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Article

A Cell Active Chemical GEF Inhibitor Selectively Targets the Trio/RhoG/Rac1 Signaling Pathway

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SUMMARY

RhoGEFs (guanine nucleotide exchange factors of the Rho GTPase family) are upstream regulators of cell adhesion and migration pathways, thus representing attractive yet relatively unexplored targets for the development of anti-invasive drugs. We screened for chemical inhibitors of TrioN, the N-terminal GEF domain of the multidomain Trio protein, and identified ITX3 as a nontoxic inhibitor. In transfected mammalian cells, ITX3 blocked TrioN-mediated dorsal membrane ruffling and Rac1 activation while having no effect on GEF337-, Tiam1-, or Vav2-mediated RhoA or Rac1 activation. ITX3 specifically inhibited endogenous TrioN activity, as evidenced by its ability to inhibit neurite outgrowth in nerve growth factor (NGF)–stimulated PC12 cells or C2C12 differentiation into myotubes. This study introduces a selective cell active inhibitor of the Trio/RhoG/Rac1 pathway and validates RhoGEFs as druggable targets.

INTRODUCTION

RhoGEFs promote the exchange of GDP with GTP on Rho GTPases and are frequently associated with membrane receptor complexes. As such, they represent major entry points allowing cross-talk between extracellular cues and Rho signaling cascades. In mammals, RhoGEFs include the Dbl-like and the Dock protein families. Dbl-like proteins, with over 70 members, all have a catalytic Dbl homology (DH) domain and a pleckstrin homology (PH) domain involved in phospholipid or protein binding; the Dock family comprises 11 members, all with a catalytic Dock homology region (DHR2) and a Ca^{2+}-dependent phospholipid binding (C2) domain (Rossman et al., 2005; Cote and Vuori, 2007).

Over the last decade, RhoGEF and Rho members have emerged as key factors in the progression of many cancer types (Ellenbroek and Collard, 2007; Vega and Ridley, 2008). This is consistent with the known roles of Rho proteins in the control of basic cell structures, in particular the respective roles of RhoA, Rac1, and Cdc42 in the formation of F-actin stress fibers, lamellipodia, and filopodia in all multicellular organisms (Jaffe and Hall, 2005; Boureux et al., 2007). Rho proteins thus modulate cell adhesion, polarity, migration, contraction, proliferation, and apoptosis in a number of normal and pathological processes, from cell movements in the developing embryo to common diseases, such as hypertension, cancer, or neurodegenerative diseases (Shirai et al., 2007; Fritz and Kaina, 2006). In contrast to Ras proteins, which are activated by somatic mutations in many tumor types (Diaz-Flores and Shannon, 2007), Rho pathways are mostly activated in tumors as a consequence of GTPase or GEF overexpression (Vega and Ridley, 2008).

Rho signaling components are therefore attractive targets for the development of inhibitory drugs (Fritz and Kaina, 2006); indeed, various chemical inhibitors have been identified acting at different points of Rho signaling: FTI (Kohl et al., 1993), which inhibits the transfer of isoprenyl lipids that anchor Rho GTPases in membranes; NSC23766 (Gao et al., 2004), which targets a Rac region involved in the binding to GEFs; and EHT 1864 (Shutes et al., 2007), which binds to Rac and destabilizes the bound nucleotide. Inhibitors that target effectors were also developed: Y27632 (Uehata et al., 1997), which inhibits the RhoA effector ROCK; and IPA-3 (Deacon et al., 2008), which inhibits the Rac/Cdc42 effector PAK kinases. Several inhibitors are already in clinical use, such as fasudil, which targets ROCK for the treatment of cerebral vasospasm and angina (reviewed in Olson, 2008), and nitrogen-containing biphosphonates, which indirectly inhibit isoprenoid biosynthesis for the control of hypercalcemia, osteoporosis, and bone cancer metastasis (reviewed in Russell et al., 2007).

Although considered to be promising therapeutic targets, the only RhoGEF inhibitors identified so far act against Trio, a multidomain protein implicated in neurogenesis, phagocytosis, and myogenesis (Lin and Greenberg, 2000; Henson, 2005; Charrasse et al., 2007; Briançon-Marjollet et al., 2008). Trio is unusual in that it contains two RhoGEF domains: one N-terminal domain (TrioN), active on RhoG/Rac1 (Blangy et al., 2000), and a C-terminal domain (TrioC), active on RhoA (Debant et al., 1996). Trio is involved in the invasive behavior of glioblastoma and breast cancers (Lane et al., 2008; Salhia et al., 2008), while Tgat, an alternative Trio isoform derived from TrioC, was identified as a new oncogene in adult T-leukemia (Yoshizuka et al., 2004). To date, two types of Trio inhibitors have been characterized: Tripα and derived peptides, which associate with and inhibit TrioC (Schmidt et al., 2002; Bouquier et al., 2009), and NPPD, a compound that is biochemically active on TrioN and that we previously identified from a screen for RhoGEF inhibitors in a permeant yeast strain (Blangy et al., 2006).

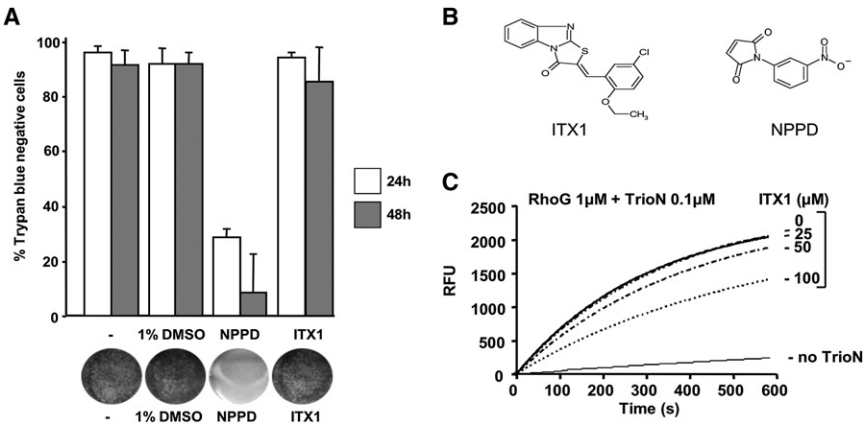

Figure 1. ITX1 Compound Is a Nontoxic Inhibitor of RhoG Activation by TrioN

(A) Upper panel: REF 52 cells were cultured for 24 hr or 48 hr in 10% FCS alone (-) or supplemented with 1% DMSO, 100 μM NPPD in 1% DMSO, or ITX1 in 1% DMSO. Histograms represent cell viability, defined as the ratio of Trypan Blue negative cells versus total cell number. Lower panel: REF 52 cells were cultured for 24 hr in the same conditions as above then stained with Crystal violet.

(B) ITX1 and NPPD chemical structures.

(C) In vitro exchange kinetics were performed by measuring the increase in fluorescence emitted with time upon incorporation of Mant-GTP into 1 μM RhoG alone (no TrioN) or with 0.1 μM TrioN in the presence of 0, 25, 50, or 100 μM ITX1 in 1% DMSO. Mant-GTP exchange is expressed as relative fluorescence units (RFU).

We show here that NPPD is toxic in mammalian cells and identify a new chemical compound isolated from the same screen. We demonstrate that this compound is active in several cell lines, blocking cellular responses to exogenously expressed TrioN or endogenous Trio, and thus disrupting various physiological processes.

RESULTS

Identification of a Nontoxic Compound, Inhibiting TrioN In Vitro GEF Activity

We previously screened a library of 3,500 chemical compounds using a GEF activity assay in permeant yeast cells and identified 1-(3-nitrophenyl)-1H-pyrrole-2,5-dione (NPPD) as a potent inhibitor of TrioN activity on RhoG and Rac1 in vitro (Blangy et al., 2006). In this study, NPPD was toxic when applied to mammalian REF-52 cells, killing 70% of the treated cells after a 24 hr treatment, and killing up to 90% of cells after 48 hr, as detected by trypan blue staining (Figure 1A). We thus assayed the toxicity of other compounds isolated from the initial screen and selected 2-(5-chloro-2-ethoxybenzylidiene) [1,3] thiazolo[3,2-a] benzimidazol-3(2H)-one (from now on referred to as ITX1, for Inhibitor of Trio eXchange 1) for its absence of toxicity in REF-52 cells (Figure 1A). ITX1 is structurally distinct from NPPD (Figure 1B). We confirmed that ITX1 indeed inhibited TrioN-mediated GTP exchange on RhoG in vitro, as demonstrated by the dose-dependent inhibition of Mant-GTP loading on RhoG (Figure 1C).

Secondary Screening of ITX1 Analogs

We next performed a search in the PubChem database to examine whether compounds showing structural features similar to ITX1 had already been used in other bioassays. Of 349 compounds with structures related to the initial compound ITX1 (CID 1919674), only 34 had been previously tested and found to be inactive in seven different bioassays (screens for inhibitors of EphA4 receptor, Hsp70, HePTP tyrosine phosphatase, MKP3 dual specificity phosphatase, Bcl-B converters, ER stress-induced apoptosis, or HIV-1 RNase H). Thus, despite their high frequency in libraries, compounds related to ITX1 have not been identified as efficient inhibitors of other targets. We collected a series of ITX1 structural analogs and tested their ability to inhibit TrioN-stimulated RhoG exchange in vitro (Figure 2). Of thirteen analogs, seven showed inhibitory activities higher than or similar to that of ITX1 (ITX2-8), whereas the other six showed little activity. Comparison of IC_{50} for the most active compounds showed that only ITX2 is statistically significantly more potent than ITX1. ITX14 showed an IC_{50} six to fifteen times lower than those of active compounds and was used from now on as a negative control analog. ITX2 was not investigated further because of its elevated background fluorescence in microscopy analysis of cultured cells. However, the higher efficacy of ITX2 suggests that substitutions on the benzene cycle of the benzylidene moiety have no positive effects (compared with ITX1 and ITX8) and may even reduce the inhibitory activity (compared with ITX10 and ITX14). Single-change variants of ITX2 will be needed for a comprehensive structure activity relationship analysis.

Biochemical Analysis of ITX Compounds

We next confirmed the dose-dependent inhibition of GTP exchange for two analogs of different strengths (ITX3 and ITX14) over a 10 min time course. ITX3 (Figure 3A, left panel) showed clear dose-dependent inhibition throughout kinetics with a higher level of efficiency than ITX1 (see Figure 1B), whereas ITX14 (Figure 3A, right panel) had little effect, as expected from the data described above (Figure 2). TrioN also promotes GTP exchange on Rac1, although to a lesser extent than on RhoG (Blangy et al., 2000); thus, we investigated whether ITX1 analogs also inhibit TrioN activity on Rac1 in a 10 min time course experiment. ITX3 and ITX1 compounds inhibited TrioN-stimulated GTP exchange on Rac1 by 45% to 50%, whereas ITX14 had no effect (Figure 3B). This finding demonstrates that ITX compounds inhibit TrioN stimulation of both GTPases to the same extent. We next examined whether ITX1 analogs have a direct effect on the affinity of RhoG for Mant-GTP, as observed for the Rac1 inhibitor EHT 1864. To this end, we analyzed the nucleotide release kinetics for RhoG preloaded with Mant-GTP (Figure 3C). In the absence of TrioN, fluorescence remained stable throughout the experiment (i.e., 900 s) and was insensitive to the presence of ITX1, ITX3, or ITX14; thus, the stability of the bound nucleotide did not seem to be

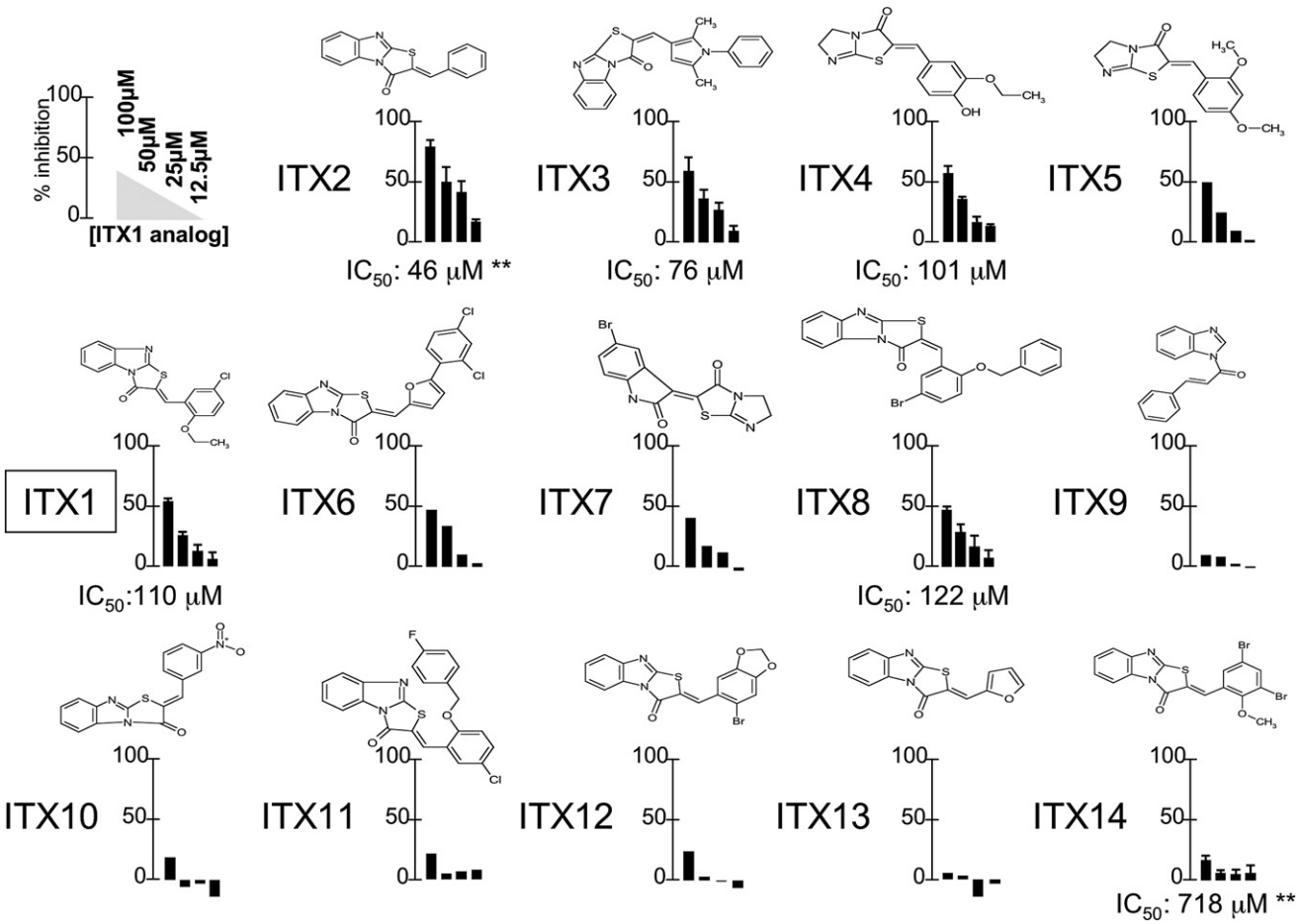

Figure 2. Secondary Screening of ITX1 Analogs

For each analog, the chemical structure and a histogram representing the percentage of inhibition of RhoG activation by TrioN are shown at various analog concentrations, as illustrated in the top left panel. Fluorescence was measured after a 15 min reaction. For ITX1-4, ITX8, and ITX14, means and standard deviations from three experiments are represented on histograms and IC_{50} are shown (** indicate IC_{50} significantly different from ITX1 IC_{50}, Mann-Whitney $p < 0.01$).

affected by these compounds. ITX3 inhibited the TrioN-stimulated release of Mant-GTP by 50%, suggesting that ITX3 affects the first steps of the exchange reaction, during which the bound nucleotide is released.

ITX3 Inhibition of TrioN Signaling In Vivo

We next examined whether the ITX1 analogs showing in vitro inhibitory activity are active on mammalian cells. We tested the effects of these compounds on TrioN-induced formation of particular F-actin membrane structures (Blangy et al., 2000). In rat embryo fibroblasts REF-52, 72% of TrioN-expressing cells showed extensive dorsal ruffling associated with loss of actin stress fibers, 28% of TrioN-expressing cells showed peripheral lamellipodia with reduced stress fiber content, and less than 1% displayed only small or no changes in F-actin structure (Figure 4A). We analyzed ITX1 and four analogs of higher (ITX3 and ITX4), similar (ITX8), or lower (ITX14) in vitro inhibition efficiency (see Figure 2). ITX5 could not be tested because of insufficient amounts available. Of the five compounds, only ITX3 significantly suppressed the TrioN-induced phenotype (Figure 4B): stress fibers were present in 15% of TrioN-expressing cells (versus <1% in untreated cells), the extent of dorsal ruffling was lower than that in untreated cells (31% versus 72%), and these protrusions appeared displaced toward peripheral lamellipodia in 54% of cells (versus 28% in untreated cells). Consistent with their biochemical effects on TrioN-stimulated GTP exchange, ITX3 inhibited the formation of TrioN-dependent cell structures in a dose-dependent manner, whereas the ITX14, which was inactive in vitro, had no significant effect (Figure 4C).

Specificity of ITX3 Inhibition

To address the specificity of ITX3 inhibition, we first examined whether the inhibition of TrioN-induced phenotypic responses by ITX3 in REF52 cells correlated with a reduced level of active Rac. TrioN activates Rac either directly or indirectly through RhoG activation (Katoh and Negishi, 2003; Gauthier-Rouviere et al., 1998). We observed higher levels of active Rac in HEK293T cells expressing GFP-TrioN than in cells expressing GFP alone (Figure 5A, compare lanes 1 and 2). These elevated levels of Rac activity were reduced by 80% in the presence of ITX3 (lane 3), whereas the inactive ITX14 analog had no effect (lane 4). We next compared the specificity of ITX3 toward other

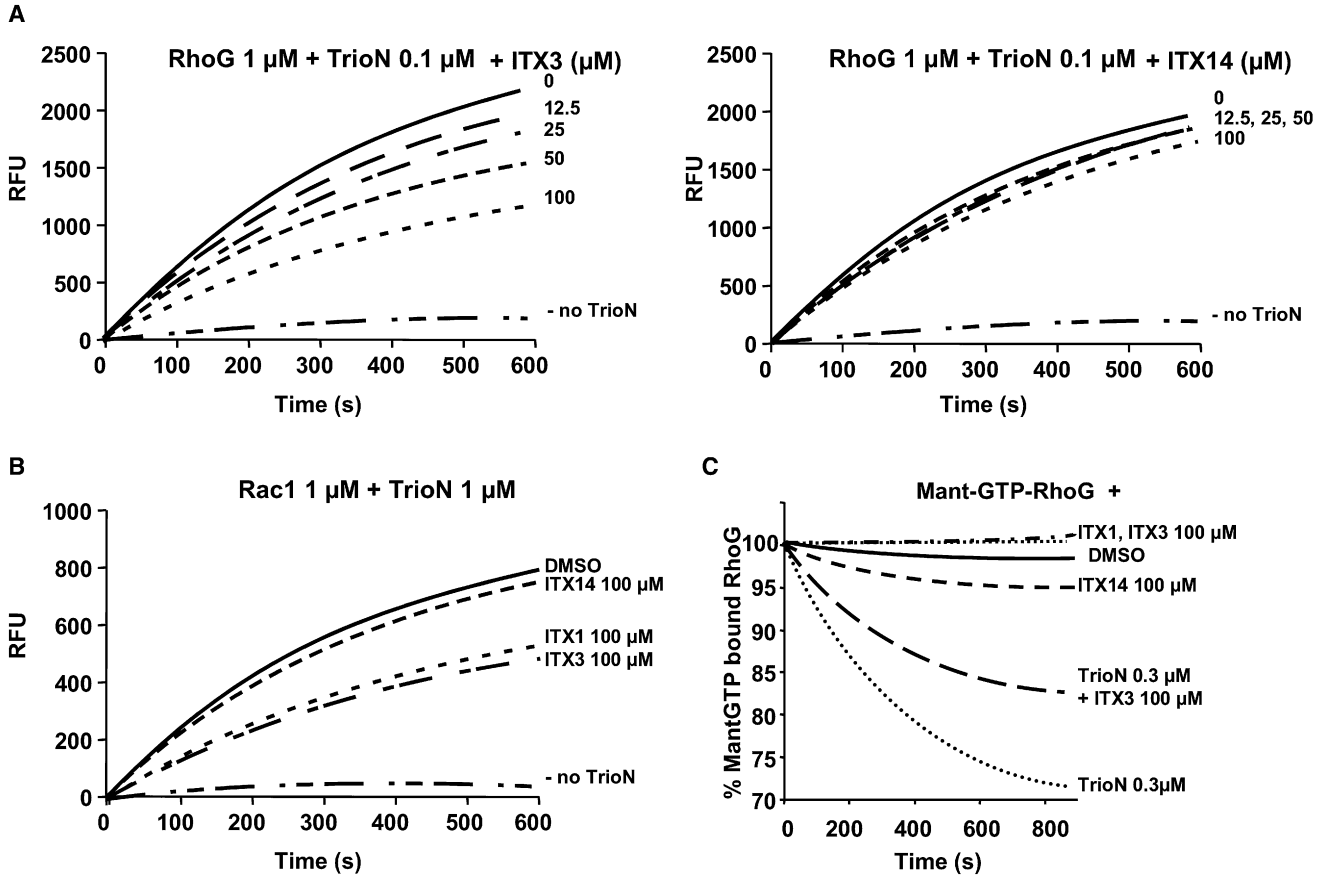

Figure 3. In Vitro Analysis of ITX3 Inhibition

(A) In vitro exchange kinetics were performed by measuring the increase in fluorescence emitted with time upon incorporation of Mant-GTP into 1 µM RhoG alone (no TrioN) or with 0.1 µM TrioN in the presence of 0, 12.5, 25, 50, or 100 µM ITX3 (left panel) or ITX14 (right panel).

(B) In vitro exchange kinetics were measured from incorporation of Mant-GTP into 1 µM Rac1 without (no TrioN) or with 1 µM TrioN in the presence of DMSO alone or containing 100 µM ITX1, ITX14, or ITX3.

(C) In vitro exchange kinetics were performed by measuring the decrease in fluorescence emitted with time from 1 µM preloaded Mant-GTP-RhoG either in the presence of ITX1, ITX3, ITX14, or DMSO or in the presence of 0.3 µM TrioN alone or in combination with 100 µM ITX3.

RhoGEFs and Rho GTPases. Among the twenty Rho family members in mammals (Boureux et al., 2007), only GEFs for RhoA-C, RhoG, Rac, and Cdc42 have been identified. We thus examined the effect of ITX3 on the exchange activity of GEF337, a RhoA-specific GEF (De Toledo et al., 2000); Tiam1, a Rac-specific GEF (Michiels et al., 1995); and Vav2, a GEF active on Rac1, RhoA, and Cdc42 (Abe et al., 2000) (Figure 5B). ITX3 treatment had no effect either on RhoA activation by GEF337 or on Rac1 activation by Tiam1 and only showed a modest effect on Rac1 activation by Vav2. ITX3 inhibition thus seems to be specific for TrioN rather than other RhoGEFs.

ITX3 Inhibition of Trio-Dependent Neurite Outgrowth

Trio is an evolutionarily conserved multifunctional protein, the depletion of which in the mouse induces secondary defects in myofibers and aberrant organization of brain tissues (O'Brien et al., 2000). Ex vivo, Trio plays essential roles in neurite outgrowth and axon guidance in insect and mammalian neuronal cells (Newsome et al., 2000; Estrach et al., 2002; Bateman et al., 2000; Briançon-Marjollet et al., 2008). NGF treatment of rat PC12 phaeochromocytoma cells stimulates neurite outgrowth through activation of the Trio/RhoG-dependent pathway, eventually leading to Rac1 and Cdc42 activation (Estrach et al., 2002). We therefore used this cell system to determine the capacity of ITX3 to inhibit GTP exchange stimulated by the TrioN domain of endogenous Trio. In a typical experiment, about 40% of NGF-treated PC12 cells produced cellular extensions (panel DMSO, Figure 6). ITX3 treatment reduced the number of cells with neurite outgrowth by 50%, whereas the inactive ITX14 analog did not affect the NGF response (histogram, Figure 6). These data therefore confirm the action of ITX3 as an inhibitor of the TrioN domain of endogenous Trio.

TrioN Requirement for Myoblast Fusion

During skeletal myogenesis, myoblasts arrest in G1 in response to external cues, express myogenic factors such as myogenin or troponinT, and then fuse to form myotubes. Trio participates in the fusion of C2C12 myoblasts; indeed, C2C12 cells expressing Trio shRNA are unable to form myotubes (Charrasse et al., 2007). However, Trio knockdown may have broader effects than simple

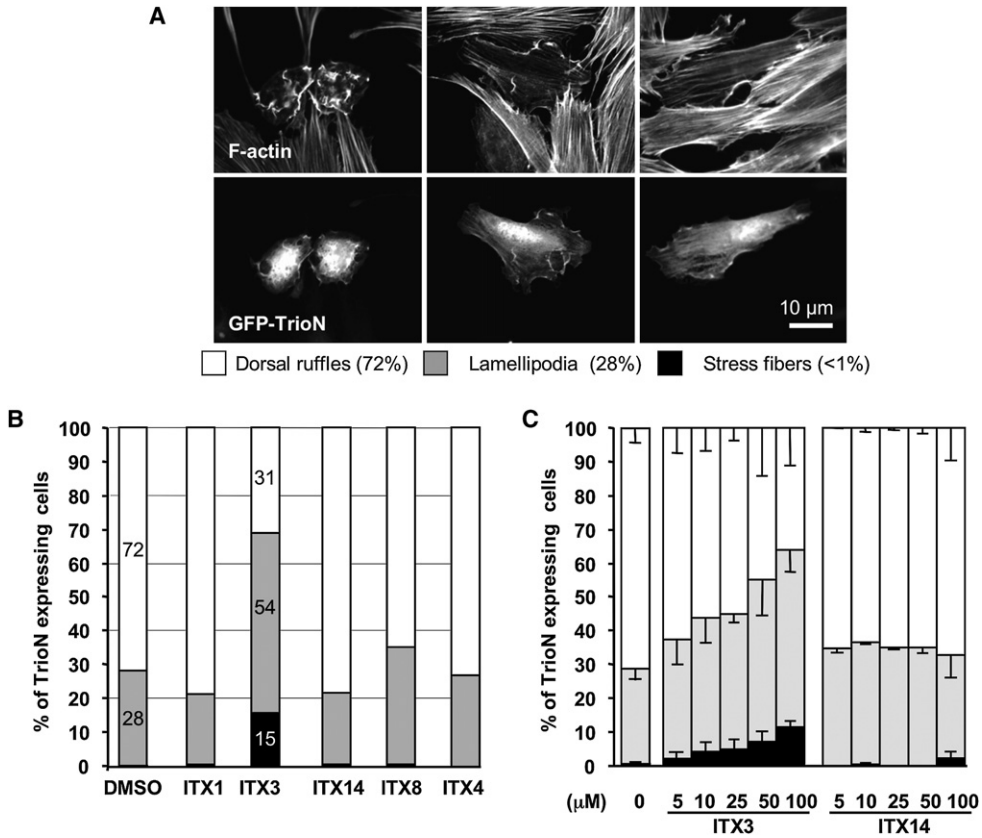

Figure 4. Inhibition of TrioN-Mediated REF52 Cell Structures by ITX1 Analogs

(A) TrioN-expressing REF52 cells were examined for F-actin distribution (upper row) and GFP fluorescence (lower row). F-actin structures and frequencies are indicated.

(B) TrioN-expressing REF52 cells were treated for 24 hr with 1% DMSO either alone (DMSO) or containing 100 μM of the indicated ITX1 analogs. Cells were stained for F-actin and scored for F-actin structures. White bars, dorsal ruffles; gray bars, peripheral lamellipodia; and black bars, stress fibers.

(C) Dose response analysis of ITX3 and ITX14. TrioN-expressing REF52 cells were treated for 24 hr with 1% DMSO containing 5, 10, 25, 50, or 100 μM ITX3 (active) or ITX14 (inactive). Cells were scored as in B. Histogram shows means and standard deviations of three independent experiments.

inhibition of TrioN activity. We therefore examined the effects of ITX3 on the fusion process. Cellular differentiation, determined by the induction of myogenin and troponin-T, did not differ between ITX3-treated C2C12 cells and control (DM) or DMSO-treated (DMSO) cells (Figure 7A). Given that these early events require RhoA activation (Wei et al., 1998), this suggests that ITX3 does not significantly affect RhoA-dependent pathways. However, myotube formation was inhibited in a dose-dependent manner in ITX3-treated cells, as detected by fusion index analysis (Figure 7B). We observed a similar extent of inhibition using 100 μM ITX3, 100 μM NSC23766 (a Rac1 inhibitor), or Trio knockdown. We then examined cell morphology; C2C12 cells incubated with 100 μM ITX3 failed to align and fuse (Figure 7C, panel b). C2C12 cells knocked down for Trio or treated with ITX3 showed a similar overall flat morphology with loose cell contacts (compare panels b and d), whereas cells treated with NSC23766 appeared refringent and more packed (panel c). NSC23766 was designed to target a surface groove in Rac1 critical for interaction with GEFs; thus, NSC23766 may target additional RhoGEFs that are involved in C2C12 cell morphology and are insensitive to ITX3. Taken together, our findings demonstrate that TrioN activity controls myotube formation during skeletal myogenesis and suggest that ITX3 acts specifically on this GEF domain.

DISCUSSION

Given the large number of Rho components encoded in the human genome (20 GTPases, over 80 GEFs, and over 50 GAPs), inhibitors targeting specific pathways would provide useful tools for basic functional studies and for subsequent development of therapeutic drugs. In this study, we identified ITX3, a compound active in cells that inhibits TrioN-induced in vitro nucleotide exchange on RhoG and Rac1, formation of TrioN-induced cellular structures in REF52 fibroblasts, and NGF-induced neurite outgrowth in PC12 cells, a process mediated by TrioN, RhoG, and Rac1. ITX3 also inhibits fusion of C2C12 myoblasts during the formation of myotubes, which evidenced that TrioN activity is required for this process. To the best of our knowledge, ITX3 has not been previously shown to be active in any other bioassay and therefore represents a new chemical compound able to inhibit Rho signaling.

The ITX3 compound showed clear specificity for the inhibition of TrioN, rather than other RhoGEFs: it had no effect on RhoA

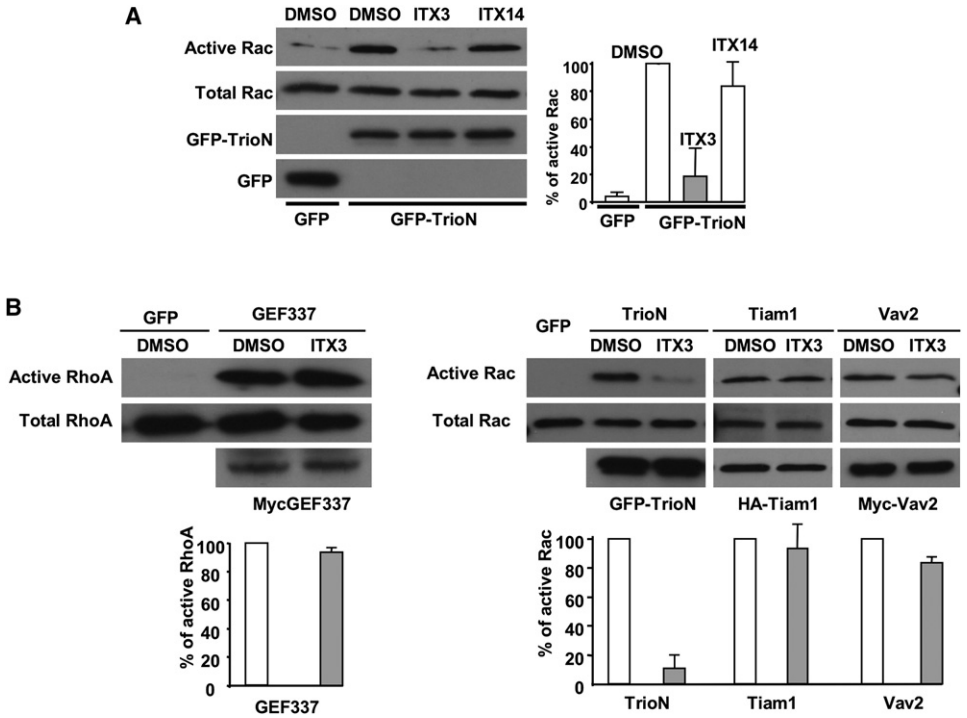

Figure 5. Specificity of ITX3 Inhibition

(A) TrioN-expressing HEK293T cells were treated for 1 hr with 1% DMSO alone or containing 50 μM ITX3 or ITX14 and then were lysed. GTP-bound Rac was affinity-purified on PAK-CRIB sepharose beads, analyzed by Western blotting, and normalized to total Rac. Expression of empty pEGFP-C1 vector was used as a negative control.

(B) Left panel: GEF337-expressing HEK293T cells were processed as in A except that GTP-bound RhoA was affinity-purified on Rhotekin-RBD sepharose beads. Right panel: TrioN-, Tiam1-, or Vav2-expressing HEK293 cells were processed as in A. For panels A and B, images show representative Western blotting experiments, and histograms represent means and standard deviations of densitometric analyses of three independent experiments.

activation by GEF337 or on Rac activation by Vav2 or Tiam1. We also demonstrated this specificity indirectly: REF52 cells expressing TrioN and treated with ITX3 were still able to form F-actin stress fibers, a process that requires Rho A-C activation. This finding strongly suggests that GEFs acting on these GTPases are not affected by ITX3. Similarly, ITX3 treatment impaired the terminal fusion process in C2C12 differentiation but did not affect myogenic commitment, an early step that requires RhoA activation and the activity of GEFT, a GEF for RhoA/Rac1/Cdc42 (Bryan et al., 2005; Guo et al., 2003; Wei et al., 1998).

We used a screening procedure designed to select inhibitors that specifically block the activation of RhoG by TrioN. As such, inhibitors were expected to target either RhoGEF, GTPase, or RhoGEF/GTPase complexes. The inhibitor EHT 1864 has a direct effect on the bound nucleotide (Shutes et al., 2007); however, this does not appear to be the case for ITX compounds, since they had no effect on stability of the GTPase-nucleotide complex in vitro. We also observed that ITX3 inhibited TrioN activity in vitro to the same extent in Mant-GTP loading assays on GDP-bound RhoG and in release assays with Mant-GTP preloaded RhoG (Figures 3A and 3C). GEF-mediated nucleotide exchange is a multistep process, which is initiated by formation of a low-affinity complex between the GEF and the nucleotide-bound GTPase (Renault et al., 2003). This initiation step promotes nucleotide release and leads to formation of a high-affinity complex between the GEF and the nucleotide-free GTPase. Our observation that ITX3 prevents Mant-GTP release from preloaded RhoG thus demonstrates that ITX3 disrupts initiation steps, impairing either formation of the low-affinity complex or release of the nucleotide from the formed complex. This is suggestive of a mechanism potentially similar to NSC23766, which binds competitively to the GEF/GTPase interface, rather than to EHT 1864, which induces destabilization of the GTPase/nucleotide complex. Our data also strongly suggest that ITX3 and NSC23766 target distinct proteins. Indeed, NSC23766 inhibits TrioN- and Tiam1-mediated Rac1 activation (Gao et al., 2004), whereas ITX3 has no effect on Tiam1 (Figure 5B). Effects of NSC23766 on both Tiam1 and TrioN are consistent with the model in which this compound targets a Rac1 groove that faces the DH domain of either Tiam1 or TrioN and is thus critical for formation of the exchange complexes (Worthylake et al., 2000; Chhatriwala et al., 2007). ITX3 specificity for TrioN and not for Tiam1 therefore implies that this compound does not target the same Rac1 interface, as it would otherwise inhibit the action of Tiam1 on Rac1. Moreover, ITX3 inhibits TrioN stimulation of RhoG and Rac1 to the same extent. These two GTPases are highly conserved (89% similar) in the region at the interface of TrioN (only 63% similarity outside this region—that is, in their 117 C-terminal amino-acid sequence). It therefore seems unlikely that ITX3 targets a region poorly conserved between RhoG or Rac, which is located outside of the TrioN interface and yet is

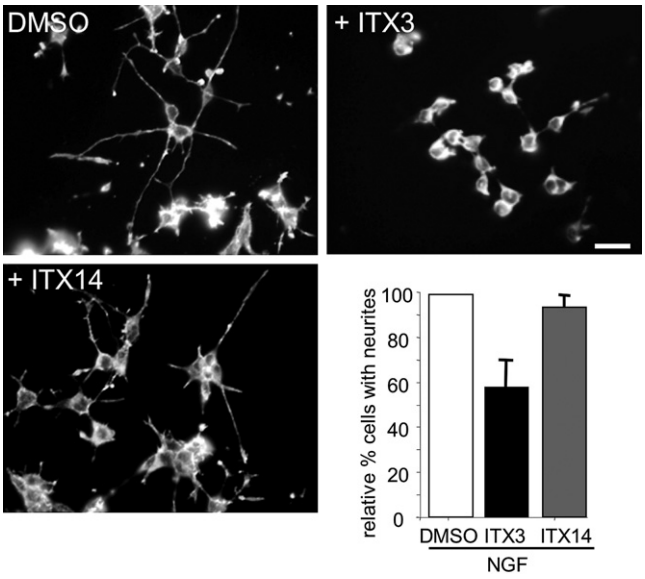

Figure 6. ITX3 Inhibition of NGF-Induced Neurite Outgrowth

GFP-expressing PC12 cells were stimulated with 50 ng·ml^{-1} NGF for 36 hr in the presence of 1% DMSO alone or in combination with 100 μM ITX3 or ITX14. Cells were counted for the presence or absence of neurites of at least twice the cell body length. Scale bar: 10 μM. Graph shows the ratio of cells with neurites relative to NGF-treated control cells, as determined from four experiments. Error bars indicate standard deviation.

essential for the stimulation by TrioN. The most parsimonious hypothesis is that ITX3 targets TrioN, consistent with the specificity for TrioN versus Tiam1 and the dual inhibition of TrioN exchange on RhoG and Rac1.

Rac1 is ubiquitously expressed and is required at early stages of development (Sugihara et al., 1998). In adults, the roles of Rac proteins have mostly been investigated in the immune system, with studies of tissue-restricted depletion of both Rac1 and Rac2 showing many resulting hematopoietic defects (Kalfa et al., 2006; Dumont et al., 2008; Ramaswamy et al., 2007). Inhibition of the overall activity of Rac proteins by drugs such as EHT 1864 can thus have major effects when used as a systemic therapeutic treatment. It should therefore be used only for specific diseases, such as breast or colorectal cancers, that produce Rac1b, an isoform that is constitutively activated in a GEF-independent manner (Stallings-Mann and Radisky, 2007; Matos and Jordan, 2008). Inhibition of Rac activation by drugs such as NSC23766 may have more specific effects, with only a subset of RhoGEFs affected. NSC23766 was indeed shown recently to attenuate the severity of disease in a murine model of p120 Bcr-Abl-induced chronic myelogenous leukemia, which involves Rac activation (Thomas et al., 2007). Suppression of GEF activity, the likely mode of action of ITX3, may have even more specific effects, particularly for GTPases such as RhoG and Rac1, which are activated by multiple GEFs. Trio preferentially activates RhoG, which, in turn, activates Rac through the ELMO/DOCK180 complex (Katoh and Negishi, 2003). The direct targeting of the TrioN domain would therefore block the downstream RhoG/Rac activation pathway without affecting activation of either GTPase by other GEFs. Such selectivity may be of interest in particular cancers (e.g., glioblastoma and breast cancers) in which Trio plays a role in their invasive behavior (Lane et al., 2008; Salhia et al., 2008).

SIGNIFICANCE

Rho signaling pathways control many aspects of cell dynamics and are frequently found activated in a variety of diseases such as hypertension and cancer. Signaling is initiated by RhoGEFs, which activate one or more Rho GTPases. These GTPases in turn activate multiple effectors to produce their cellular effects. RhoGEFs are thus attractive targets for modulating the intensity of response to specific upstream signals. However, the only Rho signaling inhibitors currently available for in vivo use target GTPases or their effectors. We used an engineered yeast strain to select for inhibitors that block RhoGEF-dependent Rho GTPase activation. We identified ITX3, a compound that is active in cells and inhibits the activity of TrioN, one of the two Dbl-like domains of Trio. ITX3 displays specificity, having no effect on RhoA activation by GEF337 or Rac1 activation by Tiam1 and Vav2. ITX3 inhibited NGF-induced neurite outgrowth, confirming the pivotal role of the TrioN domain of endogenous Trio in this process. Moreover, ITX3 treatment of differentiating C2C12 myoblasts demonstrated that TrioN activity of endogenous Trio is also needed for membrane fusion during myotube formation. These data demonstrate that the screening method we developed in yeast is suitable for identifying RhoGEF inhibitors and validate ITX3 as a lead compound for inhibiting specific endogenous RhoGEF domains in living cells. The availability of bioactive compounds targeting endogenous RhoGEF activity will open up new perspectives, allowing differential regulation of specific signaling pathways involved in disease progression.

EXPERIMENTAL PROCEDURES

Plasmids, Recombinant Proteins

Bacterial expression vectors and protein purification methods were described earlier (Blangy et al., 2006). Eukaryotic cell constructs were described elsewhere (Blangy et al., 2000; De Toledo et al., 2001; Charrasse et al., 2006; Michiels et al., 1995; Crespo et al., 1997). Tiam1 and Vav2 vectors were gifts from J. Collard and X. Bustelo, respectively.

Cell Lines, Transfection, and Immunofluorescence

Rat embryonic fibroblasts (REF-52) were cultured at 37°C in the presence of 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum (FCS) as described by Blangy et al. (2000). Cells were plated on 12 mm glass coverslips 16–24 h before being transfected using the JetPEI reagent as described by the supplier (Polyplus Transfection). Four hours after transfection, cells were incubated with fresh medium containing 1% DMSO (v/v) and chemical compounds at various concentrations. Twenty-four hours later, cells were fixed for 10 min in 3.7% (v/v) formalin in PBS. After a 2 min permeabilization in 0.1% Triton X-100 in PBS and a 30 min incubation at 20°C in 0.1% BSA in PBS, cells were stained for actin using ACMA (aminomethyl coumarin)–conjugated phalloidin (0.5 unit/ml; Sigma). Cells were washed in PBS and mounted in Mowviol (Aldrich) and observed using a DMR Leica microscope with a 63× planochromat lens. Images were recorded using a Coolsnap HQ2 CCD (charge-coupled-device) camera (Princeton Instruments) and transferred to Adobe Photoshop. Transfections were repeated at least three times and an average of 100 cells was examined.

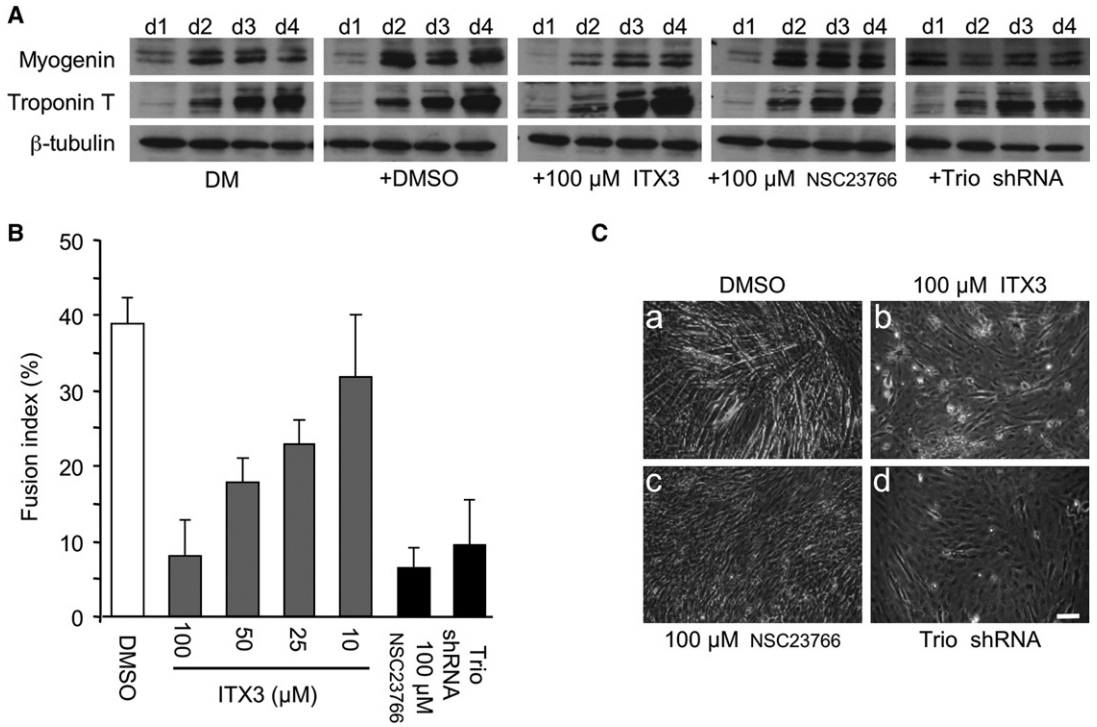

Figure 7. TrioN Implication in the Fusion of C2C12 into Myotubes

(A) Wild-type or Trio knocked-down C2C12 cells were induced to differentiate for one to four days (d1 to d4) in differentiation medium (DM), alone or in the presence of 1% DMSO, 100 μM ITX3 or 100 μM NSC23766, as indicated. Cells extracts were analyzed for Myogenin and Troponin T expression. β-tubulin was used as a loading control.

(B) C2C12 were induced to differentiate for four days in the presence of 1% DMSO alone or in combination with ITX3 at various concentrations or in the presence of 100 μM NSC23766. C2C12 cells knocked-down for Trio (Trio shRNA) were used as a control. Histogram represents the fusion index—that is, the ratio of nuclei in multinucleated myotubes to total nuclei, calculated from four independent experiments. In each experiment, at least 1000 nuclei were counted. Only cells with a minimum of three nuclei were considered as myotubes. Error bars indicate standard deviation.

(C) Phase-contrast images of C2C12 myoblasts representative of experiments described in B. Scale bar: 30 μM.

PC12 cells were seeded onto collagen type I (Sigma)–coated glass coverslips in 6-well plates at a density of 5 × 10^5 cells per well and were cultured for 18 hr in DMEM containing 5% FCS and 10% horse serum (HS). Cells were transfected for 8 hr with 1 μg of total DNA using JetPEI reagent. Transfection mix was then replaced with differentiation medium (DMEM with 50 ng·ml^{-1} NGF (Promega) and 0.5% HS) containing 1% DMSO (v/v) or 1% DMSO containing 100 μM chemical compounds for 36 hr prior to fixation.

C2C12 mouse myoblasts were grown in DMEM/Ham's F-12 (1:1) supplemented with 10% FCS. Differentiation was induced by replacing growth medium with differentiation medium consisting of DMEM/Ham's F-12 supplemented with 2% FCS. C2C12 cells stably expressing Trio shRNA (Charrasse et al., 2007) were cultured under the same conditions in medium supplemented with 200 μg·ml^{-1} Hygromycin B; 0.5% DMSO (v/v), 0.5% DMSO containing 10 to 100 μM chemical compounds, or the Rac1 inhibitor NSC23766 (Calbiochem, La Jolla, CA) were added 1 hr after addition of the differentiation medium. Medium with compounds was renewed every day for 5 days before fixation and Hoechst and F-actin staining. The fusion index was defined as the number of nuclei in multinucleated myotubes divided by the total number of nuclei. At least 1000 nuclei were counted using the MRI Cell Image Analyzer program (Baecker and Travo, 2006). All chemical compounds were purchased from ChemBridge (San Diego, CA).

Rac-GTP and RhoA-GTP Pulldown Experiments

HEK293T cells were transfected with the indicated plasmids for 24 hr, incubated for 1 hr in the presence of 1% DMSO containing or not the inhibitory compounds then lysed. For Rac1 activity assay, cells were lysed in 25 mM HEPES (pH 7.5), 1% Igepal, 5% glycerol, 100 mM NaCl, 10 mM $MgCl_2$, 5 mM NaF, 1 mM Na_3VO_4, 1mM Leupeptin/Aprotinin, and 1 mM AEBSF. Cleared lysate was incubated with 25 μg of GST-Pak CRIB bound to glutathione-coupled Sepharose beads for 45 min at 4°C. Beads were washed three times in 25 mM HEPES (pH 7.5), 0.5% Igepal, 40 mM NaCl, 30 mM $MgCl_2$, and 1 mM DTT before addition of Laemmli sample buffer. For RhoA activity assay, cells were lysed in 50 mM TrisHCl (pH 7.2), 1% Triton X-100, 500 mM NaCl, 10 mM $MgCl_2$, 1 mM Leupeptin/Aprotinin, and 1 mM AEBSF. Cleared lysate was incubated with 50 μg of GST fused RhoA-binding domain of Rhotekin (GST-RBD) bound to glutathione beads for 45 min at 4°C. Beads were then washed four times in 50 mM TrisHCl (pH 7.2) containing 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM Leupeptin/Aprotinin, and 1 mM AEBSF before addition of Laemmli sample buffer. Total cell lysates and proteins bound to beads were resolved on 15% SDS/PAGE and immunoblotted using anti-Rac1 (Transduction Laboratory) or anti-RhoA antibodies (Santa Cruz Biotechnology, Inc.). Quantification of activation was performed by densitometric analysis using the Aida/2D densitometry software (Raytest, Straubenhardt). The relative amount of active protein was determined as the amount of protein sedimented relative to the amount in whole cell lysates.

In Vitro Exchange Assays

Fluorescence-based guanine nucleotide-exchange assays were performed using Mant-GTP (Molecular Probes) in an FLX 800 microplate fluorescence reader (BioTek Instruments) at 25°C. Nucleotide exchange was measured by measuring either Mant-GTP loading or Mant-GTP release from a preloaded GTPase. Mant-GTP loading mixtures containing 0.1 μM or 1 μM GEF were incubated for 30 min at 25°C in reaction buffer (20 mM TrisHCl [pH 7.5], 50 mM NaCl, 2 mM $MgCl_2$, 1 μM Mant-GTP, and 50 μg/ml BSA) containing 5% DMSO and the inhibitor at the desired concentration. The exchange reaction was started by addition of 1 μM GTPase. The relative Mant fluorescence

(λ_{ex} = 360 nm and λ_{em} = 460 nm) was monitored for 15 min, and measurements were taken every 10 s. Alternately, GTPases were preloaded with Mant-GTP for 15 min at 25°C in reaction buffer supplemented with 2.5 mM EDTA then stabilized with 10 mM $MgCl_2$. The exchange reaction was started by addition of 0.3 μM GEF, 1 mM GTP and 5% DMSO and the inhibitor at the desired concentration. IC_{50} values were determined from triplicate data using Graphpad Prism 4 software. Sigmoidal fitting was done using built-in functions. Statistical analysis of IC_{50} values was made using the nonparametric Mann-Whitney test. The level of significance was set at $p < 0.01$.

Toxicity Assays

REF 52 cells were seeded in 24-well plates at a density of 4.10^4 cells/well in DMEM containing 10% fetal bovine serum and were cultured for 24 hr; 1% DMSO (v/v) or 1% DMSO containing 100 μM chemical compounds were added for 24 hr or 48 hr. Cells were then either trypsinized and stained with 0.2% Trypan Blue or directly stained with 1% Crystal Violet (Sigma-Aldrich). Percentage of cell viability was defined as the ratio of negative blue cells versus the total number of cells.

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