5.5 mM D-glucose (adjusted to pH 7.4 with NaOH).

Confirmation of the ability of native HEM TRPML3 to respond was provided by studies using compound SN-2, a cell impermeable compound thought to act at an unknown extracellular site (**Figure 9**, far right bars). However, in contrast to the results with TRPML3-transfected HEK cells, no significant increase in intracellular Ca^{2+} was observed in untransfected HEMs treated with 100 µM of the probe compounds (**Figure 9**). HEMs transfected with TRPML3 do show a calcium response, similar to that of transfected HEK cells.Together these results showing a lack of effect of our probes in non-recombinant HEMs provide three novel insights into native TRPML3 physiology and structure. First, that TRPML3 might be absent from the plasma membrane in native

cells (in contrast to its plasma membrane localization in the varitant waddler mutant). Second, that plasma membrane localization of TRPML3 channels is tightly regulated. Third, that the TRPML3 protein is a subunit of heteromeric channels [15, 16] that are nonresponsive to the probes. Future studies using mouse models alone and in combination with these new probes will allow us and other TRP biologists to explore these questions further.

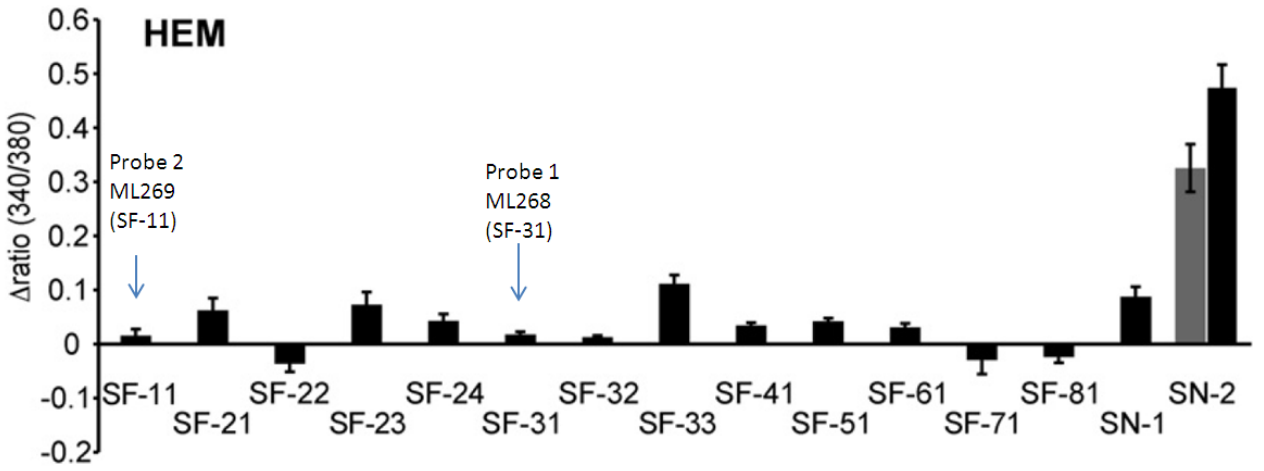


Figure 9. Lack of effect of probes on human primary skin melanocytes (100 µM). Probe 1 (SF-31) and Probe 2 (SF-11) shown along with other compounds tested. See (1) for details. SN-2 is non-cell permeable compound that exhibits TRPML3- activating activity at 30 µM and 100 µM (gray and black bars, respectively) and is hypothesized to act on an extracellular site is not yet identified. See (1) for details.

3.6 Profiling Assays

Fura-2 Assays (AID 2719 and AID 2770) Reveal Cellular Activity and TRPML3 Selectivity. We next pursued additional cell-based TRP channel profiling studies to determine whether compounds identified as selective TRPML3 agonist probe candidates also increase whole cell Ca^{2+} influx in cells transfected with human (h) TRPML3, other human, or murine (m) TRP channels, or zebrafish (dr) TRPN1. In these assays HEK293 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 were added to cells 20-25 hours after transfection (**Figure 10**). The bar diagrams represent average $[Ca^{2+}]_i$ levels 2 min after 10 µM compound application relative to the respective calcium level before application (mean values ± SEM, n ≥3 independent experiments with 20-30 cells). In contrast to our two previously reported TRPML3/2 dual agonist probes, the current probes exhibit no activity on TRPML2. As a result these new probes are a significant improvement over the prior art. Furthermore, because these probes do not increase Ca^{2+} flux in non-transfected (NT) HEK293 cells nor in HEK293 cells expressing the YFP control plasmid, it is highly unlikely that these probes activate non-TRPML3-dependent calcium pathways.

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. The MLSMR liquid sample of probe 1 (SID 14722627) was tested in 521 PubChem BioAssays and active in only 4 (3 TRPML3 agonist assays, and one ER stress assay). The liquid sample of probe 2 (SID 26731169) obtained from a commercial supplier was tested in 371 PubChem Bioassays and active only in 8 assays (3 of these are TRPML3 agonist assays). These TRPML3 agonist probes were inactive in other ion channel assays including TRPN1 (AID 1682), TRPML2 (AID 2770 the X11L calcium channel (AID 2073), and the ROM K+ channel (AID 1918).

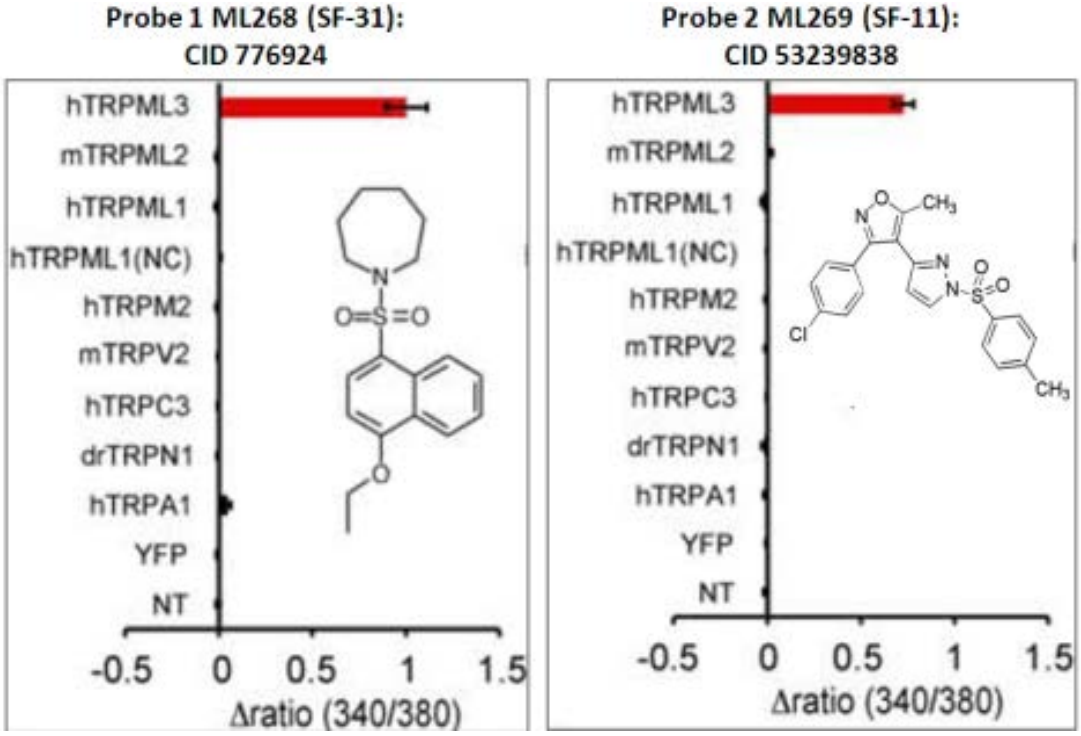


Figure 10. TRP profiling studies. $[Ca^{2+}]_i$ increases of HEK293 cells transiently expressing human (h) TRPML3, or control TRP channels in response to application of 10 µM probes. Note lack of activity of probes for other TRP channels and YFP control. TRPML1 (NC) is a TRPML1 variant that is localized to the plasma membrane. See (1) for details.

4 Discussion

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

As shown in **Table 4** , the new probes ML268 and ML269 demonstrate selective activity against TRPML3. Our prior probes ML122 and ML123 were active against both TRPML2 and TRPML3. In addition, our prior art probe ML123 exhibited activity against TRPN1. As a result, these new selective probes will allow investigations into the specific biology of the TRPML3 channel. This is significant as selective ligands such as ML268 and ML269 can be used to characterize the specific function and interactions of TRPML3. Further, there are currently no known agonists for this channel and the physiologic roles of this TRP ion channel are unknown.

Table 4. Comparison of probes to prior art.

CID/ ML#	TRPML3 EC50 (nM) [SID, AID]	Anti-Target Name	EC50* (μM) [SID, AID]	Fold Selective
Probe 1 CID 776924/ ML268	**POWDER:** 950 nM [SID 87692368, AID 2510]	TRPN1	**LIQUID:** >29.9 [SID 14722627, AID 1682] **POWDER:** >29.9 SID 87692368, AID 2583] >32-fold selectivity over TPRN1 in Fluo-8 screening assays.	**TRPML3 Selective:** **LIQUID** >29-fold **POWDER** >31-fold
		TRPML2	**POWDER:** Inactive [SID 87692368, AID 2770]	
Probe 2 CID 53239838/ ML269	290 nM (synthesis confirms structure) [SID 124349367, AID 602128]	TRPN1	**LIQUID:** >29.9 [SID 26731169, AID 1682] **POWDER:**>29.9 [SID 124349367, AID 2583] >120-fold selectivity over TPRN1 in Fluo-8 screening assays.	**TRPML3 Selective:** **LIQUID** >92-fold **POWDER** >118-fold
		TRPML2	**POWDER:** Inactive [SID 87692372, AID 2770]	
Prior Art CID 2911646/ ML123	451 nM [SID 85786753, AID 1809] 873 nM[SID 24801657,AID 1562]	TRPN1	14.0 μM [SID 85786753, AID 1809] > 29.9 μM [SID 24801657, AID 1682]	Not selective: Dual TRPML3-TRPML2 Agonist
		TRPML2	54.5%ACT	
Prior Art CID 701237/ ML122	1430 nM [SID 24787221,AID 1562] 2000 nM [powder SID 85786752, AID 1809]	TRPN1	> 29.9 μM [SID 24787221, AID 1682] > 29.9 μM [powder SID 85786752, AID 1809]	Not selective: Dual TRPML3-TRPML2 Agonist
		TRPML2	23.5% ACT	

4.2 Mechanism of Action Studies

Probes ML268 and ML269 increase TRPML3 ion channel currents. We next pursued whole cell patch clamp studies to determine whether probe compounds could increase current recordings in TRPML3 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 (**Figure 11**). These assays showed that the probes increase TRPML3 channel activity as measured by the current density. No effect was seen in YFP-transfected cells. See PubChem AIDs 2694 (TRPML3), and AID 2692 (TRPN1) for details.

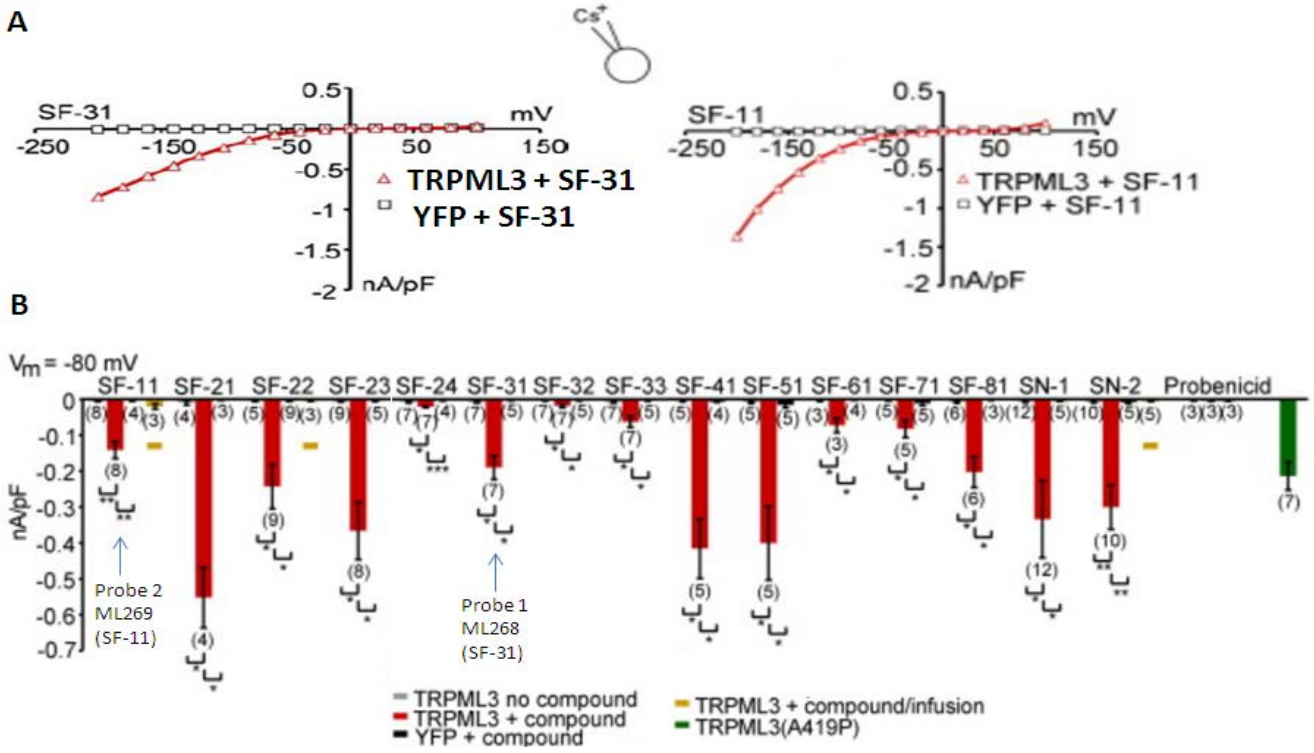


Figure 11. Patch clamp results. A, Steady-state current-voltage plots of whole-cell currents in HEK293 cells transiently transfected with human TRPML3 (triangles) or YFP control plasmid (squares) after perfusion with **Probe 1** (left panel: ML268, SF-31) or **Probe 2** (right panel: ML269, SF-11) (10 μM in standard bath solution) (1). **B,** Bar diagram shows average inward current densities at −80 mV of TRPML3-expressing HEK293 cells before (no compound) and after compound application (+ compound). Cells transfected with the constitutively active human TRPML3(A419P) isoform (green) were used as positive controls, and cells transfected with YFP were used as negative controls. In general, three bars are shown for each test series (no compound and with compound on TRPML3-expressing cells and with compound on YFP-expressing control cells). SF-31 (**Probe 1**) and SF-11 (**Probe 2**) induced current recording values of -0.14 and -0.19 nA/pF, respectively. For compounds SF-11 (Probe 2), SF-22, and SN-2, we tested intracellular application (infusion), resulting in a fourth bar, shown in amber. Compound SF-11 (Probe 2) elicited responses when applied intracellularly, which indicates that this molecule is either cell membrane permeant or that it is able to act on distinct intra- and extracellular activation sites. Compounds SF-22 and SN2 did not elicit a response when infused into the cells, indicating that the compounds act exclusively extracellularly, and suggesting that the compounds are not cell membrane permeable. The control compound probenicid did not activate TRPML3. Statistical comparisons of means were made using one-way ANOVA followed by Tukey's post test (mean values ± SEM, number in parentheses are the number of cells analyzed). ∗∗∗$p < 0.0001$, ∗∗$p < 0.001$, and ∗$p < 0.01$. Note lack of activity on YFP.

RNA Interference Assays (RNAi) to Investigate TRPML3 Heteromers

Heteromerization of TRPML channels has been postulated [15-17]. Previous reports by other groups have suggested that TRPML3 may interact with TRPML1, and that TRPML1 may regulate the plasma membrane concentrations of TRPML3 [16]. **Figure 8** showed that our probes increased calcium flux in TRPML3-transfected HEK cells, with no effect on other TRP channels nor the YFP control plasmid. We next repeated these studies in the relevant native HEM cell line (**Figure 12**, black bars) and found that these same probes had no effect on calcium flux (see **Figure 9**). We hypothesized that the proposed heteromeric channels may have very distinct features from the homomeric channels, thereby altering the calcium response in native cell types. To determine whether the effect in our HEK cells was dependent upon formation of a heteromer between TRPML3 and TRPML1 (since HEK cells express all three TRPML3 channels) we tested the effect of compounds in the presence of shRNA 1208 to block TRPML1 (**Figure 12**, red bars). Specific and efficient RNAi-mediated knock down of endogenous human TRPML1 expression has been recently reported [18, 19]. Oligonucleotide 50-GCTACCTGACCTTCTTCCACA-30 [19] was cloned into the shRNA expression vector U6 RNA Pol III (Invitrogen). HEK293 cells were cotransfected with 3 mg of shRNA to human TRPML1 and 1 mg human TRPML1-YFP plasmid using Genejammer reagent. Human epidermal melanocytes were cotransfected with 5 mg TRPML1 shRNA (1208) using Amaxa Nucleofector technology, followed by treatment with probe compounds and measurement of Fura-2-AM fluorescence ratios. These assays revealed no change in calcium flux upon loss of TRPML1, suggesting that the compounds activate TRPML3 via a mechanism independent of TRPML1. We also tested several other compounds in these assays, in particular the cell-impermeable SN-2, which has no activity on other TRP channels. SN-2 was found to dramatically increase Ca^{2+} flux in native HEMs (**Figure 12**) an effect that was also independent of TRPML1, confirming that this compound may act on an extracellular channel activation site and that heteromer formation between TRPML3 and TRPML1 is not likely in this *in vitro* system.

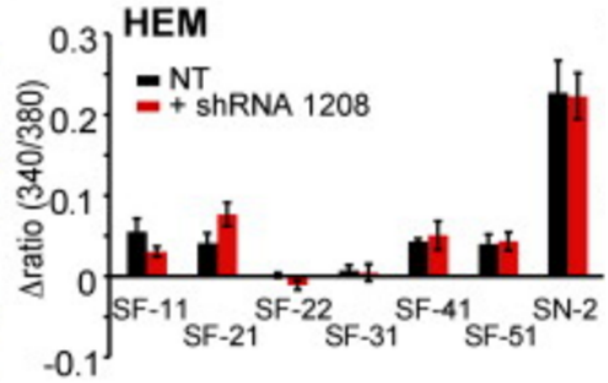


Figure 12. Ca^{2+}-imaging results showing $[Ca^{2+}]_i$ increases in primary human epidermal melanocytes (HEM) Black bars, non-transfected cells. Red bars, HEMs cotransfected with shRNA to TRPML1. Cells were treated with selected TRPML3 activating compounds from 5 different scaffolds (SF-11 (Probe 2), -21, -22(not cell permeable), -31 (Probe 1), -41, and -51) and singleton SN-2 at 30 µM (mean values ± SEM, *n* ≥ 3 independent experiments with 5-10 cells). An expression vector for YFP was cotransfected with the shRNA for identification of the transfected cells. See (1) for details.

4.3 Planned Future Studies

Due to the lack of available compounds known to act as selective TRPML3 agonists (there is no prior art), the identification of these selective probes for TRPML3 will be useful to investigate the function of TRPML3 in inner ear mechanotransduction and hearing biology. Likewise, because the other two related mucolipins TRPML1 and TRPML2 are involved in regulation of lysosomal function as well as intracellular trafficking of membrane proteins, there is a potential role for TRPML3 in these processes as well. Nevertheless, two laboratories recently reported targeted knock outs of the murine *Trpml3* gene and have not detected strong inner ear or other overt phenotypes [20, 21], which suggests that TRPML3's role in cellular physiology and disease remains obscure. It is obvious that these mice have not been characterized thoroughly because functional tests have focused on the inner ear. More experiments are certainly needed to elucidate TRPML3's function. The identified selective probes reported herein will be very useful in this process because the probes enable researchers to characterize the gating of this elusive ion channel as well as structure-function relationships with respect to interactions between probe activators and extracytosolic sodium ions.

The probes generated in this report are first in class, and thus best in class, selective agonists for TRPML3. The leads have excellent selectivity against all other TRP receptors and ion channels tested. SAR studies suggest that additional analogs may exhibit greater potency and may be superior probes than the leads selected. Analog 6 of probe #1 (which was generated after the Fura-2 assays described in Section 3.5 were performed with Probe 1 ML268) is particularly interesting in this regard, as it is the most potent of the TRPML3 agonists identified in this work. Such analogs are likely to be increasingly useful compounds for elucidating the biology of TRPML3.

5 References

1. Grimm, C., S. Jors, S.A. Saldanha, A.G. Obukhov, B. Pan, K. Oshima, M.P. Cuajungco, P. Chase, P. Hodder, and S. Heller, *Small molecule activators of TRPML3.* Chem Biol, 2010. **17**(2): p. 135-48.PMID 20189104.
2. Yamaguchi, S. and S. Muallem, *Opening the TRPML gates.* Chem Biol, 2010. **17**(3): p. 209-10.PMID 20338511.
3. Clapham, D.E., *TRP channels as cellular sensors.* Nature, 2003. **426**(6966): p. 517-24.PMID 14654832.
4. Cuajungco, M.P., C. Grimm, and S. Heller, *TRP channels as candidates for hearing and balance abnormalities in vertebrates.* Biochim Biophys Acta, 2007. **1772**(8): p. 1022-7.PMID 17300924.
5. Gillespie, P.G. and R.G. Walker, *Molecular basis of mechanosensory transduction.* Nature, 2001. **413**(6852): p. 194-202.PMID 11557988.
6. Eberl, D.F., R.W. Hardy, and M.J. Kernan, *Genetically similar transduction mechanisms for touch and hearing in Drosophila.* J Neurosci, 2000. **20**(16): p. 5981-8.PMID 10934246.
7. Qian, F. and K. Noben-Trauth, *Cellular and molecular function of mucolipins (TRPML) and polycystin 2 (TRPP2).* Pflugers Arch, 2005. **451**(1): p. 277-85.PMID 15971078.
8. Atiba-Davies, M. and K. Noben-Trauth, *TRPML3 and hearing loss in the varitint-waddler mouse.* Biochim Biophys Acta, 2007. **1772**(8): p. 1028-31.PMID 17329082.
9. Gong, Z., W. Son, Y.D. Chung, J. Kim, D.W. Shin, C.A. McClung, Y. Lee, H.W. Lee, D.J. Chang, B.K. Kaang, H. Cho, U. Oh, J. Hirsh, M.J. Kernan, and C. Kim, *Two interdependent TRPV channel subunits, inactive and Nanchung, mediate hearing in Drosophila.* J Neurosci, 2004. **24**(41): p. 9059-66.PMID 15483124.

10. Di Palma, F., I.A. Belyantseva, H.J. Kim, T.F. Vogt, B. Kachar, and K. Noben-Trauth, *Mutations in Mcoln3 associated with deafness and pigmentation defects in varitint-waddler (Va) mice.* Proc Natl Acad Sci U S A, 2002. **99**(23): p. 14994-9.PMID 12403827.
11. Nagata, K., L. Zheng, T. Madathany, A.J. Castiglioni, J.R. Bartles, and J. Garcia-Anoveros, *The varitint-waddler (Va) deafness mutation in TRPML3 generates constitutive, inward rectifying currents and causes cell degeneration.* Proc Natl Acad Sci U S A, 2008. **105**(1): p. 353-8.PMID 18162548.
12. van Aken, A.F., M. Atiba-Davies, W. Marcotti, R.J. Goodyear, J.E. Bryant, G.P. Richardson, K. Noben-Trauth, and C.J. Kros, *TRPML3 mutations cause impaired mechano-electrical transduction and depolarization by an inward-rectifier cation current in auditory hair cells of varitint-waddler mice.* J Physiol, 2008. **586**(Pt 22): p. 5403-18.PMID 18801844.
13. Grimm, C., M.P. Cuajungco, A.F. van Aken, M. Schnee, S. Jors, C.J. Kros, A.J. Ricci, and S. Heller, *A helix-breaking mutation in TRPML3 leads to constitutive activity underlying deafness in the varitint-waddler mouse.* Proc Natl Acad Sci U S A, 2007. **104**(49): p. 19583-8.PMID 18048323.
14. Brothers, S.P., S.A. Saldanha, T.P. Spicer, M. Cameron, B.A. Mercer, P. Chase, P. McDonald, C. Wahlestedt, and P.S. Hodder, *Selective and brain penetrant neuropeptide y y2 receptor antagonists discovered by whole-cell high-throughput screening.* Mol Pharmacol, 2010. **77**(1): p. 46-57.PMID 19837904.
15. Zeevi, D.A., A. Frumkin, and G. Bach, *TRPML and lysosomal function.* Biochim Biophys Acta, 2007. **1772**(8): p. 851-8.PMID 17306511.
16. Venkatachalam, K., T. Hofmann, and C. Montell, *Lysosomal localization of TRPML3 depends on TRPML2 and the mucolipidosis-associated protein TRPML1.* J Biol Chem, 2006. **281**(25): p. 17517-27.PMID 16606612.
17. Kim, J., Y.D. Chung, D.Y. Park, S. Choi, D.W. Shin, H. Soh, H.W. Lee, W. Son, J. Yim, C.S. Park, M.J. Kernan, and C. Kim, *A TRPV family ion channel required for hearing in Drosophila.* Nature, 2003. **424**(6944): p. 81-4.PMID 12819662.
18. Miedel, M.T., Y. Rbaibi, C.J. Guerriero, G. Colletti, K.M. Weixel, O.A. Weisz, and K. Kiselyov, *Membrane traffic and turnover in TRP-ML1-deficient cells: a revised model for mucolipidosis type IV pathogenesis.* J Exp Med, 2008. **205**(6): p. 1477-90.PMID 18504305.
19. Samie, M.A., C. Grimm, J.A. Evans, C. Curcio-Morelli, S. Heller, S.A. Slaugenhaupt, and M.P. Cuajungco, *The tissue-specific expression of TRPML2 (MCOLN-2) gene is influenced by the presence of TRPML1.* Pflugers Arch, 2009. **459**(1): p. 79-91.PMID 19763610.
20. Castiglioni, A.J., N.N. Remis, E.N. Flores, and J. Garcia-Anoveros, *Expression and vesicular localization of mouse Trpml3 in stria vascularis, hair cells, and vomeronasal and olfactory receptor neurons.* J Comp Neurol, 2011. **519**(6): p. 1095-114.PMID 21344404.
21. Jors, S., C. Grimm, L. Becker, and S. Heller, *Genetic inactivation of Trpml3 does not lead to hearing and vestibular impairment in mice.* PLoS One, 2010. **5**(12): p. e14317.PMID 21179200.