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Identification of a Small GTPase Inhibitor using a High-Throughput Flow Cytometry Bead-Based Multiplex Assay

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Abstract

Small GTPases are key regulators of cellular activity and represent novel targets for the treatment of human diseases using small molecule inhibitors. We describe a multiplex, flow cytometry bead-based assay for the identification and characterization of inhibitors or activators of small GTPases. Six different GST-tagged small GTPases were bound to glutathione beads each labeled with a different red fluorescence intensity. Subsequently, beads bearing different GTPase were mixed and dispensed into 384-well plates with test compounds, and fluorescent-GTP binding was used as the read-out. This novel multiplex assay allowed us to screen a library of almost 200,000 compounds and identify over 1,200 positive compounds, which were further verified by dose response analyses, using 6 to 8-plex assays. After the elimination of false positive and negative compounds, several small molecule families with opposing effects on GTP-binding activity were identified. Here we detail the characterization of MLS000532223, a general inhibitor that prevents GTP-binding to several GTPases in a dose-dependent manner and is active in biochemical and cell-based secondary assays. Live cell imaging and confocal microscopy studies revealed the inhibitor-induced actin reorganization and cell morphology changes, characteristic of Rho GTPases inhibition. Thus, high throughput screening (HTS) via flow cytometry provides a strategy for identifying novel compounds that are active against small GTPases.

Keywords

Ras; Rab and Rho GTPases; actin cytoskeleton; bead-based multiplex assay; flow cytometry; fluorescent GTP binding

INTRODUCTION

More than 170 small GTPases have been identified as monomeric molecules of 20 – 40 kDa that bind and hydrolyze guanine nucleotides. Small GTPases in general 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, transcriptional regulation, cell cycle progression, cell migration,

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cellular morphogenesis and polarization.^{1, 2} Mutant forms of small GTPases induce proliferation and transformation of a number of cell types, and differentiation of neuronal cells.^{3–5} Deregulation or abnormal activation of these proteins is also linked to disease processes.^{6, 7} For these reasons small GTPases represent a large class of potential drug targets which have not yet been intensively exploited by the pharmaceutical industry.^{8, 9} Currently, there are limited pharmacological tools targeting individual small GTPases, and most efforts have been focused on inhibiting post-translational GTPase modification by lipids, which is necessary for their 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 and creates toxicity issues.¹¹

Small GTPases exist in two interconvertible forms: GDP-bound inactive and GTP-bound active forms. GTP/GDP exchange studies usually use guanine nucleotide analogues, which behave similarly to the native species and have been modified such that they can be sensitively detected. Radiolabeled GTP analogs such as [γ -³²P] GTP and [γ -³⁵S] GTP γ S have been most commonly used. While these analogs are very sensitive, their use has obvious drawbacks. Recently developed BODIPY(4,4-difluoro-4-bora-3a,4a-diaza-s-indacene)-labeled nucleotides are therefore increasingly being adopted for characterizing of GTPase nucleotide binding activities.^{12, 13} The fluorescence emission of BODIPY-guanine nucleotides is directly affected by protein binding. Free BODIPY-nucleotides in solution exhibit quenched fluorescence, which is unquenched upon protein binding. The resulting 2–10-fold fluorescence enhancement allows real-time detection of protein-nucleotide interactions. We initially developed a bead-based flow cytometric, fluorescent GTP-binding assay that is highly sensitive and allows real-time measurements.¹⁴ Here we describe the critical adaptations that enabled its application in HTS, and formatting for a multiplexed assay that allowed simultaneous screening of six GTPase targets against nearly 200,000 compounds in the Molecular Libraries Screening Center Network library (MLSCN), resulting in the identification of small molecules which alter GTP binding to small GTPases.

MATERIALS AND METHODS

Reagents and Cell Lines

BODIPY- FL- GTP 2'-(or-3')-O-(N-(2-aminoethyl) urethane, G-12411 from Invitrogen Molecular Probes (Eugene, OR). Colorimetric G-LISA assay kit for quantifying Rac1/2/3 activation, rhodamine phalloidin, anti-Rac1 mAb and GST-GTPases (wild type (wt): Cdc42, Rac1, RhoA, H-Ras and constitutively active mutants: Cdc42Q61L, Rac1Q61L, RhoAQ63L, H-RasG12V were purchased from Cytoskeleton, Inc. (Denver, CO). GST-Rab2, GST-Rab7 were purified as described.¹⁴ GST-PAK-PBD and plasmids for GST-Rac1 and Rac2 were generously provided by Dr. G. Bokoch (Scripps Research Institute). Mouse TruBlotTM Ultra: Horseradish Peroxidase anti-mouse IgG was from eBioscience Inc. (San Diego, CA). Rac inhibitor NSC23766 was obtained from Tocris Bioscience (Ellisville, MO) and EHT1864 was provided by Dr. A. Kornienko (New Mexico Institute of Mining & Technology). Bead sets for multiplex assays were from Duke Scientific Corp. (Fremont, CA). All other reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Rat Basophilic Leukemia 2H3 (RBL) and Swiss 3T3 cells and IgE were provided by Dr. B. Wilson (University New Mexico).

Multiplexed Primary Screens

For multiplex analysis of small GTPases, we used 4 μ m diameter glutathione-beads (GSH-beads) distinguished by seven different intensities of red color (various magnitude of emission at 665 $\pm$ 10 nm with excitation at 635 nm). Each polystyrene bead set is supplied at 1.4×10^5 beads/ μ l with about 1.2×10^6 glutathione sites per bead (determined by using

GST- GFP). In preparation for protein binding, 240–250 μ l of each bead set was individually 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 1 mM EDTA (NP-HPSE) for 30 min at room temperature. Bead sets were collected by centrifugation, resuspended in 100 μ l of NP-HPSE and individually incubated with 1 μ M of a given GST-GTPase (Rab2 wt, Rab7 wt, Cdc42 wt, H-Ras wt, Rac1 wt and Rac1Q61L mutant) overnight at 4°C. Individual GTPase-coupled beads were washed twice with 100 μ l ice cold NP-HPSE buffer supplemented with 0.1% BSA and 1 mM DTT and kept in separate tubes on ice. GTPase-coupled beads were pooled together immediately prior to loading of 5 μ l of this mixture in each well of the assay plates. Next 0.1 μ l of test compounds (1 mM stock in DMSO) were added to individual wells to give a final concentration of 10 μ M compound and 1% DMSO, after which 5 μ l BODIPY- FL-GTP (200 nM stock in NP-HPSE) was added to each well. Positive controls, containing the bead mixture, 0.1 μ l DMSO (1% final) and fluorescent GTP, were included in columns 1 and 2 on each plate. Negative controls, containing the bead mixture with fluorescent GTP, 0.5 mM unlabeled GTP as a competitor, and 1% DMSO were assayed separately. Each well contained approximately 2000 beads, coupled with individual GTPases. Plates were placed on rotators and incubated for 40–45 min at 4°C. Sample analysis was conducted with a HyperCyt[®] high throughput flow cytometry platform as described previously.¹⁵ Flow cytometric light scatter and fluorescence emission at 530 $\pm$ 20 nm (FL1) and 665 $\pm$ 10 nm (FL8) were collected on a Cyan ADP flow cytometer (Beckman Coulter, Fullerton, CA). Screening of one, 384 well, plate takes ~15min and complete library screening can be performed in 2–3 weeks. The resulting time-dependent data (one file per plate) were analyzed using IDLQuery software to determine the compound activity in each well. Gating based on forward scatter (FS) and side scatter (SS) parameters was used to identify singlet bead populations. Gating based on FL8 emission distinguishes the beads coated with different proteins, and the median fluorescence per bead population was calculated. A compound was considered a “potential active” if the change in activity was greater than 20% from baseline. Baseline was calculated as described in PubChem.¹⁶

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 a total of eight times from a starting concentration of 10 mM giving a 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 Multiplexed Primary Screens. For dose–response analyses, we used one multiplex (Rab7 wt, Rab2 wt, H-Ras wt, H-RasG12V, Cdc42 wt, and Cdc42Q61L) and 3 single-plexes (for Rac1 wt, Rac1Q61L and GST-GFP).

In experiments including magnesium, we used NP-HPS buffer containing 1 mM MgCl₂. Eight GST-GTPases were assayed simultaneously in a single multiplex (Rac1 wt, Rac1Q61L, Rac2 wt, RhoA wt and RhoAL63, Cdc42 wt, Cdc42Q61L and Ral wt) and 100 nM BODIPY-FL-GTP binding was measured in the presence or absence of the serial drug dilution series. Each dose response series was run in triplicate.

Kinetic Assays

Wild-type GST-Cdc42 (4 μ M) was bound to GSH-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 NP- 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 (1% final concentration), or 10 μ M MLS000532223 and subsequently adding 50 μ l of various concentrations of ice cold BODIPY-FL-GTP. Association of the fluorescent nucleotide was

measured using a FacSCAN flow cytometer in the kinetic mode. The average number of events was 150 per sec. Data were converted to ASCII format using IDLQuery (software available free from the authors). Raw data were exported and plotted using GraphPad Prism software.

Rac Activation Assay

Swiss 3T3 cells were used to monitor Rac1 inhibition by MLS000532223 in cell based assay. Cells were serum starved overnight and treated with 1% DMSO (negative control) or 10 μ M compound in DMSO (1% final concentration) for 20–30 min. As a positive control, cells were treated with 10 ng/ml EGF for 2 min. Cell lysis, immunoprecipitation of active Rac1 with GST-PAK-PBD immobilized on GSH beads, SDS-PAGE and immunoblotting were performed as described.¹⁷

G-LISA Assay for Active Rac

Swiss 3T3 cells were cultured and starved following standard procedures (Cytoskeleton, Inc). Individual cultures grown in 6-well dishes were incubated with MLS000532223 over a concentration range from 0.1–10 μ M for 1 h and subsequently stimulated for 2 min with 10 ng/ml EGF. Cells were washed with ice cold PBS containing calcium and magnesium and further processed for protein and G-LISA assays. Positive controls included Rac1-GTP provided in kit and cell lysate prepared from control cells stimulated only with EGF. Negative controls included buffer only controls and cell lysates prepared from control cells after serum starvation.

Live Cell Microscopy

Live cell microscopy was carried out on RBL-2H3 cells. Cells were grown on coverslips overnight, washed and overlaid with Tyrode's buffer (10 mM Hepes, pH 7.4, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose and 0.1% BSA). Time lapse images were taken after addition of 10 μ M MLS000532223 (final concentration) at 60 s intervals for up to 100 min at 37°C. For ligand stimulation, IgE primed RBL cells were treated with 1 μ g/ml DNP-BSA. Imaging was performed using a Bio-Rad Radiance 2100 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 coverslips and cultured overnight in the presence or absence of 10 μ M MLS000532223. As a positive control, cells were stimulated with 1 μ g/ml DNP-BSA for 30 min as previously described.¹⁸ 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 with rhodamine-phalloidin. All incubations were 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.

β -Hexosaminidase Secretion Measurements

For measurement of β -hexosaminidase release, cells were cultured overnight with IgE in 24-well culture plates. IgE primed cells were washed and incubated for 1 h, with indicated concentrations of inhibitor in Tyrode's buffer. Aliquots (100 μ l) of the cell culture supernatants were analyzed for the spontaneous release of β -hexosaminidase. Cells were activated by exposure to DNP/BSA (0.1 μ g/ml) in Tyrode's buffer for 30 min at 37°C. Release of β -hexosaminidase was determined as described in Ortega et al.¹⁹ The values

were expressed as percent of total amount of β -hexosaminidase, determined using 1% Triton X-100 in Tyrode's buffer.

RESULTS

Identification and Verification of Rho Family GTPase Inhibitors using Multiplex Screens

For detection of GTP binding to small GTPases we used a flow cytometry-based assay for multiplex analysis of GTPase activity in a HTS of the MLSCN library. In the primary screen, each well of the microtiter plate contained seven sets of beads, with variable red fluorescence intensities. Six individual GST-GTPases representative of Rho, Ras and Rab branches of the Ras-related GTPase family (Cdc42wt, Rac1wt, Rac1Q61L, Rab2 wt, Rab7wt, and H-Ras wt) were first bound to beads of a particular fluorescence intensity (Fig. 1A–C). One bead set consisted of GSH-beads without any bound protein, and served as a “scavenger” for GST-proteins that might dissociate during the assay and precluded cross-contamination of protein-bound bead sets. The individually conjugated beads were mixed, dispensed into 384-well plates and incubated with fluorescent GTP in the presence of each test compound. Positive and negative controls contained DMSO at an identical concentration as wells containing test compounds. Protein-bound fluorescence served as the read-out and was measured using a HyperCyt flow cytometry system.²⁰ Briefly, the HyperCyt has 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 to allow individual samples to be distinguished in the flow cytometer read-out. Between 150–300 beads for each GTPase in the well. When the bead count was below 25 for a given GTPase, the result for that protein was considered “missing”. For the 194,738 total compounds screened against Cdc42 wt there were only 66 missing compounds. Figure 1D shows the time-dependent data from one 384-well plate with a single hit out of 320 test compounds on the plate. The decrease in fluorescence suggested the molecule was an inhibitor.

Primary screening of the MLSCN library (194,738 compounds) in a six-plex system, resulted in the identification of 100–500 positive compounds for each target protein. The complete results from the multiplex screen are available on PubChem¹⁶ (AID 757–761 and 764). The quality control Z' statistics were very good for each target. In particular, the average Z' was 0.87 $\pm$ 0.04 for Cdc42, 0.85 $\pm$ 0.04 for Rac1, and 0.90 $\pm$ 0.03 for Rac1 activated mutant.

The primary screening results using the described bead-based multiplex method are presented for two representative assay plates (Fig. 1E and F). In the first sample plate, only one compound out of 320, had an inhibitory effect on BODIPY-FL-GTP binding to the tested GTPases (Fig. 1E, arrow). Analysis of the raw flow cytometric data, made with IDLeQuery software, and imported into an Excel template, revealed that the inhibitory compound was MLS000532223 (1-(3-nitrophenyl)-3-phenyl-2-propyn-1-one). The primary screening data for a second sample plate (Fig. 1F) revealed one autofluorescent compound as judged by the increase in fluorescence measured for scavenger and all GTPase bead sets (arrow head), as well as a Cdc42 specific inhibitor (arrow). The Cdc42 inhibitor was identified as MLS000573151 (IUPAC: 4-[3,5-di(phenyl)-4,5-dihydropyrazol-1-yl]benzenesulfonamide).

Multi-plex and single-plex dose response assays were used to confirm the primary screen data. A GST-GFP control was used to eliminate the possibility of compound effects on the association of GST-proteins with the beads. Dose-response results are reported in PubChem (AID 1333–1337, 1339–1341)¹⁶ with compounds categorized as having activating or inhibitory effect on one or more of the GTPases tested, including Rab2 wt, Rab7 wt, and H-

Ras, Rac1, Cdc42 and RhoA wild-type and constitutively active forms. MLS000532223 behaved as a high affinity, selective inhibitor of Rho family GTPases (EC_{50} 1.6×10^{-5} – 1.2×10^{-4} M) (Fig. 2A). Less than 20% inhibition of H-Ras and Rab proteins was observed only at 10^{-4} M concentrations of the compound. MLS000573151 acted more specifically and inhibited only Cdc42 activity (EC_{50} $\sim 2 \times 10^{-6}$) (Fig. 2B) confirming the primary screen findings.

To measure the activities of MLS000532223 and MLS000573151 on RhoA and related members of the Rho GTPase family, we conducted the multiplex assay in the presence of Mg^{2+} ions, which is crucial for BODIPY-FL-GTP binding to RhoA (Fig. 2C, D). The eight-plex consisted of wild-type and constitutively active mutants of the Rho family GTPases, including Rac1, Rac 2, RhoA, and Cdc42. Ral GTPase was included on account of the known interactions between Ral and Rho family GTPases.²¹ MLS000532223 inhibited the activity of all tested GTPases of the Rho and Ral families (Fig. 2C), while MLS000573151 was a specific for Cdc42 (Fig. 2D). Thus, using flow cytometry measurements for multiplex, high throughput screening we identified novel inhibitors with micromolar affinities for the Rho-family GTPases.

MLS000532223 Modulates Rho-family GTP-binding Kinetics

Real-time assays of GTP-binding kinetics in the presence or absence of MLS000532223 were performed using wild-type Cdc42 and our established flow cytometry based assay¹⁴ (Fig. 3A–B). Assays were performed holding the inhibitor concentration constant at 10 μ M and monitoring kinetics of BODIPY-GTP binding over a >10-fold concentration range (6.5–200 nM GTP). The presence of the small molecule inhibitor decreased the B_{max} of GTP-binding, while the affinity of GTP to wild type Cdc42 remained constant (K_d = 43nM) (Fig. 3C).

MLS000532223 is Active in Cell-Based Assays and Inhibits EGF-Stimulated Rac 1 Activation

Serum starved Swiss 3T3 cells were used to monitor Rac1 activation in the presence or absence of 10 μ M MLS000532223 using a serine/threonine p-21 activated kinase (PAK) binding assay (Fig. 4A). This method takes advantage of the fact that only the active GTP-bound form of Rac is capable of binding to the PBD domain of PAK.¹⁷ The low level of active Rac1 in DMSO-treated (negative control) cells (lane 2), was completely abolished after a 30 min pretreatment of cells with 10 μ M MLS000532223 (lane 3). Cells treated with 10 ng/ml EGF for 2 min showed a robust activation of Rac1 (lane 5), while growth factor induced activation of Rac1 was completely inhibited by preliminary treatment of starved cells with 10 μ M MLS000532223 (lane 4).

In vitro measurements of BODIPY-FL-GTP binding to GST-Rac1, immobilized on beads, showed that the inhibitory effects of 10 μ M MLS000532223 and 50 μ M NSC23766 (a known Rac inhibitor²²) were similar, suggesting greater efficacy of MLS000532223. There was no inhibition of GTP binding to Rac1 in the presence of 10 μ M EHT1864 (selective inhibitor of Rac GTPases in fibroblasts²³) (Fig. 4B).

An ELISA based assay for Rac1 activation was used to monitor the dose dependent inhibition of EGF-mediated Rac activation. Cells were pretreated with varying concentrations of MLS000532223 prior to a 2 min exposure to EGF and comparatively evaluated against NSC23766. MLS000532223 effectively inhibited EGF stimulated activation of Rac1 at 3–10 μ M, while NSC23766 showed more modest inhibition even at doses up to 50 μ M. Neither compound was effective at blocking EGF-stimulated Rac activation at concentrations of 1 μ M or less (Fig. 4C).

MLS000532223 Inhibits Actin Rearrangements and Changes in Cell Morphology that are Downstream of Rho family GTPase Activation

GTPases play a key role in actin reorganization and morphological transformations of the cell. It is known that Rac1 promotes polymerization at the leading edge, orchestrating the formation of lamellipodia and membrane ruffles,²⁴ while Cdc42 and Rho are responsible for filopodia and stress fiber formations, respectively.²⁵

To assess if MLS000532223 might also affect downstream pathways regulated by Rho-family GTPases, we examined the effect of the compound on cell morphology and actin reorganization in RBL-2H3 cells following IgE receptor activation. This cell line has been widely used as a model for studies on hypersensitivity diseases that include allergy, asthma, and anaphylaxis. Activation of RBL cells via cross-linking of the high affinity IgE receptor (Fc ϵ RI) initiates cascades of biochemical events, leading to degranulation, membrane ruffling, and other physiological responses.²⁶ Cells begin to flatten within 2 min, spread on their substratum, and extend lamellipodia, an indicator of active ruffling.^{26, 27} At later stages of activation, cells secrete inflammatory mediators.²⁷ The Rho family GTPases, Rac and Cdc42, are key mediators of the actin rearrangements and morphologic changes resulting from IgE receptor stimulation of RBL cells.^{28, 29}

We examined the actin cytoskeleton in resting, ligand stimulated and MLS000532223 (10 μ M) treated RBL cells. Staining of fixed cells with rhodamine-phalloidin and confocal microscopy were used to detect the changes in cell shape, as well as reorganization of F-actin (Fig. 5A). Resting RBL cells were round and tall or modestly spread on the adherent growing surface (DMSO). Activation of RBL cells by cross-linking of Fc ϵ RI caused a remarkable transformation in cell surface topography (DNP/BSA), 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. MLS000532223 treated cells appeared round and tall, like resting RBL cells and failed to flatten after cross-linking of IgE receptors (MLS000532223 $\pm$ DNP/BSA).

Stress fiber formation is a hallmark of Rho activation and has been well-documented in EGF treated fibroblasts such as Swiss 3T3 cells.²⁴ Quiescent, serum-starved Swiss 3T3 fibroblasts exhibit filamentous actin staining largely at the cell periphery, corresponding to the cortical actin (Fig. 5B, DMSO). Treatment of cells with EGF activates Rho and leads to the robust formation of numerous stress fibers within 5–10 min after stimulation (EGF). Brief pre-treatment of cells for 30 min with 10 μ M MLS000532223 completely inhibited formation of stress fibers upon EGF stimulation (MLS000532223 $\pm$ EGF).

Live cell imaging studies were used to monitor the dynamic responses to MLS000532223 in real time. Cross-linking of IgE receptors induced cell flattening, ruffling and formation of lamellipodia and filopodia within a minute after stimulation, and surface ridges were enhanced after 15–20 min (Fig. 6B), as compared to DMSO treated cells which remained spherical (Fig. 6A). The presence of 10 μ M MLS000532223 completely abrogated normal cell shape changes in response to ligand-mediated activation (Fig. 6D), while resting RBL-2H3 cells treated with MLS000532223 showed no change in morphology (Fig. 6C).

Thus, in two different cell lines actin rearrangements and changes in cell morphology in response to receptor mediated activation of Rho family GTPases were completely abrogated. The data confirm that MLS000532223 acts on upstream Rho family GTPase targets (Rac1, Cdc42 and RhoA) and thereby broadly inhibits downstream responses such as lamellipodia, filopodia and stress fiber formation as expected.

MLS000532223 Inhibits Ligand-Stimulated β -hexosaminidase Release

In RBL-2H3 cells, ligand stimulated activation of $Fc_\epsilon RI$ and Rho family GTPases stimulates both actin remodeling and β -hexosaminidase secretion. Therefore, the compound was also tested for an ability to affect β -hexosaminidase secretion in resting and stimulated RBL-2H3 cells. In the absence of ligand stimulation, there was no β -hexosaminidase secretion with 10 μM MLS000532223 compound (Fig. 7A), indicating that compound is not toxic to cells. In ligand-stimulated cells, the presence of 10 μM MLS000532223 reduced secretion by 50% (Fig. 7B).

NSC23766 and EHT1864 were recently reported to selectively inhibit Rac GTPases in fibroblasts^{22, 30}. Therefore, it was of interest to compare available inhibitors to MLS000532223 in the hematopoietic cells studied here. In contrast to MLS000532223, 10 μM EHT1864 failed to inhibit RBL-2H3 cell shape changes and β -hexosaminidase secretion in response to ligand-stimulation (Fig. 6E and Fig. 7A). NSC23766 was toxic for RBL-2H3 cells, inducing extensive β -hexosaminidase spontaneous release and cell death (Fig. 7A). The data suggest that different Rac inhibitors may exhibit cell type specificity, which should be carefully evaluated.

DISCUSSION

For detection of GTP binding to small GTPases we used a flow cytometry-based assay¹⁴. This method was found to be very sensitive, and allowed us to measure a dose response of GTP binding to individual GST-tagged small GTPases even though different conditions were required for optimal activity of individual GTPases. BODIPY-GTP γNH was identified as a better substrate for some of the small GTPases such as RhoA wt, Rac1wt, Cdc42, while others (Rab proteins¹⁴ and Ras) preferred binding to BODIPY-FL-GTP. Mg^{2+} ions were crucial for Rho enzyme activity measurement, 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 chose specific conditions, which though not optimal for all GTPases, allowed us to simultaneously measure GTP binding of several small GTPases. Experiments comparing GTP-binding to individual GTPases in multiplex or single-plex assay format were identical, therefore a multi-plex flow cytometric assay was used for HTS of a MLSCN library. Approximately 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. A total of 100–500 positive compounds for each member of multiplex were identified in the primary screen (the results from this multi-plex screen were published on PubChem AID 757, 758, 759, 760, 761, 764). False positives such as autofluorescent compounds and compounds affecting GST-protein binding to GSH-beads were eliminated, and ~1200 compounds were selected and tested in secondary dose-response assays of which 18 % of the compounds judged to be active in the primary screen were confirmed in dose response analysis. Here we identified Rho family selective (MLS000532223) and Cdc42 specific (MLS000573151) inhibitors and characterized the cellular activity of MLS000532223 in detail. The pull-down assay of active Rac1 showed that MLS000532223 completely inhibited EGF induced Rac1 activation in Swiss 3T3 cells. Live cell imaging and confocal microscopy studies demonstrated the inhibition of actin reorganization and cell morphology changes downstream of Rho family GTPase activation.

Inhibitors of Rho family GTPases are of significant interest as targets for drug therapy. Hyperactivated small GTPases are implicated in 30% of all human cancers and are particularly prevalent in myeloid leukemia, pancreatic, lung and colon carcinomas.^{4, 5} Rho

family GTPases have been found central to the control of gene regulation, cell proliferation and cell migration.² Thus, it is not surprising that based on overexpression or hyperactivation they are increasingly associated with cancer cell metastasis and invasion, as well as uncontrolled proliferation and loss of growth control³¹. Based on the inhibitory activity of MLS000532223 on Rac1, Rac2 and Cdc42 in hematopoietic cell types, it will be of interest to further develop this probe for possible use to control the growth of leukemic cells.

Kinetic experiments revealed that MLS000532223 did not affect the K_d of GTP binding and since its activity was not reversible (data not shown), suggesting that it is not a competitive inhibitor for the GTP binding site. The observed incomplete inhibition of GTP binding could represent an allosteric binding site, limited molecular solubility, or protein heterogeneity which has not been resolved by the experiments performed to date.

Comparison of MLS000532223 against two other Rac1 specific inhibitors that have been developed, NSC23766 and EHT1864, reveals the importance of biological context.^{22, 30} NSC23766 was developed using rational drug design and found to specifically inhibit guanine nucleotide exchange and Rac1 activation without affecting Cdc42 or RhoA both in vitro and in cell-based assays.²² At the doses (50 μ M) used on NIH 3T3 cells, NSC23766 proved toxic to RBL-2H3 cells and at lower doses (10 μ M) it failed to inhibit the activated Fc ϵ R1-induced cell spreading and lamellipodia formation. EHT1864, identified as an inhibitor of amyloid precursor processing, interacts with Rac1 and related isoforms. Although EHT1864 blocked lamellipodia formation in NIH 3T3 fibroblasts following platelet derived growth factor stimulation³⁰, as shown here it did not block activated Fc ϵ R1-induced cell spreading and ruffling in RBL-2H3 cells. Together the data suggest that MLS000532223 is an effective Rho GTPase inhibitor for hematopoietic cells and that cell-type specific inhibition of Rho family GTPases may be possible and a worthwhile therapeutic strategy.

In sum, our data indicate that multi-plex bead-based assay could successfully be used via flow cytometry for HTS of libraries. 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 enabled the analysis, comparison and detection of the specific activators/inhibitors of individual GTPases in a systematic and previously unparalleled manner. The identified small molecules provide a new chemical platform for the rationale design of selective inhibitors of key small G protein members that could represent a boon for understanding the biology and pharmacology of small GTPases.

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ABBREVIATIONS

BODIPY	4,4-difluoro-4-bora-3a,4a-diaza-s-indacene or <u>d</u> ipyrrromethene- <u>b</u> oron-difluoride
BSA	bovine serum albumin

DMSO	dimethyl sulfoxide
DNP	dinitrophenol
EGF	epidermal growth factor
FITC	fluorescein isothiocyanate
GFP	green fluorescence protein
GSH	glutathione
GST	Glutathione-S-transferase
mAb	monoclonal antibody
MLSMR	Molecular Libraries Small Molecule Respository (MLSMR)
NMMLSC	New Mexico Molecular Libraries Screening Center
PAK	p-21 activated kinase
PBD	protein binding domain
RBL-2H3	rat basophilic leukemia cells
UNMCMD	UNM Center for Molecular Discovery

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Fig. 1. High throughput screen identifies small molecule inhibitors of small GTPases
(A) Schematic diagram of the fluorescent GTP binding to GST-GTPases immobilized on GSH-beads. Beads of varying red fluorescence intensities are used as identifiers for individual protein-conjugated bead sets. **(B)** Plot of forward scatter versus a logarithm of side scatter. Singlet beads were selected by drawing a gate as shown. **(C)** 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. **(D)** 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 (Y axis: fluorescence intensity, X axis: time). Arrow denotes positive well containing inhibitor, inset 2x magnification of positive 'hit' in primary screen. **(E)** The raw data were parsed in IDLeQuery to produce annotated fluorescence summary data for each well. The parsed data were then processed through an Excel template file constructed specifically for the assay to segregate data for each target and the fluorescence scavenger in the multi-plex. Only one compound out of 320 inhibits all GTPases (arrow); **(F)** Excel file shows one fluorescent compound (arrow head) and one Cdc42 specific inhibitor (arrow)

Fig. 2. Dose dependent inhibition of BODIPY-FL-GTP binding to GTPases by small molecules MLS000532223 and MLS000573151 inhibit GTP-binding in the presence of (A and B) 1mM EDTA and (C and D) 1mM MgCl₂; (n=3). Final concentration of fluorescent GTP was 100 nM.

Fig. 3. Small molecule inhibitor affects Cdc42 GTP-binding kinetics

Time course of BODIPY-FL-GTP binding to Cdc42 wt in the (A) absence or (B) presence of 10 μM ML5000532223 was measured over 3 min, as described in Materials and Methods. (n=4) (C) Values of Cdc42-bound fluorescent nucleotide (after 3 min of incubation) were plotted versus the concentration of BODIPY-FL-GTP. Graphs were analyzed using GraphPad Prism curve-fitting.

Fig. 4. Active Rac1 assays

(A) Active Rac1 from serum starved Swiss 3T3 cells isolated with GST-PAK-PBD beads. Bottom blot shows total Rac 1 in lysate as a protein loading control. Beads bound GST-PAK-PBD only as negative control (lane 1). Swiss 3T3 cell lysates from DMSO treated control cells (lane 2), cells treated with 10 μ M MLS000532223 (lane 3), 10 μ M MLS000532223 pretreated cells activated with EGF (lane 4), 10 ng/ml EGF alone as a positive control (lane 5). (B) In vitro bead based assay of Rac1 binding to 100 nM fluorescent GTP in the presence of 10 μ M MLS000532223, 50 μ M NSC23766 or 10 μ M EHT1864. (C) G-LISA assay of active Rac in starved Swiss 3T3 cells performed as described in Materials and Methods.

Fig. 5. MLS000532223 modulates actin remodeling in mast cells

(A) 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 (DMSO), DNP-stimulated cells (DNP/BSA), cells treated with 10 μM MLS000532223 (MLS000532223) and cells activated with 1 μg/ml DNP/BSA after inhibitor treatment (MLS000532223 – DNP/BSA). (B) Quiescent, serum-starved Swiss 3T3 (DMSO), starved Swiss 3T3 cells after activation with 10 ng/ml EGF for 2 min (EGF), fibroblasts treated with 10 μM MLS000532223 for 30 min (MLS000532223), and cells activated with EGF after treatment with inhibitor pretreatment (MLS000532223-EGF). Bars 5 μm

Fig. 6. MLS000532223 inhibits IgE induced morphological changes in 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 1 $\mu\text{g/ml}$ DNP-BSA. **(C)** RBL-2H3 cells incubated with 10 μM MLS000532223. **(D)** RBL cells pretreated with 10 μM MLS000532223 and then activated with 1 $\mu\text{g/ml}$ DNP/BSA. **(E)** IgE primed RBL-2H3 cells incubated with 10 μM EHT 1864 and treated with 1 $\mu\text{g/ml}$ DNP/BSA. Bars 10 μm

Fig. 7. MLS000532223 inhibits ligand-stimulated β -hexosaminidase secretion of RBL cells (A) RBL cells were grown in RPMI medium overnight in 6-well plate. After washing attached cells were incubated with Tyrode's buffer containing 1% DMSO, 10 μ M MLS000532223; 20 μ M EHT 1864 or 50 μ M NSC 23766 for 1h. β -Hexosaminidase released into the supernatant was determined as described by Ortega.¹⁹ Total enzyme activity was measured on wells in which the cells were lysed with 0.1% Triton X-100. (B) β -Hexosaminidase activity in the supernatants of resting and IgE activated RBL cells, treated with various concentrations of MLS000532223, was determined and the percentage of enzyme released was calculated as described in Materials and Methods.

Table 1

Summary of Primary and Confirmatory Screening Outcomes.

GTPase target	Primary Screen Actives ^a	Secondary Screen Actives ^b	Inhibitors	Activators
Cdc42 wt	398	33	20	10
Cdc42 act	ND	40	25	10
Rab2	365	19	5	14
Rab7	107	13	6	7
Rac1 wt	521	48	28	17
Rac1 Q61L	219	54	35	18
H-Ras wt	267	9	5	4
H-Ras G12V	ND	7	5	2

^aTotal number of compounds screened in primary screen: 194,635.

^bTotal number of compounds screened in secondary screens: 1281 for Rac1 wt and Rac1 Q61L; 1262 for all other GTPases. Compounds were categorized as activators or inhibitors if they increased or decreased GTP-binding, respectively, by greater than 20%. A small number of active compounds could not be specifically designated as inhibitors or activators based on the GTP-binding assay.

ND not determined.