

UNMCMD Probe Report

Title: Three small molecule pan activator families of Ras-related GTPases

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Assigned Assay Grant #: MH081231-01

Screening Center Name & PI: UNM Center for Molecular Discovery (UNMCMD) formerly NM Molecular Libraries Screening Center (NMMLSC)

Chemistry Center Name & PI: UNMCMD, Larry Sklar

Assay Submitter & Institution: Angela Wandinger-Ness, University of New Mexico

PubChem Summary Bioassay Identifier (AID): 1333, 1334, 1335, 1336, 1337, 1339, 1340, 1341

Probe (1) Structure & Characteristics:

Compound ID 888706

Substance ID 57578335

Molecular Weight 259.32352 [g/mol]

Molecular Formula C₁₄H₁₃NO₂SH-Bond Donor 1

H-Bond Acceptor 3

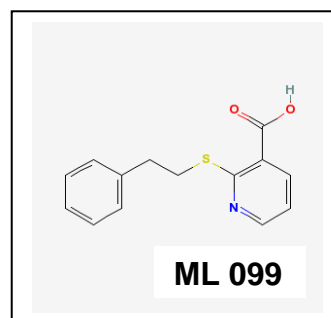
Rotatable Bond Count 5

Exact Mass 259.066699

Topological Polar Surface Area 50.2

Heavy Atom Count 18

Chemical name 2-(2-phenylethylsulfanyl)pyridine-3-carboxylic acid [ML 099]



CID**/ ML #	Target Name [†]	IC50/EC50 (nM) [SID, AID] [†]	Anti-target Name(s) [†]	IC50/EC50 (nM) [SID, AID] [†]	Selectivity*	Secondary Assay(s) Name: IC50/EC50 (nM) [SID, AID] [§]
888706/ ML 099	cell division cycle 42 (GTP binding protein, 25kDa) activated mutant	58.88 [57578335, 1333]	glutathione S- transferase	>100000 [57578335, 1769]	>1000	12.5 [57578335,1762]
888706/ ML 099	cell division cycle 42 (GTP binding protein, 25kDa) wild type	100.00 [57578335, 1334]	glutathione S- transferase	>100000 [57578335, 1769]	>1000	10.5 [57578335,1758]
888706/ ML 099	Ras protein wild type	141.25 [57578335, 1335]	glutathione S- transferase	>100000 [57578335, 1769]	>500	10.8 [57578335,1759]
888706/ ML 099	GTP-binding protein (rab7)	181.97 [57578335, 1336]	glutathione S- transferase	>100000 [57578335, 1769]	>500	5.8 [57578335,1760]
888706/ ML 099	Ras-related protein Rab-2A	354.81 [57578335, 1337]	glutathione S- transferase	>100000 [57578335, 1769]	>200	6.2 [57578335,1763]
888706/ ML 099	Rac1 protein activated mutant	25.42 [57578335, 1339]	glutathione S- transferase	>100000 [57578335, 1769]	>1000	nd
888706/ ML 099	Rac1 protein wild type	20.17 [57578335, 1340]	glutathione S- transferase	>100000 [57578335, 1769]	>1000	nd
888706/ ML 099	Ras protein activated mutant	95.50 [57578335, 1341]	glutathione S- transferase	>100000 [57578335, 1769]	>1000	16.6 [57578335,1761]

Probe (2) Structure & Characteristics:

Compound ID 7345532

Substance ID 57578337

Molecular Weight 309.35906 [g/mol]

Molecular Formula C19H19NO3

XLogP3-AA 3.9

H-Bond Donor 1

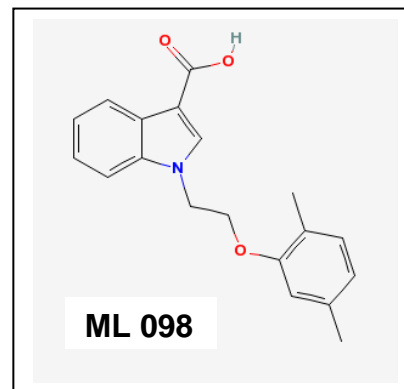
H-Bond Acceptor 3

Rotatable Bond Count 5

Topological Polar Surface Area 51.5

Heavy Atom Count 23

Chemical name 1-[2-(2,5-dimethylphenoxy)ethyl]-1H-indole-3-carboxylic acid [ML 098]



CID**/ ML #	Target Name [‡]	IC50/EC50 (nM) [SID, AID] [†]	Anti-target Name(s) [‡]	IC50/EC50 (nM) [SID, AID] [†]	Selectivity*	Secondary Assay(s) Name: IC50/EC50 (nM) [SID, AID] [§]
7345532/ ML 098	cell division cycle 42 (GTP binding protein, 25kDa) activated mutant	354.81 [57578337, 1333]	glutathione S-transferase	>100000 [57578337, 1769]	>200	nd
7345532/ ML 098	cell division cycle 42 (GTP binding protein, 25kDa) wild type	588.84 [57578337, 1334]	glutathione S-transferase	>100000 [57578337, 1769]	>100	nd
7345532/ ML 098	Ras protein wild type	346.74 [57578337, 1335]	glutathione S-transferase	>100000 [57578337, 1769]	>200	nd
7345532/ ML 098	GTP-binding protein (rab7)	77.62 [57578337, 1336]	glutathione S-transferase	>100000 [57578337, 1769]	>1000	nd
7345532/ ML 098	Ras-related protein Rab-2A	158.48 [57578337, 1337]	glutathione S-transferase	>100000 [57578337, 1769]	>500	nd
7345532/ ML 098	Rac1 protein activated mutant	223.87 [57578337, 1339]	glutathione S-transferase	>100000 [57578337, 1769]	>200	nd
7345532/ ML 098	Rac1 protein wild type	794.32 [57578337, 1340]	glutathione S-transferase	>100000 [57578337, 1769]	>100	nd
7345532/ ML 098	Ras protein activated mutant	194.98 [57578337, 1341]	glutathione S-transferase	>100000 [57578337, 1769]	>500	nd

Probe (3) Structure & Characteristics:

Compound ID 2160985

Substance ID 57578338

Molecular Weight 307.13934 [g/mol]

Molecular Formula C₁₄H₁₁BrO₃

XLogP3-AA 3.6

H-Bond Donor 1

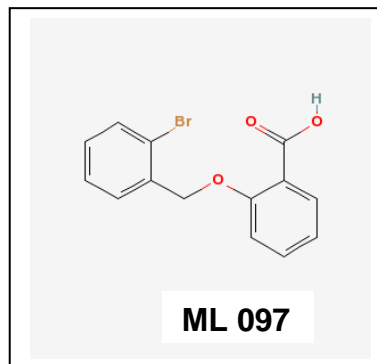
H-Bond Acceptor 3

Rotatable Bond Count 4

Topological Polar Surface Area 46.5

Heavy Atom Count 18

Chemical name 2-[(2-bromophenyl)methoxy]benzoic acid [ML 097]



CID**/ ML#	Target Name [‡]	IC50/EC50 (nM) [SID, AID] [†]	Anti-target Name(s) [†]	IC50/EC50 (nM) [SID, AID] [†]	Selectivity*	Secondary Assay(s) Name: IC50/EC50 (nM) [SID, AID] [§]
2160985/ ML 097	cell division cycle 42 (GTP binding protein, 25kDa) activated mutant	50.11 [57578338, 1333]	glutathione S- transferase	>100000 [57578338, 1769]	>1000	nd
2160985/ ML 097	cell division cycle 42 (GTP binding protein, 25kDa) wild type	102.32 [57578338, 1334]	glutathione S- transferase	>100000 [57578338, 1769]	>500	nd
2160985/ ML 097	Ras protein wild type	109.64 [57578338, 1335]	glutathione S- transferase	>100000 [57578338, 1769]	>500	nd
2160985/ ML 097	GTP-binding protein (rab7)	20.41 [57578338, 1336]	glutathione S- transferase	>100000 [57578338, 1769]	>500	nd
2160985/ ML 097	Ras-related protein Rab- 2A	Inactive [57578338, 1337]	glutathione S- transferase	>100000 [57578338, 1769]		nd
2160985/ ML 097	Rac1 protein activated mutant	81.28 [57578338, 1339]	glutathione S- transferase	>100000 [57578338, 1769]	>1000	nd
2160985/ ML 097	Rac1 protein wild type	151.35 [57578338, 1340]	glutathione S- transferase	>100000 [57578338, 1769]	>500	nd
2160985/ ML 097	Ras protein activated mutant	93.32 [57578338, 1341]	glutathione S- transferase	>100000 [57578338, 1769]	>1000	nd

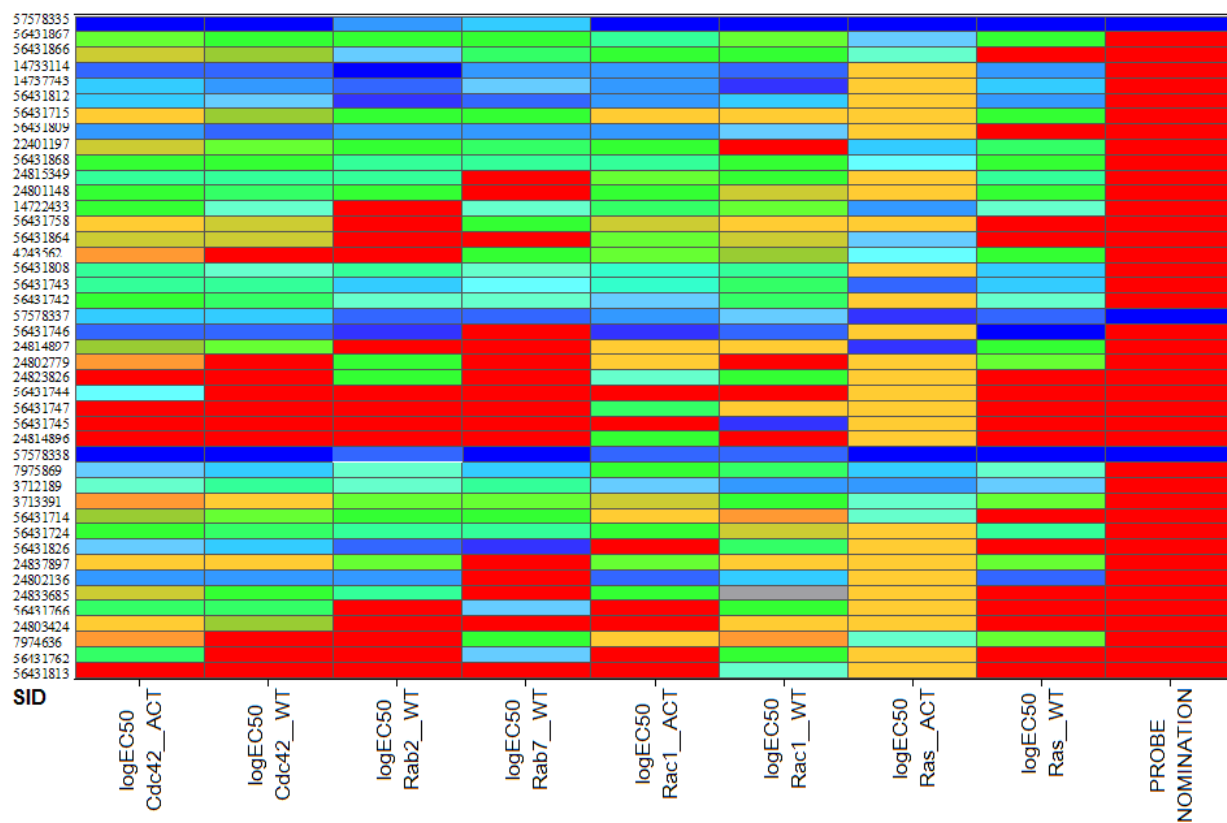
[‡]Short descriptive name of target or pathway (similar for antitarget, if applicable).

[†]IC50/EC50 value in nM along with the PubChem SID and AID where this value can be found.

[§]For secondary assay provide the following information: Name, IC50/EC50 [SID, AID]

*Selectivity = antitarget IC50/target IC50

**Provide the PubChem CID for the probe.



Biological activity (logEC50) heatmap of the 43 compounds across 8 assays. Shades of blue reflect better biological activity, whereas green-to-red reflects reduced activity. The right column highlights the 3 candidates for chemical probe nomination.

Probe	Pub Chem Assays tested	PubChem Assays active	All assays other than GTPases	Confirmatory Assays
1 (CID 888706)	312	24	4 (nuclear factor kappa-B, subunit 1; Allosteric Modulators of D1 Receptors ; β -glucocerebrosidase; small molecule inhibitors of MT1-MMP transcription)	1 (MT1-MMP EC50 = 20 μ M)
2 (CID 7345532)	152	12	0	0
3 (CID 2160985)	151	10	1 (Beta lactamase)	0

This table shows that these probe molecules, taken together, have only been found active in 1 PubChem confirmatory assay, and that the activity is 200 times lower than reported here. The first probe is reported in the patent literature only as a reactive intermediate.

Summary of Recommended Usage and State of the Art of G Protein Activators

Abnormal changes in the activation of small G proteins promote transformation of cells towards malignancy and other disease states. The mutated forms of small GTPases induce proliferation and transformation of a number of cell types as well as differentiation of neuronal cells. These findings have attracted the attention of scientists in cancer research as well as in variety of other fields, emphasizing importance of the identification of regulators of the activity of small GTPases. Recent studies have mainly been focused on the identification of GTPase inhibitors. To our knowledge, small molecule activators of low molecule weight GTPases, with the possible exception of aluminum tetrafluoride which activates the GDP bound form are unknown.

There is considerable data which suggests that many diseases are related to decreased activity of small GTPases (1-15). The only known activation mechanism is via guanine nucleotide-exchange factors (GEFs). These multidomain proteins accelerate the exchange of GDP by GTP by several orders of magnitude. GEFs catalyze the dissociation of the nucleotide from the G protein by modifying the nucleotide-binding site such that the nucleotide affinity is decreased and the GDP is released and replaced with GTP (since the cellular concentration of GTP is ten times higher compared to GDP). The molecules described in this report function by increasing the affinity of the GTPases for guanine nucleotides. We propose that in biochemical systems, these novel small activators should prove useful: 1) in defining the mechanism by which GEF regulates GTPase activity; 2) in defining protein-protein interactions between GEFs and GTPases; 3) defining the sites of GTPase regulation in crystal structures.

The activity of GTPases in cellular systems has been extensively characterized:

A) Rho GTPases: Rac2 is selectively expressed in immune cells and human Rac2 mutations result in immunodeficiency (8). Therefore, activators of Rac2 or other Rac family members might be of benefit in these diseases. Rac1 is important for maintaining hair follicle integrity and a rac1 deficiency in the skin causes nearly complete hair loss in an animal model (9). Could a topically applied Rac1 activator prevent hair loss? Cdc42 is crucial for maintenance of epithelial cell polarity (10). Activators of Cdc42 function might have a beneficial function in solid tumors and ameliorate faciogenital dysplasia where Cdc42 GEF is mutant. Rho GTPases affect cell proliferation and migration (11). In some cases underexpression is associated with cancer. Could an activator be beneficial in such cases?

Rab GTPases: Mutations in Rab7 cause neurologic disease Charcot-Marie-Tooth disease, an autosomal dominant disease (12). Thus, if the activity of the functional wild-type protein could be increased selectively, it might ameliorate disease pathogenesis. Mutations in the Rab8 effector optineurin cause open angle glaucoma (13). Activators of Rab8 might provide a bypass to the effector or enable an inefficient effector to function and improve patient outcomes. Point mutations in the Rab3a nucleotide binding domain with reduced nucleotide binding activity cause anomalies in circadian behavior and homeostatic response to sleep loss (14) Thus, activators can be used to test the impact of selectively increasing the GTP-bound pool of proteins bearing a point mutant on learning and memory and emotion control.

C) Ras GTPases: KRas is implicated in cardiac development and can be substituted for by HRas except in cardiovascular homeostasis (15). Activators of KRas may be useful in treatment of cardiomyopathy.

In cellular systems, some potential uses of the new activators could represent: 1) ameliorating mutations in GTPases and GEF proteins, thereby increasing the function of the wild-type or mutant GTPases; 2) however, because the activators described are not selective, one might imagine using the small molecules in conjunction with siRNA knockdowns of the target GTPase, evaluating cell function before and after activator administration.

References for Summary

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1. Scientific Rationale for Project

More than 150 small G proteins (small GTPases) have been identified as monomeric molecules with molecular masses of about 20 - 40 kDa that bind and hydrolyze guanine nucleotides. Functions for many of the small GTPases remain to be discovered, but it is obvious that they are very important intracellular signaling proteins that control diverse cellular functions including cell proliferation, survival and apoptosis, cell-to-cell and cell-to-extracellular matrix adhesion, cytoskeleton organization, transcription regulation, cell cycle progression, cell migration, cellular morphogenesis and polarization (1,2).

The mutated forms of small GTPases induce proliferation and transformation of the number of cell types, and differentiation of neuronal cells (3-5). Today, it is well established that deregulation or abnormal activation of these proteins are linked to disease processes (mostly promote malignant transformation of cell) (6,7). For this reason small GTPases represent a large class of potential drug targets which have been intensively pursued by the pharmaceutical industry (8,9). Currently, there are limited pharmacological tools targeting small GTPases, and most of the efforts have been focused on inhibiting the lipid modifications of GTPases, necessary for their plasma membrane localization and activation (10). Unfortunately, these inhibitors and drugs are not specific to GTPases and affect other cell signaling pathways, which complicate the interpretation of results.

According to molecular structures, small GTPases exhibit two inter-converting forms: GDP-bound inactive and GTP-bound active forms. GTP/GDP exchange studies usually require guanine nucleotide analogues, which behave similarly to the native species and have been modified such that they can be sensitively detected. The most common modification involves insertion of radiolabeled isotopes of phosphorous or sulfur to yield [γ -³²P] GTP and [γ -³⁵S] GTP γ S. While these analogs are very sensitive, their use has obvious drawbacks for HTS or other discovery approaches. For this reason, usage of fluorescently labeled nucleotides became more popular. Recently developed BODIPY dye-labeled nucleotides are excellent tools for characterization of nucleotide binding of proteins (11,12).

The fluorescence of BODIPY-guanine nucleotides are directly affected by protein binding. Unbound nucleotides exhibit quenched fluorescence, which is disrupted upon binding to proteins, resulting in a 2-10-fold fluorescence enhancement that allows real-time detection of protein-nucleotide interactions. For this reason, a sensitive fluorescence method is very useful for kinetic and high-throughput screening (HTS) studies. We had recently developed a bead-based flow cytometric, fluorescent GTP-binding assay which is highly sensitive and allows real-time measurements (13) that was applicable for HTS.

Here, for the first time we extended the novel bead-based format for a multiplexed target assay allowed us to perform a screen of the 200,000 compound MLSMR for identification of small molecules which alter GTP binding to small GTPases. The unique multiplexing capabilities of flow cytometry enabled the simultaneous quantitative analysis of the activation or inhibition of six or more GTPases immobilized on color-coded beads, leading to more than 1.2 million measurements. Three small molecule families with activating effects on GTP-binding activity were identified and reported here. High-throughput cytometry screening results were confirmed by biochemical and cell-based secondary screening data. Pull-down assay of active Rac1 showed that an activator increased Rac1 activity in Swiss 3T3 cells. Live cell imaging and confocal microscopy studies revealed the activator-induced actin reorganization and cell morphology changes, characteristic to Rho GTPase family activation. Families of pan GTPase activators have the potential of being used broadly to determine when small GTPases are participating in any cellular signaling process of interest.

2. Project Description

a. Describe the original goal for probe characteristics as identified in the CPDP¹.

Overview. This target, which grew out of a collaboration between the Center and the target provider was assigned in MLSCN Cycle 5, prior to the time when a CPDP was required. As the first large multiplex performed by our Center on the complete MLSMR available at that time, it provided an extraordinarily rich data set which has continued to be evaluated through the so-called MLSCN Pilot Phase “Phase-out” period. A multiplex target has the potential to yield small molecules that are activators or inhibitors of individual targets, the entire set of targets, or a subset of targets. The primary screen and dose-response yielded apparently non-selective activators, and inhibitors with varying degrees of selectivity. Unfortunately, convenient cell-based secondary assays were not part of the R03 submission and the Center and the target provider worked hard to develop novel secondary assays with variable, but low throughput once the screen had been performed.

The characteristics of the novel small molecule activators of GTPases include the ability to modulate GTP-binding and protein-protein interaction activities of members of the GTPase families represented by the series of targets included in this multiplex, with a potency of at least 100 nM. While 3 families of probe scaffolds have been identified, we chose to characterize in extensive detail the probe identified for the first family in the extended project description below. (Figures, legends, and detailed methods are provided at the end of this section. The compound referred to in this section as MLS00088004 is Probe 1 also known by Compound ID 888706 or Substance ID 57578335.)

However, in the MLSCN Phase-out period, a CPDP for a selective GTPase inhibitor probe has also been developed with the Kansas University Chemistry Specialty Center. KUSCC is currently evaluating the results presented in this probe report for the possibility of synthetically creating selective activators. We have retained the lead probes from all three scaffold families to provide an optimal opportunity should we agree to proceed to selectivity.

Compound identification and verification using multiplex screen. For detection of GTP binding to small G-proteins we used a flow cytometry-based assay¹³ that allowed us to measure a dose response of GTP binding to individual GST-tagged small G-proteins.

It was observed that different conditions were required for the optimal activity of different GTPases. BODIPY-GTP γ NH was identified as a better substrate for some of the small GTPases such as RhoA wt, Rac1wt; Cdc42, while others (Rab proteins and Ras) prefer binding to BODIPY -FL-GTP. Mg²⁺ ions were crucial for the Rho enzyme activity, while Rab proteins and H-Ras more effectively bound fluorescent GTP in the presence of EDTA. Activity of H-Ras was completely inhibited in the presence of 0.01% dodecyl maltoside in the assay, while this detergent had negligible effect on GTP binding by other GTPases.

Based on these preliminary results, we had chosen specific conditions which are not optimal for all GTPases, but allow us to simultaneously measure the GTP binding of several small GTPases. This approach was used for multiplex analysis of GTPase activity and, therefore, HTS of the MLSMR. This is a low-cost, time saving and highly efficient method for the simultaneous measurements of the activity for the number of proteins. Moreover, it simplifies analysis, comparison and detection of the specific activators/inhibitors of individual GTPases.

In primary screening, each well contained beads of seven different red fluorescent intensities, coupled with individual GST-GTPases. This mixture was incubated for 45 min with 100 nM

fluorescent GTP in the presence of 1 μ M compound, and protein binding fluorescence was measured using a flow cytometer with the beads delivered by a HyperCyt system²⁰. Briefly, HyperCyt consists of an autosampler connected to a peristaltic pump: the autosampler sips ~2 μ l of suspension from each of the wells of a multi-well plate, leaving an air gap between samples.

The primary screening of one plate of the MLSMR library using bead-based multiplex method is presented in Fig. 1. Fig. 1B demonstrates that only one compound out of 320, had an effect on BODIPY -FL-GTP binding. Analysis of the row data with IDLeQuery software, GraphPad Prism and Excel template revealed that this activator was MLS000088004 (2-[(2-phenylethyl)thio]nicotinic acid. IUPAC Name: 2-(2-phenylethylsulfanyl)pyridine-3-carboxylic acid) which non-specifically activated small G-proteins (Fig. 1D).

The MLSCN library of 194,738 compounds was screened in a multiplex for potential activators and inhibitors of fluorescent GTP binding to six different GTPase proteins: Cdc42, Rac1, Rac1 activated mutant, Rab2, Rab7, and Ras. The results from the multiplex screen were published on PubChem (<http://pubchem.ncbi.nlm.nih.gov/>) with PubChem Bioassay identification numbers of 761, 757, 764, 758, 759, respectively. The quality control Z' statistics were very good for each target. In particular, the average Z' was 0.87 +/-0.04 for Cdc42, 0.85 +/- 0.04 for Rac1, and 0.90 +/- 0.03 for Rac1 activated mutant (Fig. 1C).

Dose response analysis. To verify the primary screen results, we used dose response assays with one multiplex (Rab7 wt, Rab2 wt, H-Ras wt, constitutively active mutant H-RasG12V, Cdc42 wt, and constitutively active mutant Cdc42Q61L) and 3 single-plexes (for Rac1 wt, constitutively active mutant Rac1Q61L and GST-GFP). GST-GFP single-plex was used to eliminate the possibility that compound affects GST-protein binding to beads.

Fig. 1 shows that MLS00088004 behaved as a non-selective, high affinity activator of eight different GTPases (Fig. 1E; EC₅₀ 1.6 x 10⁻⁸ – 1.4 x 10⁻⁷ M), though Rho-family GTPases (H-Rac1 and Cdc42) showed the greatest sensitivity to the compound. Table 1 shows that EC₅₀, we have identified a novel compound with nanomolar affinity for the Ras-family GTPases and particular affinity for Rac1 and Cdc42 relative to members of the Ras and Rab subfamilies.

Small molecules modulate Rho-family GTP-binding kinetics and *in vitro* activation. When assays were performed at a fixed concentration of fluorescent GTP and optimal activator concentration, the magnitude of activation appeared to vary widely among the GTPase families, potentially because the K_d of the interaction between fluorescent GTP and/or the activator varies for individual GTPases. To explore this possibility, we varied the concentration of the fluorescent GTP as shown in Fig. 2A, B and quantified in the Table (Fig. 2C) where the apparent B_{max} is shown to increase (1.2 to 1.8 fold) and the apparent K_d in the presence or absence of MLS000088004 were performed using wild-type Cdc42 and our established flow cytometry based assay 28 (Fig. 2D). The results are consistent with the activator increasing the affinity of nucleotide binding. All of the families in this report have been shown to exhibit an increase in affinity (data not shown). Real-time assays of GTP-binding kinetics of dissociation typically exhibits a corresponding decrease (1.2-3.1 fold).

In vitro detection of active Rac 1. Two different approaches were used for detection of Rac 1 activation *in vitro*. Both of these methods are based on so-called pull down assay of Rac. This method is well known and widely used for detection of active Rac1 and Cdc42. It is based on the fact that only active, GTP bound form of Rac is capable of binding to protein binding domain of PBD of the serine/threonine kinase PAK, and activating this enzyme. To measure the *in vitro* activation of Rac1 by MLS000088004, His-Rac1 was incubated with increasing concentrations of the compound (0-12.5 μ M) in the presence of GTP γ S to prevent hydrolysis during the assay. The fraction of active His-Rac1 was identified by binding to GST-PAK-PBD immobilized on

glutathione beads and immunoblot analysis of bound Rac1. The precipitated GTPase was then quantified by Western blotting, using specific antibodies¹⁶. A dose dependent increase in Rac1 activation in the presence of increasing amounts of MLS000088004 was clearly evident (Fig. 3A). In second set of experiments, Swiss 3T3 cells were used to analyze the effect of MLS000088004 on Rac1 activation. For positive control, the cells were treated with 10 ng/ml EGF for 2 min. As shown in Fig. 3B, resting starved 3T3 cells treated with DMSO had low levels of active Rac1 (lane 1). Active Rac1 was dramatically elevated following treatment with MLS000088004 (lane 2).

Cell-based assay. GTPases play a key role in actin re-organization and morphological transformations of the cell. Rac1 promotes polymerization at the leading edge, orchestrating the formation of lamellipodia and membrane ruffles²¹, while Cdc42 is responsible for filopodia formation²². As was mentioned above, MLS000088004 behaved as a non-selective activator, but Rho-family GTPases, Rac1 and Cdc42 showed the greatest sensitivity to the compound. For this reason, the cell-based assay was used to examine the effect of the compound on cell morphology and actin re-organization in RBL 2H3 cells. This cell line has been widely used as a model for studying of hypersensitivity diseases that include allergy, asthma, and anaphylaxis.

RBL cells can be activated via several pathways (IgE cross-linking or aggregation of GPI-anchored proteins²³. Cross-linking of the high affinity IgE receptor (Fc ϵ RI) activates the main pathway, which initiates cascades of biochemical events leading to degranulation, membrane ruffling and other physiological responses²⁴. Early stages of activation are characterized by an increase of tyrosine phosphorylation of proteins (key enzymes are Syk and Lyn), while secretion of inflammatory mediators is observed at later stages of activation. In addition to secretion, activated cells begin to flatten (within 2min), spread on their substratum, and extend lamellipodia which show active ruffling^{24, 25}.

Recent studies indicate that the same GTPases are involved in activation of the RBL cells. Stable expression of dominant negative Rac and Cdc42 in RBL cells inhibits Fc ϵ RI-mediated degranulation²⁶. The lethal toxin of *Clostridium sordellii* which inactivates Rac, and possibly Cdc42 (but not Rho) inhibits Fc ϵ RI-mediated tyrosine phosphorylation of phospholipase C, inositol phosphate formation, calcium mobilization and secretion of hexosaminidase suggesting a crucial role of Rac/Cdc42 in RBL cell signaling²⁷.

Treatment of resting RBL cells with MLS000088004 induces ruffling, spreading and flattening. We examined the actin cytoskeleton structures of the resting, ligand stimulated and MLS000088004 treated RBL cells. Cells were grown overnight with or without 10 μ M MLS000088004. Immunofluorescence staining with rhodamine-phalloidin and confocal microscopy were used to detect the changes in cell shape, as well as reorganization of F-actin. Resting RBL cells were round and tall or modestly spread on the adherent growing surface (Fig. 4A). Activation of RBL cells by cross-linking of Fc ϵ RI caused a remarkable transformation in cell surface topography, and the cell height decreased compared to DMSO controls. Cells lost their spherical shape and became spread and elongated on the substratum with actin becoming localized to the cell perimeter, leading edge and surface ruffles (Fig. 4). The same reorganization of actin and cell shape (dramatic increase in cell spreading and flattening) was observed after treatment of resting RBL cells with MLS000088004.

Live cell imaging. Live cell imaging studies revealed that whereas DMSO-treated control resting cells maintained a spherical shape with few protrusions (Fig. 5A), ligand-stimulated (cross-linking of IgE receptors) cells began flattening, ruffling and forming lamellipodia and filopodia within a minute after stimulation, and surface ridges were enhanced after 15-20 min (Fig. 5B). Addition of 10 μ M activator to resting RBL cells induced formation of lamellipodia and microspikes within 10 min, and by 25 min cells were flattened and surface area was significantly increased (Fig. 5C).

Taken together, our observations demonstrate that small GTPase activator MLS000088004 induced similar morphological changes as cell activation via IgE receptor cross-linking. The magnitudes of those reorganizations are comparable. Live cell image figures show that formation of lamellipodia and filopodia after addition of GTPase activator occurred rapidly and on a similar timescale as receptor-mediated activation. All of the probe families have been shown to induce morphological changes (data not shown) and quantitative dose-response analysis is currently being undertaken.

Signaling pathways upregulated by MLS000088004 are different from those induced by IgE receptor activation. To determine if morphological changes induced by MLS000088004 involve the same signaling pathway as IgE receptor cross-linked stimulation, we examined the effect of this compound on Syk phosphorylation and β -hexosaminidase release (early and late phase activation markers, respectively). Syk tyrosine kinase is tyrosine-phosphorylated in the early stages of RBL cell activation via several pathways (IgE cross-linking, or aggregation of GPI-anchored proteins)^{18, 28, 29} and plays key role in activation of RBL cells. To determine the effect of MLS000088004 on Syk phosphorylation, resting RBL cells were treated with indicated concentration of the activator (Fig. 6A). Immunoprecipitation with anti-Syk polyclonal antibody and immunoblot with phosphotyrosine-specific antibody indicated that treatment of RBL cells with MLS000088004 did not initiate Syk tyrosine-phosphorylation (Fig. 6A).

Last, we examined the effect of MLS000088004 on RBL cell activation, characterized by β -hexosaminidase secretion. Fig. 6B (black bars) shows that in the absence of ligand stimulation there was no β -hexosaminidase release in MLS000088004-treated cells, indicating that compound is not toxic. In RBL cells activated via IgE-receptor cross-linking, the presence of MLS000088004 had no further stimulatory effect on secretion (Fig. 6B open bars). These data indicate that, MLS000088004 compound induces morphological changes of RBL cells via activation of small GTPases Rac and Cdc42. However, these changes do not involve traditional, Fc ϵ RI -induced signaling pathway.

Summary. A multiplex flow cytometric assay was used for HTS of MLSCN library. Nearly 200,000 compounds were screened for identification of potential activators and inhibitors of GTP binding to six different small GTPases. This approach allowed us to collect and analyze more than 1.2 million data points in the primary screen. 100-500 positive compounds for each member of multiplex were identified by primary screening. (The results from this multiplex screen were published on PubChem <http://pubchem.ncbi.nlm.nih.gov/>, with PubChem Bioassay identification numbers of 761, 757, 764, 760, 758, 759, respectively). False positives such as autofluorescent compounds and compounds affecting GST-protein binding to glutathione-beads were eliminated, and ~1000 compounds were selected and tested in secondary dose-response assays. Dose-response results were reported in PubChem (AID 1333-1337, 1339-1341) with compounds categorized as having activating or inhibitory effect on one or more of the GTPases tested, including Ras (wild-type and activated), Rab2, Rab7, Rac1 (wild-type and activated), Cdc42 (wild-type and activated), and RhoA. Flow cytometry data of primary screening were confirmed with dose-response analysis and various biochemical and cell-based assays. Pull-down assay of active Rac1 shows that MLS88004 increases Rac1 activity in Swiss 3T3 cells. Live cell imaging and confocal microscopy studies revealed that the activator-induced actin reorganization and cell morphology changes characteristic of Rho GTPase activation.

On the following pages are Figures, Legends, and detailed Materials and Methods for Section 2a.

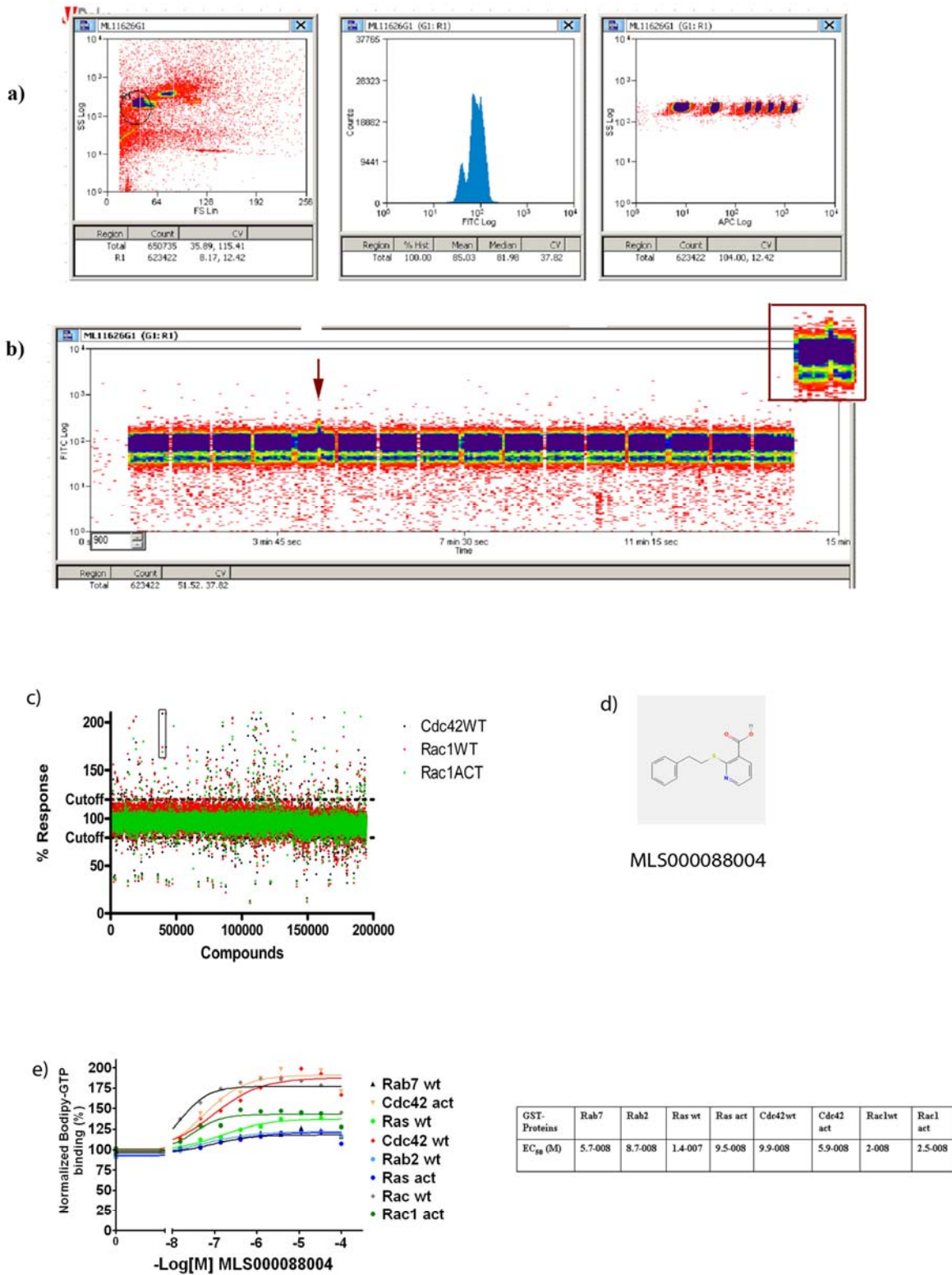
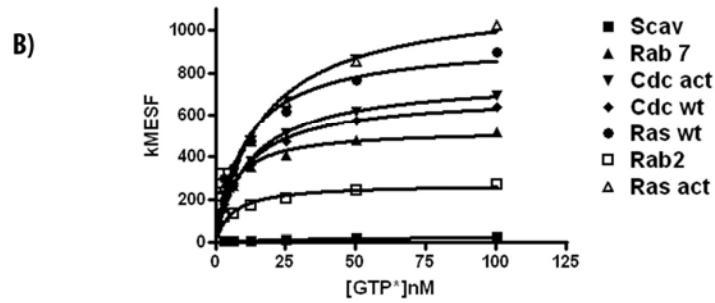
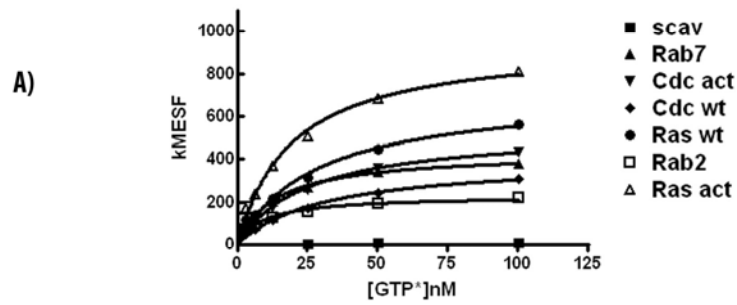
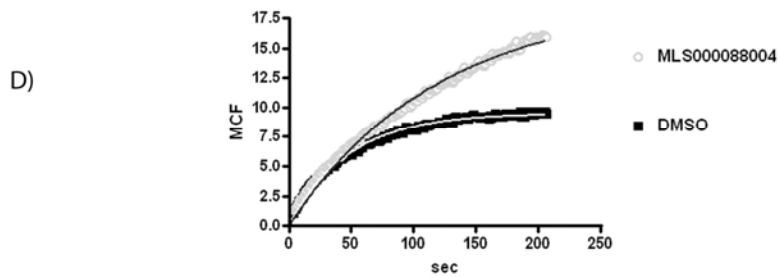


Fig 1



C)

		Rab7 wt	Cdc42 wt	Cdc 42 act	H-Ras wt	H-Ras act	Rab2 wt
BMAX (kMESF)	DMSO	433.8 ± 9.2	397.9 ± 13.4	541.3 ± 13.6	713.1 ± 37.2	949.9 ± 29.3	231.3 ± 7.4
	88004	530.2 ± 11.3	694.1 ± 11.5	773.8 ± 14.5	948.9 ± 34.6	1160 ± 41.5	272.8 ± 7.9
KD (nM)	DMSO	13.9 ± 0.9	30.6 ± 2.5	25.3 ± 1.6	28.6 ± 3.7	19.4 ± 1.6	9.6 ± 1.1
	88004	5.8 ± 0.5	10.5 ± 0.6	12.5 ± 0.7	10.8 ± 1.3	16.6 ± 1.7	6.2 ± 0.7



	DMSO	MLS000088004
Best-fit values		
YMAX	9.499	19.01
K	0.02137	0.008424
HalfLife	32.43	82.28
Std. Error		
YMAX	0.03104	0.2166
K	0.0002403	0.0001772

Fig 2

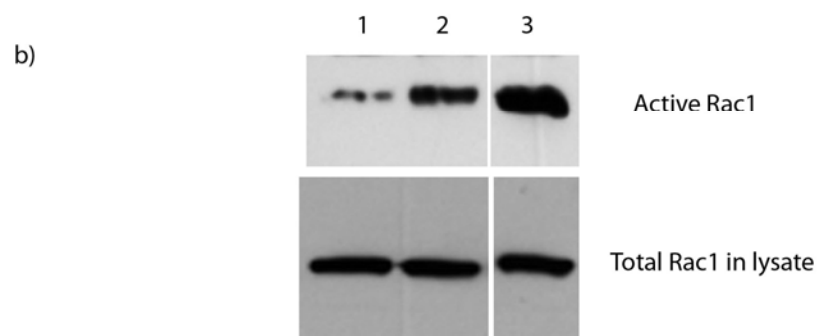
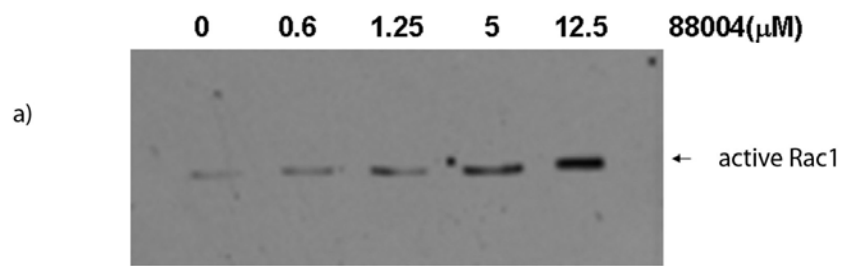


Fig 3

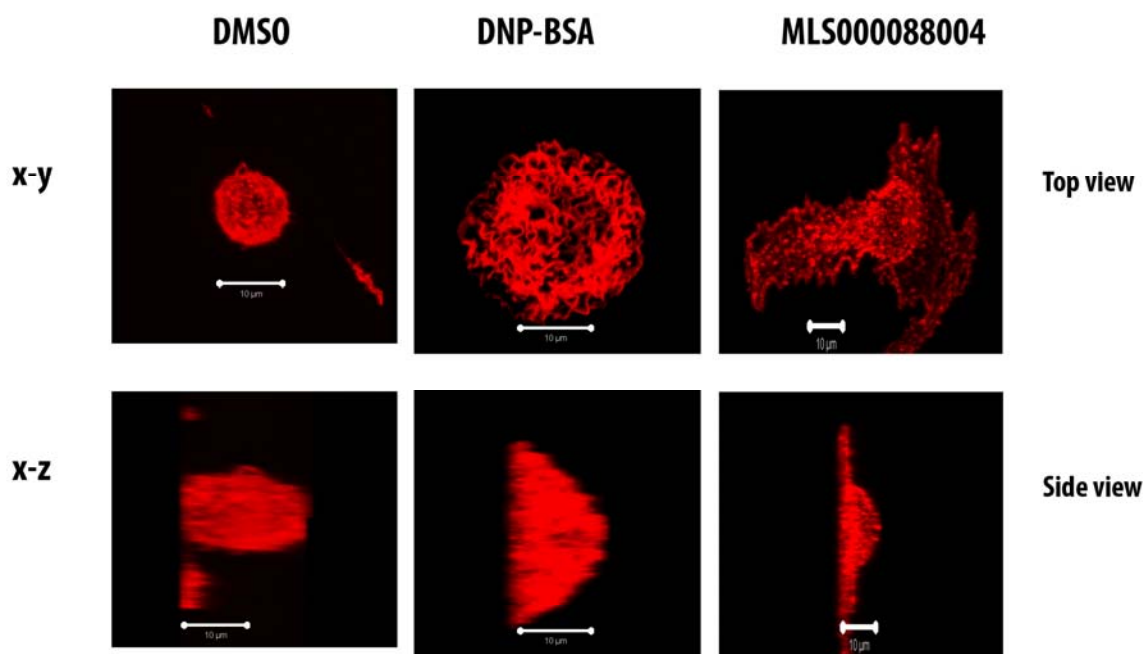


Fig 4

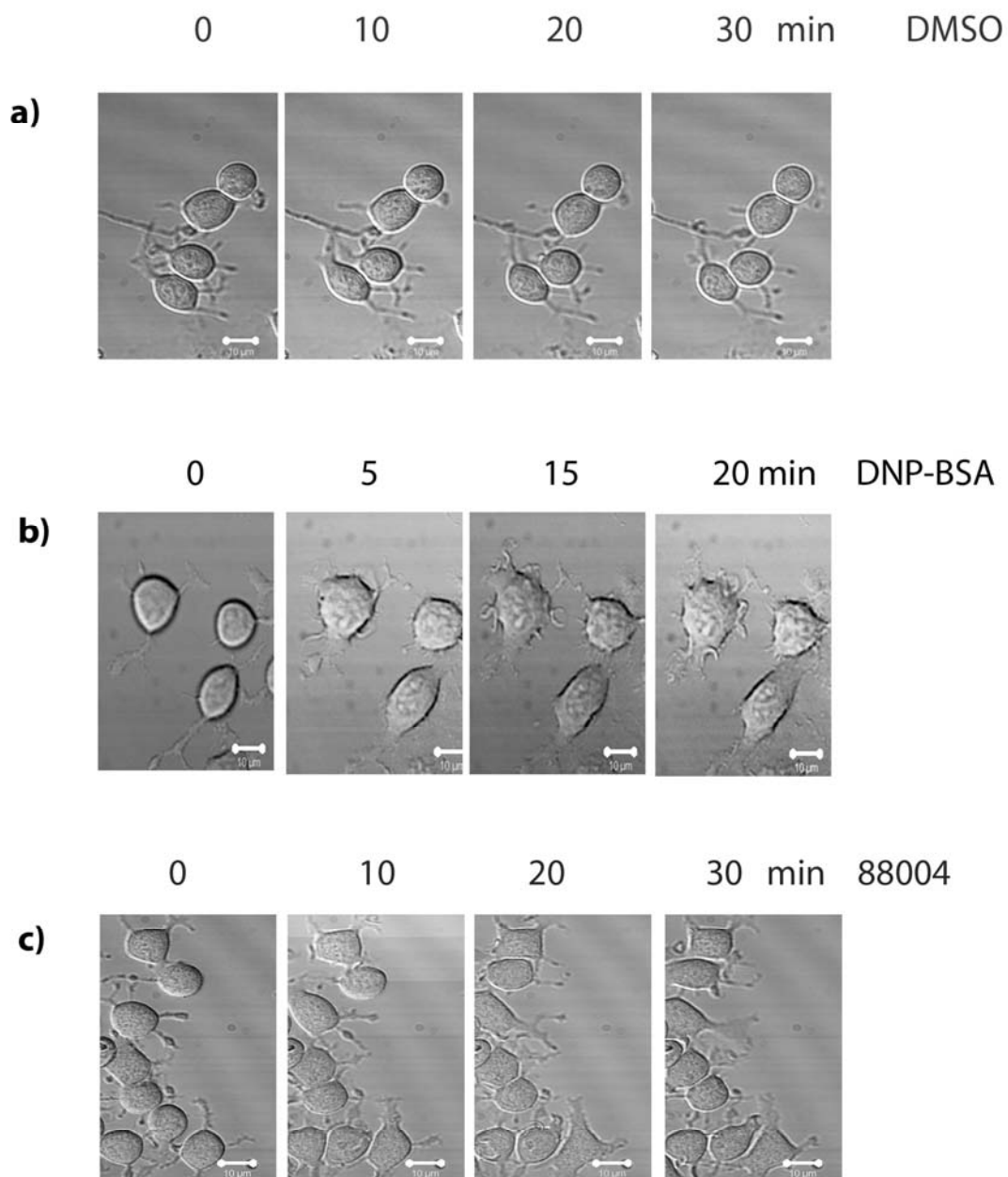


Fig 5

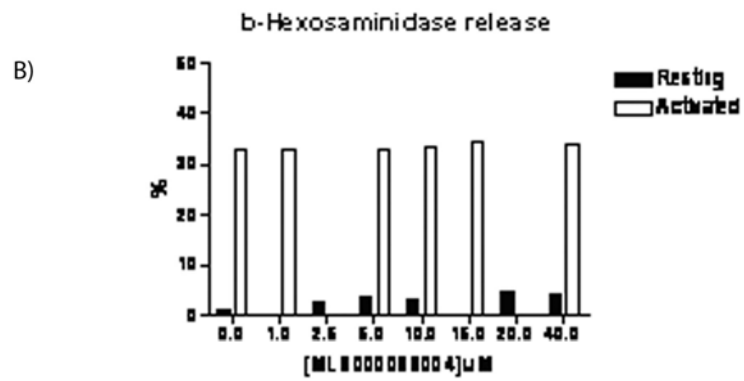
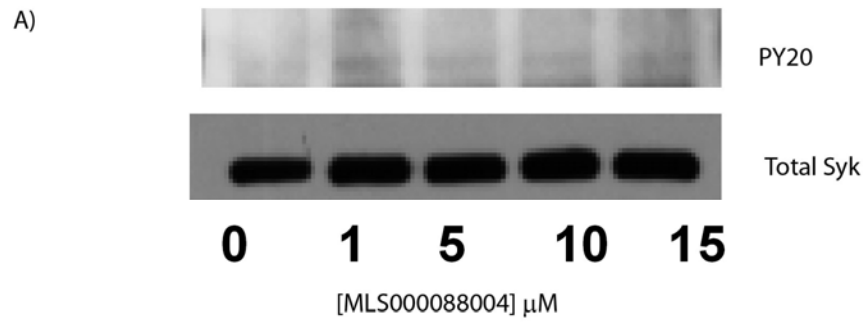


Fig 6

Figures Legends for Section 2a.

Figure 1: High throughput screen identifies small molecule activators and inhibitors of Ras GTPase superfamily. (A) Left panel shows a plot of forward scatter versus a logarithm of side scatter. Singlet beads are selected by drawing a gate as shown. Middle panel shows a plot of the logarithm of green fluorescence versus counts. Right panel shows a plot of the logarithm of red fluorescence versus logarithm of side scatter. The seven different bead sets are obvious and gates were drawn about each set. (B) GST-GTPases were conjugated to fluorescent GSH-beads and assays were initiated with the addition of BODIPY-GTP and library compounds in 384 well plates. HyperCyt time-resolved data (time versus logarithm of green fluorescence) collected in the FL8 red fluorescence channel are shown for one 384-well plate. Each block represents a group of 20 samples, separated by blank wells. Each sample contains data for 6 targets and a GSH-GFP control (Y axis: fluorescence intensity, X axis: time). Arrow denotes positive well containing MLS000088004, inset 2x magnification of positive 'hit' in primary screen. (C) Graph depicting the MLSCN screen of 194,738 compounds' effect on Cdc42WT (black), Rac1WT (red) and Rac1ACT (green). Percent response was calculated by the following equation, % Response = $100 \times (\text{TestSample} - \text{Blocked Control}) / (\text{Unblocked Control} - \text{Blocked Control})$, which lead to 100 % response corresponding to compounds with no effect. Compounds designated as potential hits were those with % Response beyond the cutoff: % Response > 120% for activators and % Response < 80 % for inhibitors, see dotted line in graph. Encircled with a black rectangle are the calculated response values corresponding to MLS000088004; 209 % response for Cdc42, 174 % response for Rac1, and 152 % response for Rac1 activated mutant. (D) Dose dependent activation of GTPases by MLS000088004 with corresponding table of reported results. n=3.

Figure 2: Effects of small molecule activator and inhibitor on Cdc42 GTP-binding, kinetics and in vitro Rac1 activation. (A) Simultaneous binding analysis of six G proteins and one control as a function of the concentration of fluorescent GTP in the presence of DMSO. n=4. (B) Simultaneous binding analysis of six G proteins and one control as a function of the concentration of fluorescent GTP in the presence of 10 μM MLS000088004. (C) Summary table of binding constants (B_{max} and K_d). (D) MLS000088004 increases Cdc42 B_{max} and apparent affinity for GTP, while MLS000532223 decreases Cdc42 B_{max} and apparent affinity for GTP relative to control measurements in DMSO. Comparative measurements were made using 12.5 nM BODIPY-GTP. n=4.

Figure 3: (A) Purified His-Rac1 (80 ng) was briefly preincubated with MLS000088004 (0-12.5 μM), 125 nM GTP \square S was added and active Rac1 was isolated by binding to 5 μg GST-PAK-PBD immobilized on GSH-beads. Bound active Rac1 was detected by immunoblotting. n=2. (B) Swiss 3T3 cells were serum starved overnight, treated with DMSO (control, lane 1), MLS000088004 (lane 2), or EGF alone (lane 3). Top blot shows active Rac isolated on GST-PAK-PBD beads and bottom blot shows total Rac 1 in lysate as a protein loading control.

Figure 4: MLS000088004 modulate actin remodeling in mast cells. RBL-2H3 cells were treated as indicated, fixed, stained with rhodamine phalloidin and imaged on Zeiss LSM510. Individual panels show x-y and x-z views of resting cells, DNP-stimulated cells and cells treated with 10 μM MLS000088004. Bars 10 μM

Figure 5: MLS000088004 induces cell morphology changes comparable to ligand-stimulated mast cells. Live RBL-2H3 cells were monitored by DIC microscopy on a Zeiss inverted microscope for up to 30 min. (A) Resting RBL-2H3 cells. (B) RBL-2H3 cells stimulated with DNP-BSA. (C) RBL-2H3 cells incubated with 10 μM MLS000088004. Bars 10 μM . All of the families have been shown to induce morphological changes and quantitative dose-response analysis is currently being undertaken.

Figure 6: MLS000088004 has no effect tyrosine phosphorylation of Syk and ligand-stimulated β -hexosaminidase secretion of RBL cells. **A)** RBL cells after treatment of indicated concentrations of MLS000088004 were lysed, and Syk kinase was immunoprecipitated its tyrosine phosphorylation was detected as described¹⁸. Top blot shows tyrosine phosphorylation of Syk kinase after IP with Syk polyclonal antibody, and bottom blot shows total Syk in lysate as a protein loading control. **B)** Media was harvested from resting or ligand-stimulated RBL-2H3 cells and assayed for β -hexosaminidase activity. (Black bars) Percent secreted β -hexosaminidase activity from resting cells incubated with DMSO or compound. (Open bars) Percent secreted β -hexosaminidase activity from ligand stimulated cells incubated with 0-15 μ M compound.

Materials and Methods for Section 2b.

Reagents and Cell Lines. (BODIPY FL GTP 2'-(or-3')-O-(N-(2-aminoethyl) urethane, G-12411 from Invitrogen Molecular Probes (<http://probes.invitrogen.com>). GST-GTPase chimeras were purchased from commercial vendors (Cytoskeleton Inc. Denver, CO) or purified from *E. coli* (GST-Rab2, GST-Rab7) as described¹³. Bead sets for multiplex assays were from Duke Scientific Corp., Fremont, CA. Anti-Syk polyclonal antibody was obtained from Dr. P.Draber (Institute of Molecular Genetics, Prague). All other reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. RBL and Swiss 3T3 cells were generously provided by Dr. B. Wilson (University New Mexico).

Multiplexed Primary Screens. For multiplex analysis of small GTPases, we used 4 μ m size glutathione-beads distinguished by seven different intensities of red color (various magnitude of emission at 665 +/-10 nm with excitation at 635 nm). Bead sets were coated with individual GST-GTPase proteins, blocked with 0.1% BSA in Buffer NP-HPS (0.01% (vol/vol) NP-40; 30 mM HEPES pH 7.5; 100 mM KCl; 20 mM NaCl) containing 1mM EDTA (NP-HPSE) and incubated overnight at 4°C. Individual GTPase coupled beads were washed in NP-HPSE buffer (NP-HPS supplemented with 0.1% BSA and 1mM DTT) and pooled together and this mixture used for multiplex analyzes of GTP binding. The assay was conducted in 384-well microplates in a total well volume of 10.1 μ l (5 μ l protein-coated beads in NP-HPSE, 0.1 μ l of test compound, and 5 μ l 200 nM BODIPY- FL-GTP (in NP-HPSE for a final concentration of GTP of 100 nM). Positive controls, containing bead mixture and fluorescent GTP but no test compound, were located in columns 1 and 2 on each plate. Negative controls, containing bead mixture with fluorescent GTP and 0.5 mM unlabeled GTP, were assayed separately. Plates were placed on rotators and incubated for 40-45 min at 4°C. Sample analysis was conducted with the HyperCyt[®] high throughput flow cytometry platform as described previously^{14, 15}. Flow cytometric light scatter and fluorescence emission at 530 +/- 20 nm (FL1) and 665 +/- 10 nm (FL8) were collected on a Cyan ADP flow cytometer (Beckman Coulter, Fullerton, CA). The resulting time-dependent data (single file per plate) were analyzed using IDLQuery software to determine the compound activity in each well. Gating based on FL8 emission distinguishes the beads coated with different proteins, and the median fluorescence per bead population was calculated.

Dose Response Measurements Test compounds identified for further analysis after the primary screen were cherry-picked from compound storage plates, then serially diluted 1:3 eight times from a starting concentration of 10 mM for a total of 9-point dilution series in DMSO. The final concentrations in the assay ranged from 10 nM to 100 μ M. Beads were coated with proteins as described under Multiplex Primary Screens. For dose-response analysis, we used one multiplex (Rab7 wt, Rab2 wt, H-Ras wt, constitutively active mutant H-RasG12V, Cdc42 wt, and constitutively active mutant Cdc42Q61L) and 3 single-plexes (for Rac1 wt, constitutively active mutant Rac1Q61L and GST-GFP).

Kinetic Assays Wild-type GST-Cdc42 (4 μ M) was bound to glutathione beads overnight at 4°C. Cdc42 on GSH-beads was depleted of nucleotide by incubating with 10 mM EDTA containing buffer for 20 min at 30°C, washing twice with 0.01% NP-40 containing HPS buffer, then resuspending in the same buffer containing 1 mM EDTA, 1 mM DTT and 0.1% BSA. Kinetic assays were performed by incubating 50 μ l of GST-Cdc42-GSH-bead suspension for 2 min with either DMSO, or 10 μ M MLS000088004 and subsequently adding 50 μ l of various concentration ice cold BODIPY-GTP. Association of the fluorescent nucleotide was measured using a FacSCAN flow cytometer in the kinetic mode. Data were converted to ASCII format using IDLQuery (software available free from the authors). Raw data were exported and plotted using GraphPad Prism software (www.graphpad.com).

***In vitro* and *In vivo* Rac1 Activation** For *in vitro* Rac1 activation measurements, DMSO or an equal volume of MLS000088004 in DMSO was incubated for 10 min at 30°C with 80 ng His-Rac1 protein and 125 nM GTP γ S in 50 μ l of 10 mM EDTA-containing buffer (NP-HPS with 1mM DTT). Further nucleotide exchange was inhibited with the addition of MgCl₂ to a final concentration of 60 mM and transferring tubes on ice. Activated Rac1 protein was isolated from solution by adding 5 μ g GST-PAK-PBD and glutathione beads. The suspension was rotated at 4°C for 1 h. The beads bearing activated Rac1 bound to GST-PAK-PDB were collected by centrifugation at 14,000 x g (2 min, 4°C), washed with NP-HPS buffer, mixed, boiled with Laemmli buffer, and subjected to SDS-PAGE (12% gels), followed by transfer of proteins onto nitrocellulose membranes. Immunoblot analysis was performed using a mAb directed against Rac1 (Cytoskeleton, Inc.). Protein bands were visualized with an ECL reagent (Pierce Biotechnology, Rockford, IL). Swiss 3T3 cells were used to monitor *in vivo* Rac1 activation by MLS000088004. Cells were serum starved overnight and treated with DMSO (1%, negative control) or 10 μ M compound in DMSO for 20-30 min. As a positive control cells were treated with 10 ng/ml EGF for 2 min. Lysis, immunoprecipitation of active Rac1 with GST-PAK-PBD immobilized on GSH beads, SDS-PAGE and immunoblotting was performed as in ¹⁶.

Live Cell Microscopy. Live cell microscopy was carried out on a rat basophil leukemia cell line (RBL-2H3). For experiments RBL-2H3 cells were grown on cover slips overnight, washed and overlaid with Tyrode's buffer (10mM Hepes, pH7.4, 130mM NaCl, 5mM KCl, 1.4mM CaCl₂, 1mM MgCl₂, 5.6mM Glucose and 0.1% BSA). Time lapse images were taken after addition of 10 μ M MLS000088004 (final concentration) at intervals of 60 s for up to 100 min. For ligand stimulation IgE primed RBL cells were treated with 1 μ g/ml DNP-BSA. Imaging was performed using a Bio-Rad confocal microscope equipped with a 60x 1.4 NA oil immersion objective equipped with Lasersharp3000 software.

Immunofluorescence Staining and Microscopy. RBL-2H3 cells were grown on cover slips and cultured overnight in the absence or presence of 10 μ M MLS000088004. As a positive control, cells were stimulated with 1 μ g/ml DNP-BSA for 30 min as previously described ¹⁷. Cells were washed with phosphate buffered saline, fixed with 3% paraformaldehyde, permeabilized for 5 min with 0.1% Triton X-100 in Tyrode's buffer, blocked for 1 h with 1% BSA in Tyrode's buffer, and stained for 1 h ea with rhodamine phalloidin (Cytoskeleton Inc.) according to manufacturer's instructions. All incubations were performed at room temperature. For imaging, samples were mounted on glass slides using ProLong® Gold antifade reagent (Invitrogen). A Zeiss LSM 510 microscope, 40x objective was used to collect images.

Syk immunoprecipitation IP of Syk and Western Blot analysis performed as described in ¹⁸.

β -Hexosaminidase Secretion Measurements. For β -hexosaminidase release, cells were seeded in 24-well culture plates and incubated for 1 h, with or without indicated concentrations of activators. Hexosaminidase release was determined as described ¹⁹. The values were expressed as percent of total amount of β -hexosaminidase, determined using 1% Triton X-100 in Tyrode's buffer.

b. For each assay implemented and screening run please provide

i. PubChem Bioassay Name(s), AID(s), Assay-Type (Primary, DR, Counterscreen, Secondary)

AID	Bioassay name	Assay Type
757	HTS to identify specific small molecule inhibitors of Ras and Ras-related GTPases specifically Rac wildtype	Primary Screen
758	HTS to identify specific small molecule inhibitors of Ras and Ras-related GTPases specifically Rab7	Primary Screen
759	HTS to identify specific small molecule inhibitors of Ras and Ras-related GTPases specifically Ras wildtype	Primary Screen
760	HTS to identify specific small molecule inhibitors of Ras and Ras-related GTPases specifically Rab2 wildtype	Primary Screen
761	HTS to identify specific small molecule inhibitors of Ras and Ras-related GTPases specifically Cdc wildtype	Primary Screen
764	HTS to identify specific small molecule inhibitors of Ras and Ras-related GTPases specifically Rac activated mutant	Primary Screen
1333	Multiplex dose response to identify specific small molecule inhibitors of Ras and Ras-related GTPases specifically Cdc42 activated mutant	Confirmatory Dose Response
1334	Multiplex dose response to identify specific small molecule inhibitors of Ras and Ras-related GTPases specifically Cdc42 wild type	Confirmatory Dose Response
1335	Multiplex dose response to identify specific small molecule inhibitors of Ras and Ras-related GTPases specifically Ras wild type	Confirmatory Dose Response
1336	Multiplex dose response to identify specific small molecule inhibitors of Ras and Ras-related GTPases specifically Rab 7 wild type	Confirmatory Dose Response
1337	Multiplex dose response to identify specific small molecule inhibitors of Ras and Ras-related GTPases specifically Rab2 wild type	Confirmatory Dose Response
1339	Multiplex dose response to identify specific small molecule inhibitors of Ras and Ras-related GTPases specifically Rac activated mutant	Confirmatory Dose Response
1340	Multiplex dose response to identify specific small molecule inhibitors of Ras and Ras-related GTPases specifically Rac wild type	Confirmatory Dose Response
1341	Multiplex dose response to identify specific small molecule inhibitors of Ras and Ras-related GTPases specifically Ras activated mutant	Confirmatory Dose Response
1758	Activator binding kinetics on Ras and Ras-related GTPases, specifically Cdc42 wild type	Secondary
1759	Activator binding kinetics on Ras and Ras-related GTPases, specifically Ras wild type	Secondary
1760	Activator binding kinetics on Ras and Ras-related GTPases, specifically Rab 7 wild type	Secondary
1761	Activator binding kinetics on Ras and Ras-related GTPases, specifically Ras activated mutant	Secondary
1762	Activator binding kinetics on Ras and Ras-related GTPases, specifically Cdc42 activated mutant	Secondary
1763	Activator binding kinetics on Ras and Ras-related GTPases, specifically Rab2 wild type	Secondary
1769	Profiling assay to determine GST-GSH interactions in multiplex bead-based assays	Counterscreen
Submitted, AID to be assigned	Summary Report for Multiplex dose response to identify specific small molecule inhibitors of Ras and Ras-related	Summary

	GTPases	
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ii. Assay rationale and descriptions, table of reagents and source

Bioassay name	Assay Rationale/Description	Reagents/source
HTS to identify specific small molecule inhibitors of Ras and Ras-related GTPases: all 6 targets (AIDs 757-761, and AID 764)	This is a no-wash fluorescent GTP-binding assay adapted to multiplexed, high-throughput measurements whereby multiple GTPases are simultaneously screened against the MLSCN library. The specificity is based on the observation that individual GTPases including wt and activated forms exhibit measurably distinct affinities for Bodipy-FI-GTP vs GTP. The multiplex assay involves the binding of fluorescent GTP to G protein-GST fusion proteins on GSH beads. A set of six G proteins (Rac 1 wt, Rab7 wt, Rac 1 activated, Ras wt, Rab 2 wt., CDC wt) are arrayed under conditions of divalent molecule depletion. The average Z' for the screen was 0.85 +/- 0.04.	Bodipy FI GTP: Invitrogen, USA GST-GTPase chimeric proteins: Cytoskeleton, USA or purified from <i>E. coli</i> (GST-Rab2, GST-Rab7) Bead sets: Duke Scientific, USA
Multiplex dose response to identify specific small molecule inhibitors of Ras and Ras-related GTPases: all 6 targets (AIDs 1333-1337, and 1339-1341)	The assay is the same as the primary HTS against the MLSCN library compounds in a 9-point dose response curve over an assay concentration range of 10 nM to 100 µM.	Same reagents as the HTS
Activator binding kinetics on Ras and Ras-related GTPases: all 6 targets (AIDs 1758-1763)	Real-time binding kinetics between GTP and each of the protein targets were characterized in a multiplex assay. The resulting binding of fluorescent GTP after 3 minutes was utilized for determining Bmax and Kd for each target in the presence of 10 microM small molecule activator compound MLS000088004 versus DMSO as control.	Same reagents as the HTS
Profiling assay to determine GST-GSH interactions in multiplex bead-based assays (AID 1769)	Compounds were evaluated for the ability to interfere with the binding of the GST fusion proteins to the GSH beads (thus producing potential false positive results). Dose response experiments were conducted to test an assay concentration range of 10 nanoM to 100 microM.	GST-green fluorescent protein: purified at UNMCMD GSH beads: Duke Scientific, USA

iii.Center Summary of Results

Bioassay name	Compounds Tested / Actives
HTS to identify specific small molecule inhibitors of Ras and Ras-related GTPases: all 6 targets (AIDs 757-761, and AID 764)	194,635 tested / 1,877 active
Multiplex dose response to identify specific small molecule inhibitors of Ras and Ras-related GTPases: all 6 targets (AIDs 1333-1337, and 1339-1341)	1,275 tested / 964 active
Activator binding kinetics on Ras and Ras-related GTPases: all 6 targets (AIDS 1758-1763)	1 compound tested (activator probe candidate binding equilibrium kinetics against all targets)
Profiling assay to determine GST-GSH interactions in multiplex bead-based assays (AID 1769)	1117 tested / 397 active

c. Probe Optimization

- i. Describe SAR & chemistry strategy (including structure and data) that led to the probe.

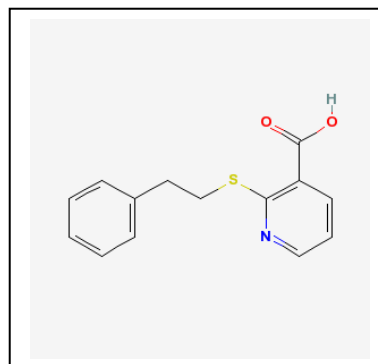
This information is provided for each probe scaffold in Section 3.

3. Probes

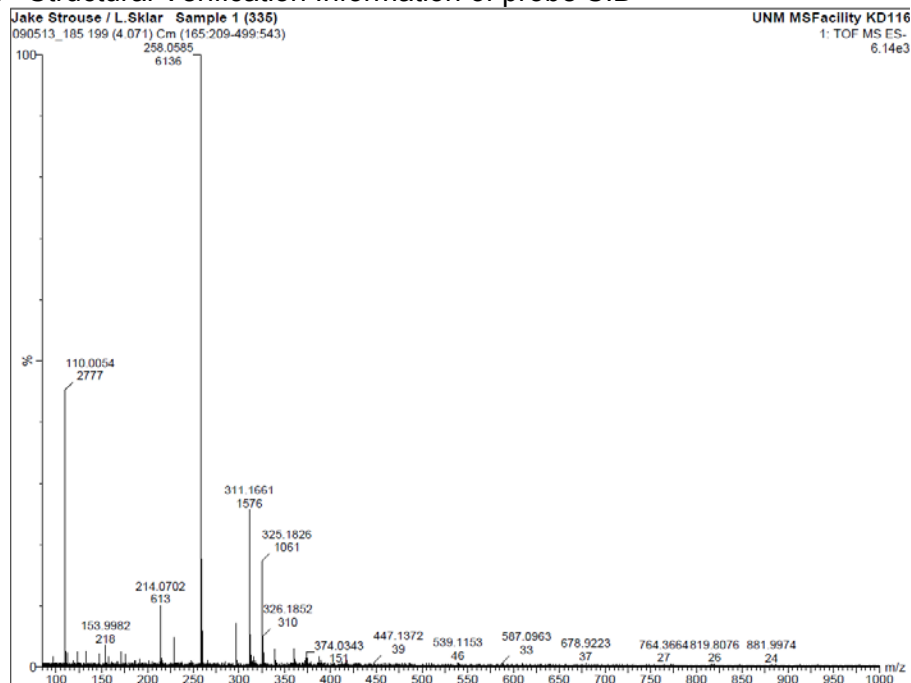
3.1: Probe 1

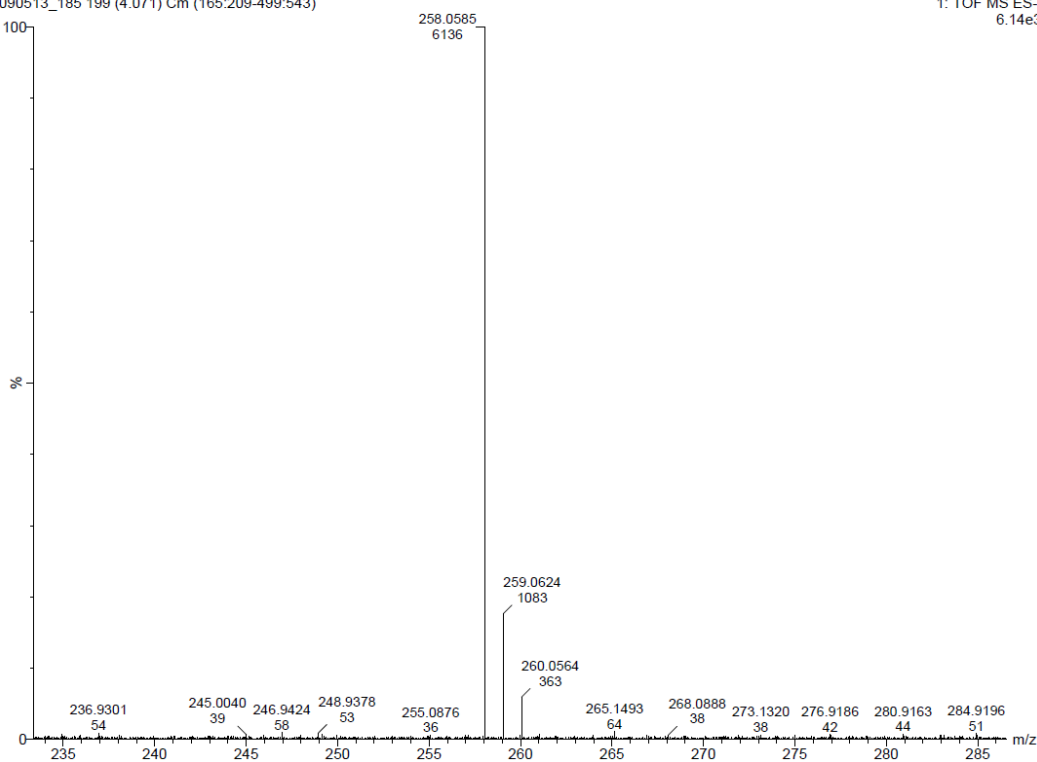
- a. Chemical name of probe compound: 2-(2-phenylethylsulfanyl)pyridine-3-carboxylic acid **[ML099]**
- b. Probe chemical structure including stereochemistry if known

Molecular Weight 259.32352 [g/mol]
Molecular Formula C₁₄H₁₃NO₂S
XLogP3-AA 3.2
H-Bond Donor 1
H-Bond Acceptor 3
Rotatable Bond Count 5
Exact Mass 259.066699
Topological Polar Surface Area 50.2
Heavy Atom Count 18
Solubility Sparingly Soluble (0.080 g/L calc in water RT)
Molar Volume 200.6±5.0 cm³/mol
pKa 3.01±0.36



c. Structural Verification Information of probe SID





Elemental Composition Report

Page 1

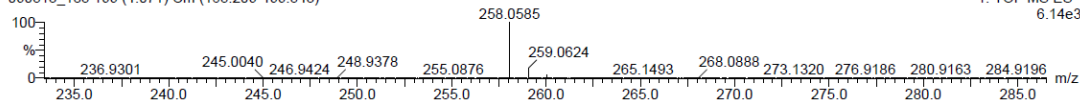
Single Mass Analysis

Tolerance = 10.0 PPM / DBE: min = -1.5, max = 50.0

Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions

90 formula(e) evaluated with 4 results within limits (all results (up to 1000) for each mass)



Minimum: -1.5
Maximum: 50.0

Mass	Calc. Mass	mDa	PPM	DBE	Score	Formula
258.0585	258.0582	0.3	1.1	0.5	2	C6 H16 N3 O4 S2
	258.0589	-0.4	-1.5	9.5	1	C14 H12 N O2 S
	258.0598	-1.3	-5.2	1.5	4	C9 H17 N O2 23Na S2
	258.0565	2.0	7.9	6.5	3	C12 H13 N O2 23Na S

HRMS analysis of 57578335. The results are consistent with the presence of the [M-H]⁻ of C₁₄H₁₃NO₂S with a -1.5 ppm difference from the expected m/z of 258.0589 (observed mass = 258.0585)

- d. PubChem CID (corresponding to the SID): Compound ID 888706; Substance ID 57578335
- e. If available from a vendor, please provide details. ChemBridge ID 7360080
- f. Provide MLS# that verifies the submission of probe molecule and five related samples that were submitted to the SMR collection: Compound is in the MLSMR; not submitted

g. Describe mode of action for biological activity of probe:

As shown in Fig. 2 above, the activators appear to increase the affinity of binding of the fluorescent GTP analogs to the GTPases. To understand the action and binding mode of these small molecule activators against the GTPase proteins, docking simulations were performed on Rac1 and Cdc42 using the Surflex-dock program, as implemented in Sybyl 8.0. On the basis of the mechanism of recruitment and activation of GTPases by GEFs (guanine nucleotide exchange factors are GTPase binding regulator) a working hypothesis has been developed. The assumption is that activators interact with the GTPases in their inactive form (GDP-bound state) and bind to an allosteric binding site localized between switch regions I and II (Fig 7).

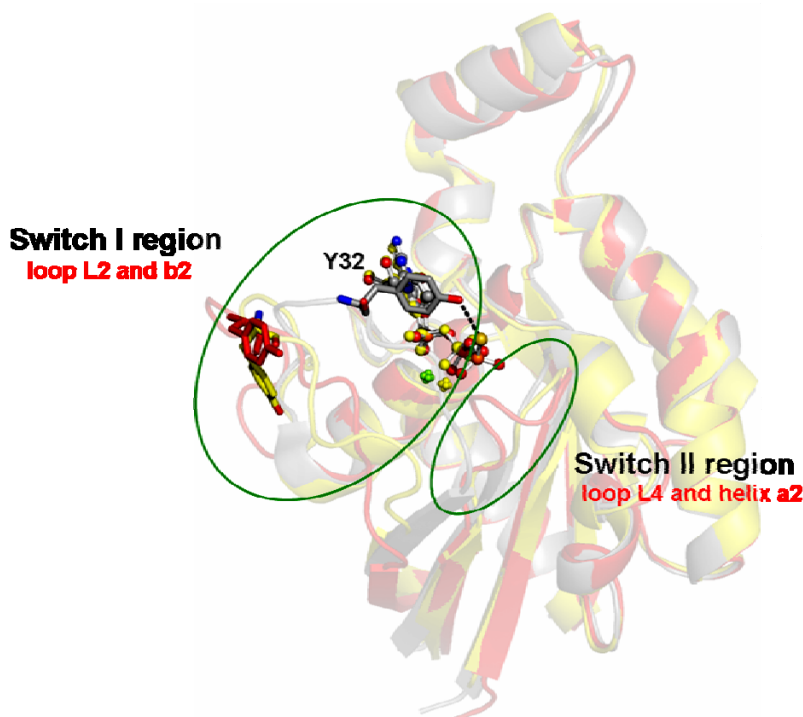


Figure 7. Common architecture of GTPase: green circles indicate the two flexible regions switch I and II. Red and yellow cartoons represent GTPases in free and GDP-bound states, respectively. The GTP-active form is rendered as grey cartoon with the Tyr32 involved in hydrogen interaction with the γ -phosphate of GTP. GDP and GTP are represented in ball and stick while the tyrosines are rendered in stick.

The sequence identity and similarity between Rac1 and Cdc42 is about 70% and 81% respectively, and the sequence alignment indicated only one difference in the binding site of these proteins. At position 56 there is a Trp in the binding site of Rac1 and a Phe for the corresponding position from Cdc42 (Figure 8). This similarity between the binding sites could explain the lack of selectivity of these activators with respect to Rac1 and Cdc42.

```

sp|P63000|RAC1_HUMAN      MQAIRCVVVG DGAVGKTCLLISYTTNAPFGEYIPTVFDNYSANVMVDGKP 50
sp|P60953|CDC42_HUMAN    MQTIRCVVVG DGAVGKTCLLISYTTNKFPSEYVPTVFDNYAVVMIGGEP 50
    *:*****:*****:*****:*****:*****:*****:*****:*****:*****:

```

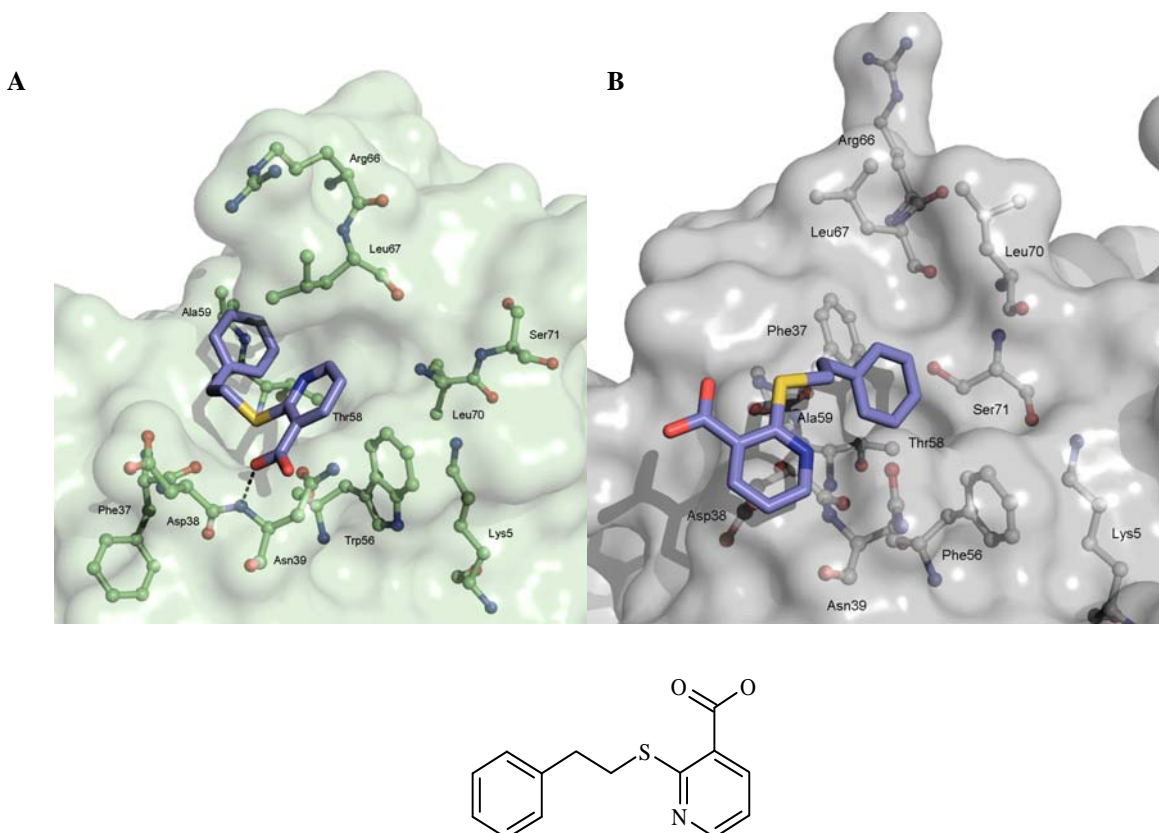
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sp|P63000|RAC1_HUMAN      VNLGLD DTAGQEDYDLRLRPLSYPQTDVFLICFSLVSPASFENVRAKWYPE 100
sp|P60953|CDC42_HUMAN    YTLGLD DTAGQEDYDLRLRPLSYPQTDVFLVCFVSVSPSSFENVKEKWPYE 100
    *:*****:*****:*****:*****:*****:*****:*****:*****:

```

Figure 8. Sequence alignment of human Rac1 and Cdc42. The cyan boxes emphasize residues involved in the interaction with GEF regulator while grey boxes show the amino acids selected to define the binding cavity in docking calculations

This binding mode of these compounds involves favorable hydrophobic interactions with surrounding amino acids, specifically the residues from position 56, Trp for Rac1 and Phe for Cdc42. The views obtained from the docking of MLS00088004 at Rac1 and Cdc42 are depicted in Fig. 9. The binding mode of this compound in Rac1 binding cavity (Fig. 9A) shows the carboxylic group oriented towards the NH group of Asn39 backbone and involves in H-bond interactions. Additional hydrophobic contacts are favored by the close contact between the pyridine moiety of the compound and the indole side chain of Trp56, while the peripheral phenyl group is oriented towards the hydrophobic region delimited by the side chain of Leu67. In the Cdc42 active site (Fig. (B)), the binding mode is different. The terminal phenyl group of the compound is oriented towards a hydrophobic favourable region delimited by the benzyl ring of Phe37, while the carboxylic group is exposed to the solvent and the H-bond interactions are lost. The presence of Phe at position 56 instead of Trp triggers a different orientation of the amino acids in the binding site which result in a shallower cavity compared with the binding cavity of Rac1. The consequent loss of the favorable interactions with Asn39 and Leu67 could explain the decrease of biological activity of MLS00088004 for Cdc42 in comparison with Rac1.



MLS00088004

Figure 9. The binding mode of MLS00088004 (Rac-pEC₅₀ = 7.70; Cdc42-pEC₅₀ = 7.23). The figure shows the docking views of this compound in A) the binding cavity of Rac1, depicted in light green and B) Cdc42 represented in grey. The relevant amino acids in both binding sites are rendered in ball-stick. The hydrogen bonds are represented with dash line.

h. Detailed synthetic pathway for making probe

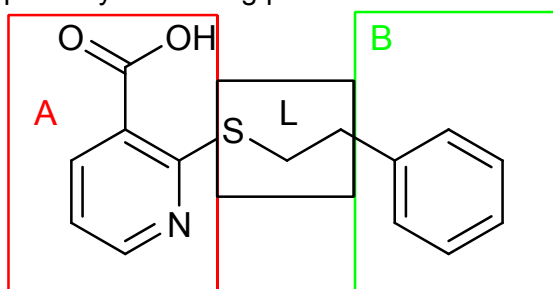
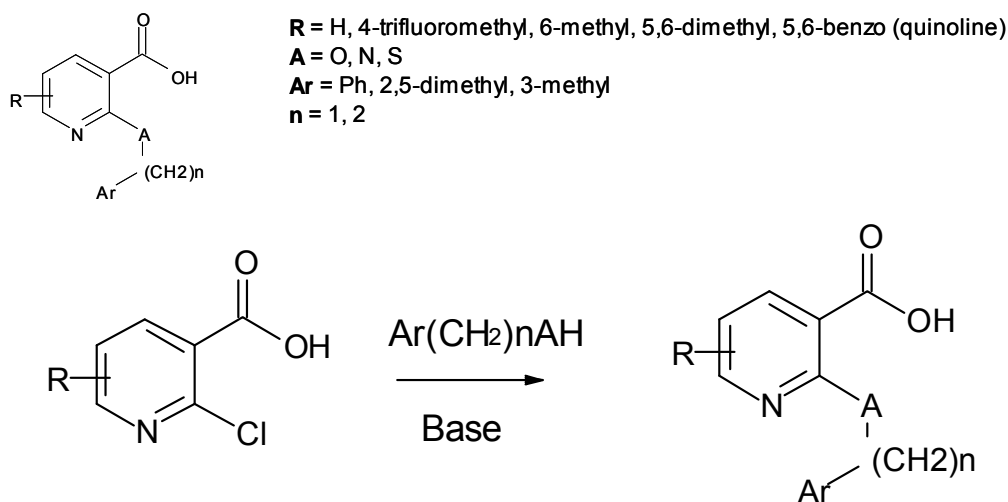


Fig. 10. SID 57578335

The original hit compound (SID 57578335) was identified during the primary HTS campaign and confirmed in dose response screen to have a $\log EC_{50}$ =7.7 on Rac1 wild type, $\log EC_{50}$ =7.59 on Rac1 activated mutant, $\log EC_{50}$ =7.23 on cell division cycle 42 (GTP binding protein, 25kDa) activated mutant, $\log EC_{50}$ =7.00 on cell division cycle 42 (GTP binding protein, 25kDa) wild type, $\log EC_{50}$ =6.45 on Ras-related protein Rab-2A, $\log EC_{50}$ =6.74 on GTP-binding protein (rab7), $\log EC_{50}$ =7.02 on Ras protein activated mutant, $\log EC_{50}$ =6.85 on Ras protein wild type. The activity was confirmed by purchasing the compound from a commercial source (ChemBridge ID 7360080). The chemical optimization strategy involved modification of section A, B, and L (Fig. 10). Analogs of nicotinic acid were available commercially and were purchased. A chemical synthesis project was also done by ChemDiv, The following variations were investigated: linker section sulfur atom was substituted with nitrogen or oxygen atoms, linker length was varied from 1 to 3 atoms; section A ring small substituents were added and the carboxylic group was preserved in all variations because primary data in close analogs to SID 57578335 indicated that it is essential for biological activity; ring B section was varied by introduction of small substituents on phenyl ring. Based on these modifications, 9 compounds are synthetic derivatives, 8 compounds were commercially purchased, and 8 compounds were cherry picked from MLSMR. The synthetic analogs around SID 57578335 were synthesized by ChemDiv in an attempt to improve activity (Schema 1).



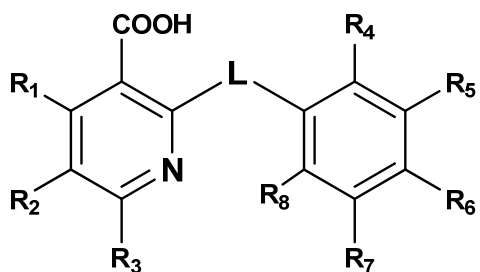
Probe 1. Representative synthetic scheme. Reagents and conditions

SAR analysis led to the following conclusions:

- Pyridine ring (section A): for active compounds at position 2 is carboxyl group, while R_1 through R_3 are hydrogen i.e. unsubstituted.
- Phenyl ring (section B): active compounds can have small non-polar substituents like: methyl, methoxy, halogen. Polar substituents like nitro or aldehyde groups render the compound inactive.

- Linker (section L): is between 1 and 3 atoms the first linker atom attached to pyridine ring can be sulfur, oxygen or nitrogen.

The detailed SAR table and biological activities are presented in the following:



Supplier ID	PubChem SID	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	R ₈	Linker
MLS000088004	57578335									
Z494-0019	56431867					CH ₃				
Z494-0017	56431866				CH ₃				CH ₃	
MLS000522071	14733114					CH ₃				
MLS000522069	14737743					CH ₃			CH ₃	
K783-0843	56431812						OCH ₃			
0412-0036	56431715									
K781-5215	56431809					F				
MLS000394214	22401197						CH ₃		CH ₃	
Z494-0020	56431868							CH ₃		
MLS000673633	24815349							Cl		
MLS000715411	24801148								Cl	
MLS000122880	14722433						CH ₃			
8012-6709	56431758						Cl			
Z494-0015	56431864				CH ₃				CH ₃	
MLS000046237	4243562									
K781-0014	56431808				CH ₃					
Z494-0011	56431862			CH ₃		CH ₃				
R052-0595	56431824				OCH ₃				CHO	
8017-3981	56431763						CH ₃			
Z494-0007	56431859			CH ₃	CH ₃				CH ₃	
Z494-0006	56431858				CH ₃				CH ₃	
2189-1384	56431719						NO ₂			
Z494-0016	56431865			CH ₃	CH ₃				CH ₃	
Z494-0010	56431861					CH ₃				

Active Inactive Inconclusive

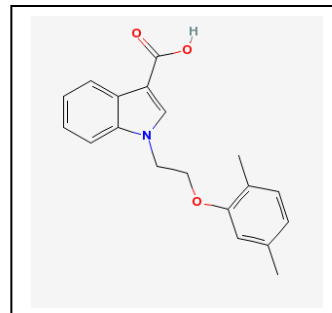
Supplier ID	PubChem SID	logEC ₅₀ Cdc42_ACT	logEC ₅₀ Cdc42_WT	logEC ₅₀ Rab2_WT	logEC ₅₀ Rab7_WT	logEC ₅₀ Rac1_ACT	logEC ₅₀ Rac1_WT	logEC ₅₀ Ras_ACT	logEC ₅₀ Ras_WT
MLS000088004	57578335	7.23	7.00	6.45	6.74	7.59	7.70	7.02	6.85
Z494-0019	56431867	5.79	5.66	5.65	5.76	6.12	5.66	5.75	5.61
Z494-0017	56431866	5.51	5.30	6.23	5.87	5.88	5.72	5.45	
MLS000522071	14733114	6.72	6.52	7.13	6.97	6.76	7.14		6.37
MLS000522069	14737743	6.44	6.39	6.68	6.54	6.77	7.29		6.13
K783-0843	56431812	6.39	6.18	6.90	7.05	6.75	6.34		6.41
0412-0036	56431715	5.38	5.29	5.49	5.62	5.33	5.04		5.30
K781-5215	56431809	6.62	6.63	6.58	6.88	6.79	6.15		
MLS000394214	22401197	5.50	5.48	5.17	5.97	5.95		5.96	5.73
Z494-0020	56431868	5.94	5.72	5.85	6.00	6.14	5.80	5.63	5.64
MLS000673633	24815349	6.06	5.86	5.89		5.82	5.71		5.76
MLS000715411	24801148	5.91	5.75	5.61		5.85	5.41		5.62
MLS000122880	14722433	5.95	6.07		6.33	5.98	5.70	6.07	5.96
8012-6709	56431758	5.27	5.17		5.39	5.41	5.00		
Z494-0015	56431864	5.54	5.08			5.78	5.39	5.82	
MLS000046237	4243562	4.85			5.31	5.74	5.51	5.70	5.64
K781-0014	56431808	6.10	6.03	5.99	6.27	6.22	5.92		6.18

- i. Center summary of probe properties (solubility, absorbance/fluorescence, reactivity, toxicity, etc.). See Section 3.1b above
- j. A tabular presentation summarizing known probe properties. Section 3.1c above

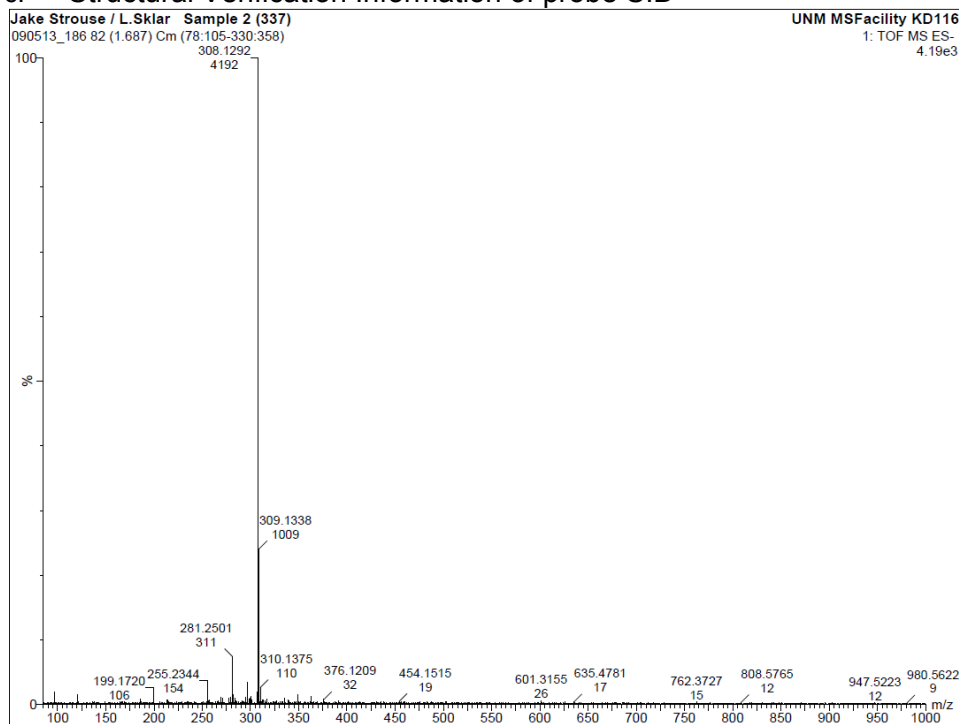
3.2: Probe 2

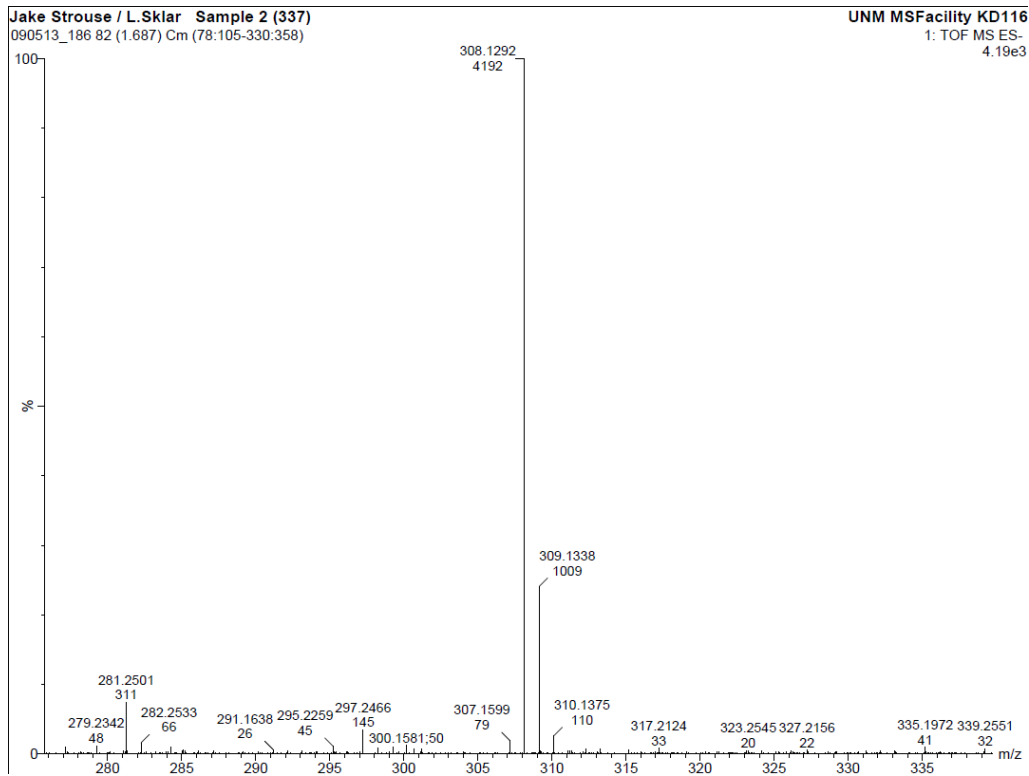
- Chemical name of probe compound: 1-[2-(2,5-dimethylphenoxy)ethyl]-1H-indole-3-carboxylic acid **[ML098]**
- Probe chemical structure including stereochemistry if known

Molecular Weight 309.35906 [g/mol]
Molecular Formula C₁₉H₁₉NO₃
XLogP3-AA 3.9
H-Bond Donor 1
H-Bond Acceptor 3
Rotatable Bond Count 5
Topological Polar Surface Area 51.5
Heavy Atom Count 23
Solubility Sparingly Soluble (3.7E-3 g/L calc in water RT)
Molar Volume 263.0±7.0 cm³/mol
pKa 4.07±0.10



c. Structural Verification Information of probe SID





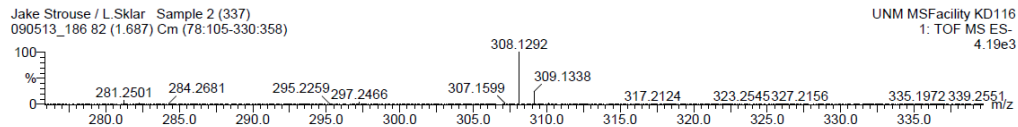
Single Mass Analysis

Tolerance = 10.0 PPM / DBE: min = -1.5, max = 50.0

Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions

158 formula(e) evaluated with 4 results within limits (all results (up to 1000) for each mass)

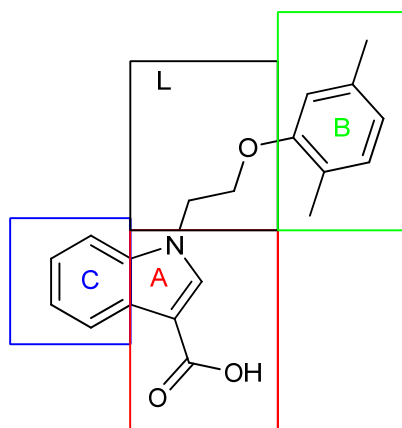


Minimum: -1.5
Maximum: 50.0

Mass	Calc. Mass	mDa	PPM	DBE	Score	Formula
308.1292	308.1296	-0.4	-1.4	3.5	4	C14 H23 N O3 23Na S
	308.1287	0.5	1.7	11.5	1	C19 H18 N O3
	308.1320	-2.8	-9.2	6.5	2	C16 H22 N O3 S
	308.1263	2.9	9.5	8.5	3	C17 H19 N O3 23Na

HRMS analysis of 57578337. The results are consistent with the presence of the [M-H]⁻ of C₁₉H₁₉NO₃ with a 1.7 ppm difference from the expected m/z of 308.1287 (observed mass = 308.1292).

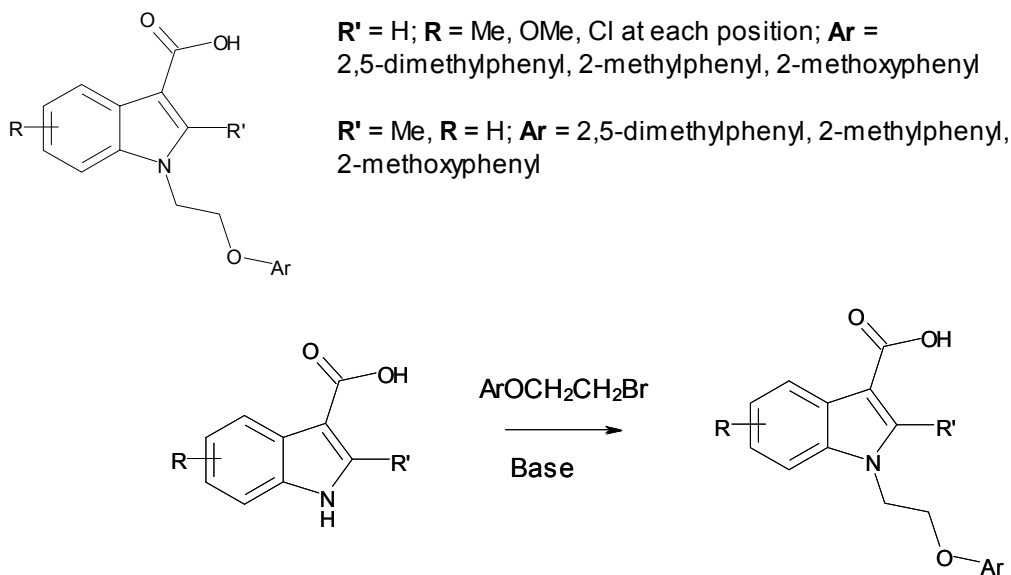
- d. Pub chem. CID (corresponding to the SID): Compound ID 7345532; Substance ID 57578337
- e. If available from a vendor, please provide details: ChemDiv ID 7815-0263
- f. Provide MLS# that verifies the submission of probe molecule and five related samples that were submitted to the SMR collection: Compound is in the MLSMR, not submitted.
- g. Describe mode of action for biological activity of probe. Section 3.1g above.
- h. Detailed synthetic pathway for making probe



SID 57578337

Figure 11.

The original hit compound (SID 57578337) was identified during the primary HTS campaign and confirmed in a dose response screen to have a $\log EC_{50}$ =6.82 on Rac1 wild type, $\log EC_{50}$ =7.09 on Rac1 activated mutant, $\log EC_{50}$ =7.30 on cell division cycle 42 (GTP binding protein, 25kDa) activated mutant, $\log EC_{50}$ =6.99 on cell division cycle 42 (GTP binding protein, 25kDa) wild type, $\log EC_{50}$ =6.80 on Ras-related protein Rab-2A, $\log EC_{50}$ =6.74 on GTP-binding protein (rab7), $\log EC_{50}$ =6.71 on Ras protein activated mutant, $\log EC_{50}$ =6.46 on Ras protein wild type. The activity was confirmed by purchasing the compound from a commercial source (ChemDiv ID 7815-0263). The chemical optimization strategy involved modification of section A, B, C, and L (Figure 11). Analogs of substituted indole scaffold were available commercially and were purchased and chemical synthesis project was also done by ChemDiv. The following variations were investigated: linker section oxygen atom was substituted with carbon atom, linker length was varied from 1 to 4 atoms; section A ring small substituents were varied by introduction of methyl group; ring B section was varied by introduction of methyl, methoxy, halogen, tert-butyl, and iso-propyl substituents at various positions; section C ring was varied by introduction of methyl, methoxy, and halogen substituents at various positions. Based on these modifications a total of 14 compounds are synthetic derivatives and 14 compounds were commercially purchased, 4 compounds were cherry picked from MLSMR. The synthetic analogs around SID 57578337 were synthesized by ChemDiv in an attempt to improve activity (Schema 2).

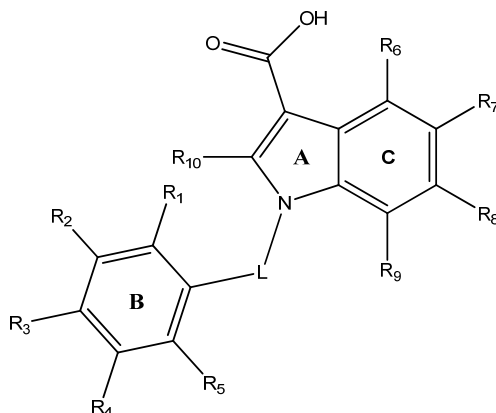


Probe 2. Representative synthetic scheme. Reagents and conditions

SAR analysis led to the following conclusions:

- Ring B: small substituents like methyl, methoxy, chlorine, bromine, fluorine are allowed. Larger hydrophobic substituents like tert-butyl, iso-propyl lead to inactive compounds.
- Ring A: no substituents allowed
- Ring C: no substituents allowed
- Linker: size between 1 to 3 atoms; one atom can be heteroatom like oxygen. Linker size larger than 3 leads to inactive compounds

Some compounds like 7815-0268, 7815-0267, 7815-0265 have noisy data or low response and were marked as inconclusive. The best active compound MLS000689786 has ring B substituted with methyl in position R₁ and R₄ and linker size 3



Supplier ID	PubChem SID	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	R ₈	R ₉	R ₁₀	Linker
7815-0253	56431743			F								A-B
7815-0252	56431742	F										A-B
MLS000689786	57578337	CH ₃			CH ₃							A-B-O
7815-0259	56431746			Br								A-B
MLS000689788	24814897	Cl										A-B-O
MLS000716591	24802779	OCH ₃										A-B-O
MLS000689785	24823826	CH ₃										A-B-O
7815-0255	56431744	Cl										A-B
7815-0260	56431747											A-B-O
7815-0256	56431745			Cl								A-B
MLS000689787	24814896			CH ₃								A-B-O
Z494-0049	56431894										CH ₃	A-B-O
Z494-0047	56431892	OCH ₃							Cl			A-B-O
Z494-0051	56431896	CH ₃								CH ₃		A-B-O
Z494-0050	56431895	CH ₃					CH ₃					A-B-O
Z494-0048	56431893	OCH ₃								Cl		A-B-O
Z494-0046	56431891	OCH ₃						Cl				A-B-O
Z494-0045	56431890	OCH ₃					Cl					A-B-O
Z494-0044	56431889	OCH ₃								CH ₃		A-B-O
Z494-0043	56431888	OCH ₃							CH ₃			A-B-O

Z494-0042	56431887	OCH ₃						CH ₃					
Z494-0041	56431886	OCH ₃					CH ₃						
Z494-0039	56431885	CH ₃								OCH ₃			
Z494-0038	56431884	CH ₃						OCH ₃					
Z494-0037	56431883	CH ₃					OCH ₃						
7815-0272	56431752			t-Butyl									
7815-0276	56431753	CH ₃											
7815-0277	56431754	CH ₃			CH ₃								
7815-0265	56431748		CH ₃	CH ₃									
7815-0267	56431749			Cl									
7815-0278	56431755		CH ₃	Cl									
7815-0271	56431751	i-Propyl			CH ₃								
7815-0268	56431750		CH ₃	Cl									

Active Inactive Inconclusive

Supplier ID	PubChem SID	logEC ₅₀ Cdc42_ACT	logEC ₅₀ Cdc42_WT	logEC ₅₀ Rab2_WT	logEC ₅₀ Rab7_WT	logEC ₅₀ Rac1_ACT	logEC ₅₀ Rac1_WT	logEC ₅₀ Ras_ACT	logEC ₅₀ Ras_WT
7815-0253	56431743	6.13	5.87	6.30	6.46	6.22	5.89	6.33	6.09
7815-0252	56431742	5.90	5.84	6.11	6.29	6.36	5.88	6.71	5.96
MLS000689786	57578337	6.45	6.23	6.80	7.11	6.65	6.10	6.71	6.46
7815-0259	56431746	6.78	6.53	6.84		7.16	6.91		6.83
MLS000689788	24814897	5.56	5.52			5.26	5.09	6.60	5.57
MLS000716591	24802779	4.32		5.15		5.06			4.55
MLS000689785	24823826			5.48		6.29	5.77		
7815-0255	56431744	6.24							
7815-0260	56431747					6.07	5.26		
7815-0256	56431745						7.35		
MLS000689787	24814896					5.94			

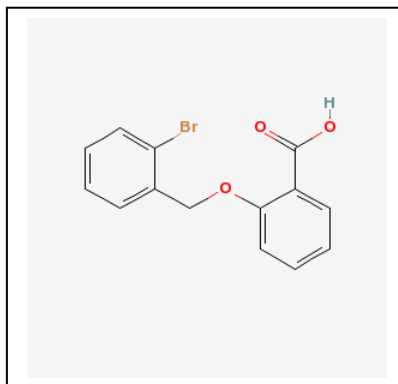
- i. Center summary of probe properties (solubility, absorbance/fluorescence, reactivity, toxicity, etc.) See Section 3.2b above.
- j. A tabular presentation summarizing known probe properties See Section 3.2b above

3.3: Probe 3

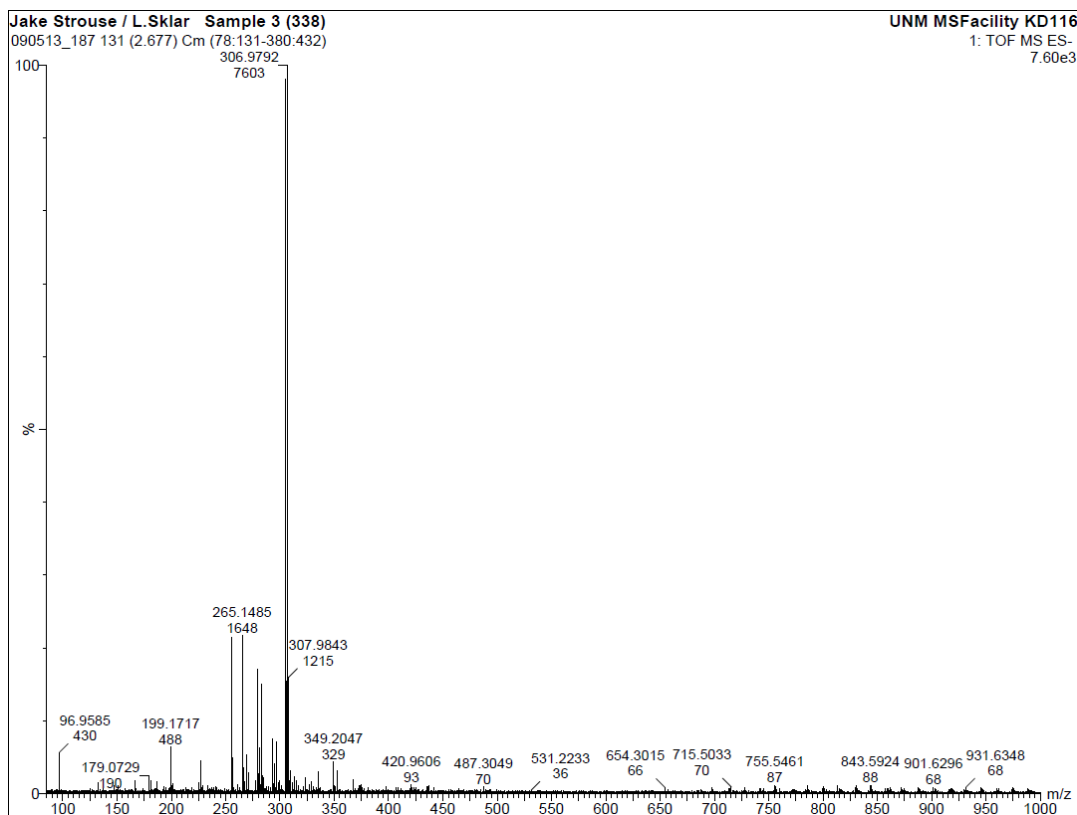
a. Chemical name of probe compound: 2-[(2-bromophenyl)methoxy]benzoic acid
[ML097]

b. Probe chemical structure including stereochemistry if known

Molecular Weight 307.13934 [g/mol]
Molecular Formula C₁₄H₁₁BrO₃
XLogP3-AA: 3.6
H-Bond Donor: 1
H-Bond Acceptor: 3
Rotatable Bond Count: 4
Topological Polar Surface Area: 46.5
Heavy Atom Count: 18
Solubility Sparingly Soluble (0.046 g/L calculated in water RT)
Molar Volume: 202.8±3.0 cm³/mol
pKa: 3.38±0.36

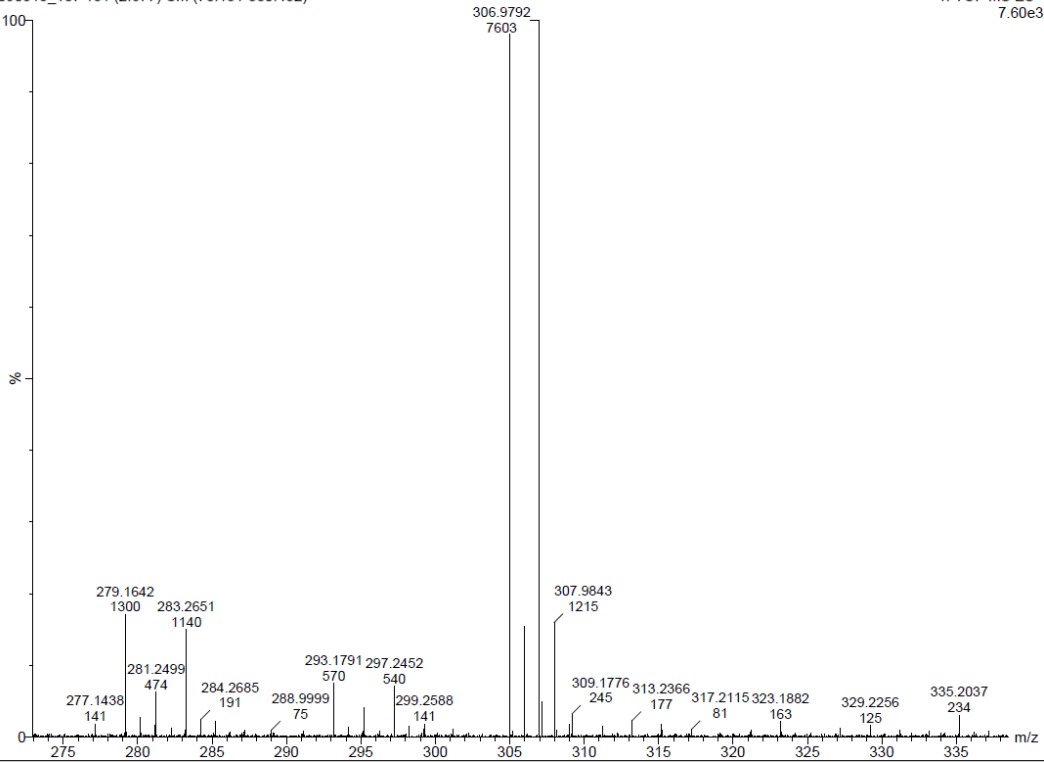


c. Structural Verification Information of probe SID



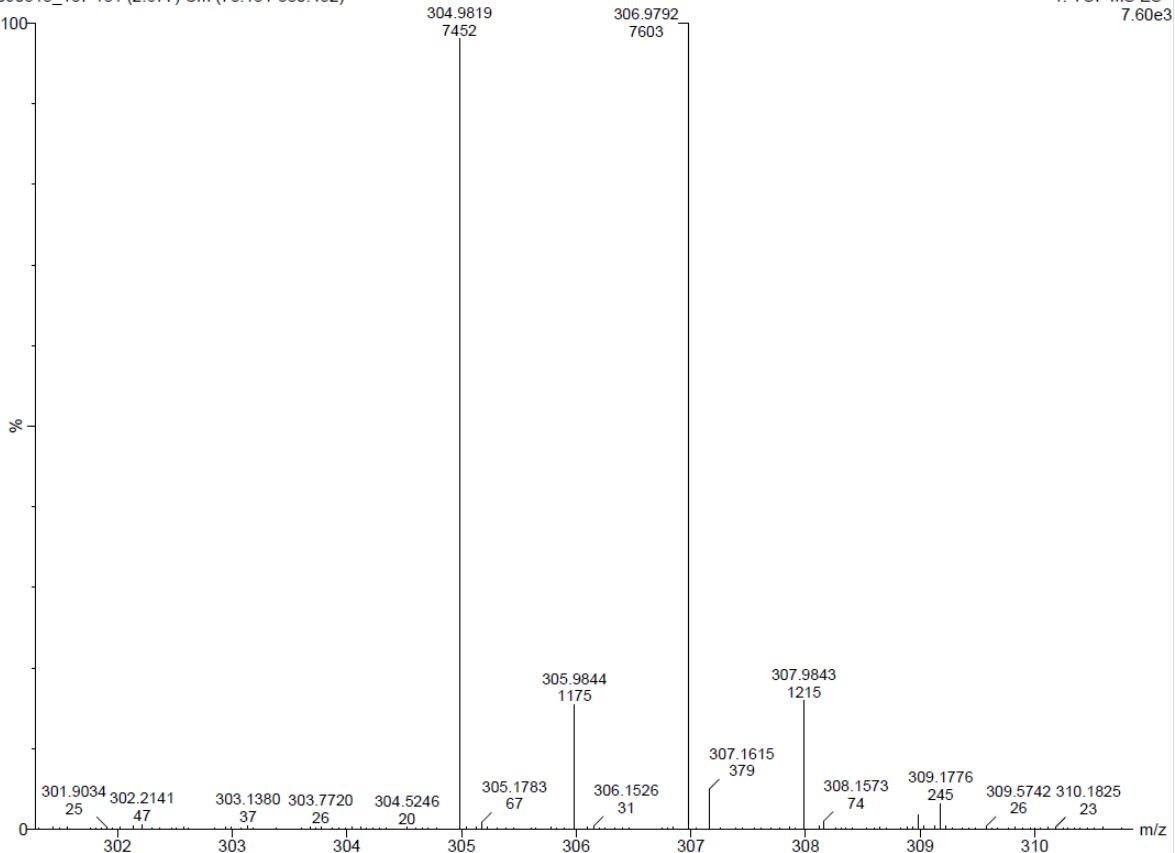
Jake Strouse / L.Sklar Sample 3 (338)
090513_187 131 (2.677) Cm (78:131-380:432)

UNM MS Facility KD116
1: TOF MS ES-
7.60e3



Jake Strouse / L.Sklar Sample 3 (338)
090513_187 131 (2.677) Cm (78:131-380:432)

UNM MS Facility KD116
1: TOF MS ES-
7.60e3



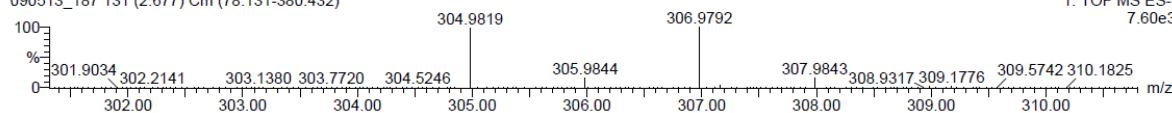
Single Mass Analysis

Tolerance = 10.0 PPM / DBE: min = -1.5, max = 50.0

Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions

248 formula(e) evaluated with 5 results within limits (all results (up to 1000) for each mass)

Jake Strouse / L.Sklar Sample 3 (338)
090513_187 131 (2.677) Cm (78:131-380:432)UNM MS Facility KD116
1: TOF MS ES-
7.60e3

Minimum: -1.5
Maximum: 200.0 10.0 50.0

Mass	Calc. Mass	mDa	PPM	DBE	Score	Formula
304.9819	304.9813	0.6	1.9	9.5	4	C14 H10 O3 79Br
	304.9813	0.6	2.0	-1.0	5	C8 H19 N O 79Br 81Br
	304.9810	0.9	2.9	3.0	3	C7 H11 N3 O4 23Na 81Br
	304.9834	-1.5	-5.0	6.0	1	C9 H10 N3 O4 81Br
	304.9789	3.0	9.8	6.5	2	C12 H11 O3 23Na 79Br

HRMS analysis of 57578338. The results are consistent with the presence of the $[M-H]^-$ of $C_{14}H_{11}BrO_3$ with a 1.9 ppm difference from the expected m/z of 304.9813 for the 79Br isotope (observed mass = 304.9819).

- PubChem CID (corresponding to the SID) Compound ID 2160985; Substance ID 57578338
- If available from a vendor, please provide details. Ryan Scientific ID T5225985
- Provide MLS# that verifies the submission of probe molecule and five related samples that were submitted to the SMR collection. Compound is in the MLSMR; not submitted.
- Describe mode of action for biological activity of probe. See Section 3.1g above.
- Detailed synthetic pathway for making probe

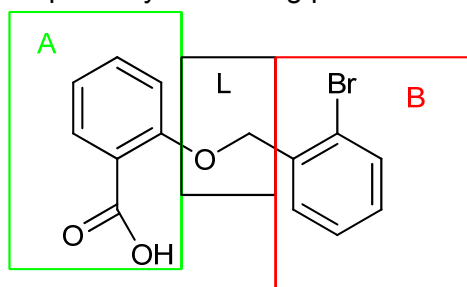
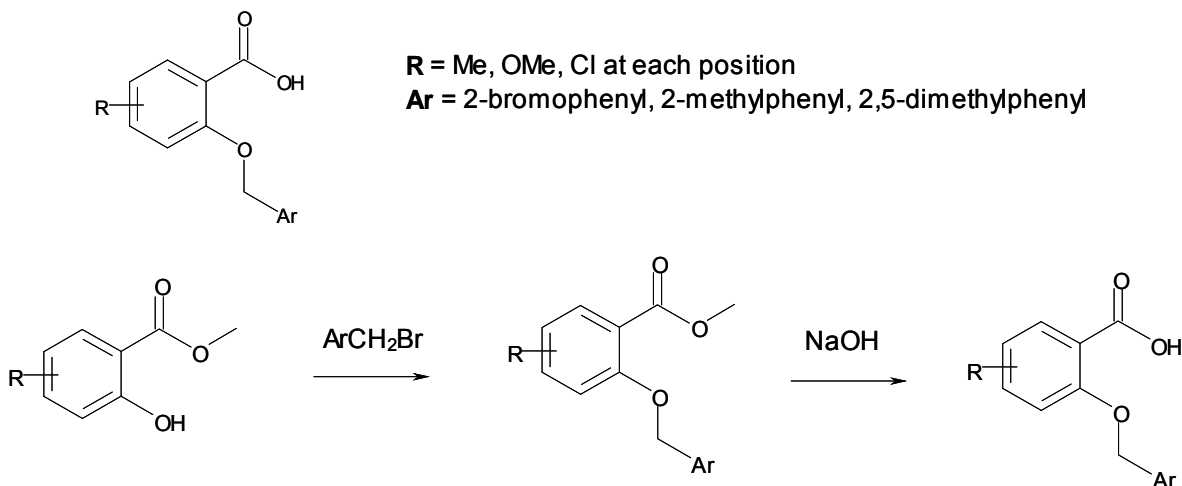


Figure 12 SID 57578338

The original hit compound (SID 57578338) was identified during the primary HTS campaign and confirmed in dose response screen to have a $\log EC_{50}$ =6.82 on Rac1 wild type, $\log EC_{50}$ =7.09 on Rac1 activated mutant, $\log EC_{50}$ =6.60 on cell division cycle 42 (GTP binding protein, 25kDa) activated mutant, $\log EC_{50}$ =6.84 on cell division cycle 42 (GTP binding protein, 25kDa) wild type, $\log EC_{50}$ =6.84 on GTP-binding protein (rab7), $\log EC_{50}$ =6.53 on Ras protein activated mutant, $\log EC_{50}$ =6.47 on Ras protein wild type. The activity was confirmed by purchasing the compound from a commercial source (Ryan Scientific ID T5225985). The chemical optimization strategy involved modification of section A, B, and L (Fig. 12). Analogs of salicylic acid were available commercially and were purchased, chemical synthesis project was also done by ChemDiv, the following variations were investigated: linker section ether oxygen moiety was substituted with carboxylic, carbonyl amine, amide, and thioether moieties, linker length was varied from 1 to 4 atoms; section A ring small substituents were varied by introduction of methyl, methoxy,

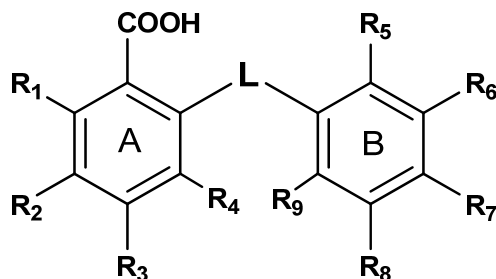
halogen, nitro, heteroalkyl, and amine substituents at various positions, group; ring B section was varied by introduction of methyl, methoxy, halogen, alkyl, and nitro substituents at various positions. Based on these modifications a total of 15 compounds are synthetic derivatives and 9 compounds were commercially purchased. 17 compounds were cherry picked from MLSMR. The synthetic analogs around SID 57578338 were synthesized by ChemDiv in an attempt to improve activity (Schema 3).



Probe 3. Representative synthetic scheme. Reagents and conditions

SAR analysis led to the following conclusions:

- Ring A: No substitutions R_1 - R_4 are allowed, any substitutions will render the compound inactive.
- Ring B: Small hydrophobic substituents are allowed like: methyl, methoxy, halogen, isopropyl.
- Linker: Length is between 1-4 atoms, with the following functional groups: amine, ether, carbonyl, carboxyl.
- Steric effects seems to play a role in the case of compounds: MLS000760373, MLS000714513 and R092-0019, the last compound (R092-0019) is not substituted at positions R_5 and R_9 whereas MLS000760373 and MLS000714513 are substituted with methyl. The R_5 and R_9 methyl induce a preferential out of ring B plane conformation which can explain the observed bioactivity for MLS000760373 and MLS000714513 and no activity for R092-0019.



Supplier ID	PubChem SID	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	R ₈	R ₉	Linker
MLS000761515	57578338									Br	
MLS000104449	7975869										
MLS000081810	3712189										

MLS000081844	3713391							OCH ₃			
0384-0034	56431714										
4478-5409	56431724					F		F			
R152-1213	56431826							Cl			
MLS000760373	24837897						CH ₃	CH ₃			
MLS000715316	24802136								OCH ₃		
MLS000775662	24833685							OCH ₃			
8524-6914	56431766							Cl			
MLS000714513	24803424					CH ₃	CH ₃		CH ₃	CH ₃	
MLS000111718	7974636									OCH ₃	
8016-5752	56431762					C(CH ₃) ₂			CH ₃		
K783-0963	56431813					Cl					
MLS000043585	4243978	F	F	F	F				OCH ₃		
K783-0702	56431811					Cl				Cl	
MLS000689738	24814013	F	F	F	F			CH ₃		CH ₃	
MLS000689734	24814024	F	F	F	F			CH ₃			
MLS000775808	24831309		NO ₂							Cl	
MLS000693348	24795918			Cl		NO ₂					
MLS000663391	24797131			Cl				OCH ₃			
Z494-0034	56431880		OCH ₃			CH ₃				CH ₃	
Z494-0023	56431870	OCH ₃				Br					
Z494-0022	56431869	CF ₃									
Z494-0025	56431872			OCH ₃						Br	
Z494-0028	56431875		CH ₃			CH ₃					
8017-4247	56431764		NH ₂					Cl			

Z494-0033	56431879	OCH ₃				CH ₃				CH ₃	
Z494-0029	56431876			CH ₃						CH ₃	
Z494-0031	56431878			OCH ₃						CH ₃	
Z494-0024	56431871		OCH ₃			Br					
MLS000760107	24835967		NO ₂								
Z494-0036	56431882					OCH ₃	CH ₃			CH ₃	
Z494-0027	56431874	CH ₃									
Z494-0026	56431873					OCH ₃				Br	
Z494-0035	56431881			OCH ₃		CH ₃				CH ₃	
R092-0019	56431825							CH ₃			
Z494-0030	56431877					CH ₃	CH ₃				
Z494-0002	56431857	CF ₃									
MLS000621480	24794372		NO ₂								
MLS000621480	24794372		NO ₂								

Active Inactive Inconclusive

Supplier ID	PubChem SID	logEC ₅₀ Cdc42_ACT	logEC ₅₀ Cdc42_WT	logEC ₅₀ Rab2_WT	logEC ₅₀ Rab7_WT	logEC ₅₀ Rac1_ACT	logEC ₅₀ Rac1_WT	logEC ₅₀ Ras_ACT	logEC ₅₀ Ras_WT
MLS000761515	57578338	6.60	6.84	6.61	6.76	7.09	6.82	6.53	6.47
MLS000104449	7975869	6.26	6.23	6.18	6.59	5.90	5.86	5.86	5.89
MLS000081810	3712189	6.17	5.97	6.14	6.01	6.41	6.73	6.09	5.99
MLS000081844	3713391	4.86	4.87	4.46	4.63	5.37	5.77	4.84	4.90
0384-0034	56431714	5.65	5.47	5.55	5.46	5.11	4.84	5.47	
4478-5409	56431724	5.97	5.79	5.94	6.07	5.97	5.40		5.78
R152-1213	56431826	6.25	6.30	6.75	7.36		5.84		
MLS000760373	24837897	5.32	4.97	4.93		5.76	5.21		4.91
MLS000715316	24802136	6.56	6.44	6.55		6.95	6.34		6.46
MLS000775662	24833685	5.55	5.58	5.97		5.86			
8524-6914	56431766	6.05	5.81		6.47		5.82		
MLS000714513	24803424	5.31	5.23				5.21		
MLS000111718	7974636	5.05			5.57	5.08	4.80	5.20	4.95
8016-5752	56431762	5.99			6.50		5.81		
K783-0963	56431813						6.05		

- i. Center summary of probe properties (solubility, absorbance/fluorescence, reactivity, toxicity, etc.) See Section 3.3c above.
- j. A tabular presentation summarizing known probe properties. See Section 3.3c above.

4. Appendices (in a tabular form): Data for each probe in sections 3.1, 3.2, 3.3

- a. Comparative data on (1) probe, (2) similar compound structures (establishing SAR) and (3) compounds representing “best in class” probes prior to this probe project (to demonstrate that the probe molecule is an “improvement on the current state of the art” in the same assay under the same conditions).
- b. Comparative data showing probe specificity for target

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