
**Mechanisms of Signal Transduction:
Specificity and Mechanism of Action of
EHT 1864, a Novel Small Molecule
Inhibitor of Rac Family Small GTPases**

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Specificity and Mechanism of Action of EHT 1864, a Novel Small Molecule Inhibitor of Rac Family Small GTPases*

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There is now considerable experimental evidence that aberrant activation of Rho family small GTPases promotes the uncontrolled proliferation, invasion, and metastatic properties of human cancer cells. Therefore, there is considerable interest in the development of small molecule inhibitors of Rho GTPase function. However, to date, most efforts have focused on inhibitors that indirectly block Rho GTPase function, by targeting either enzymes involved in post-translational processing or downstream protein kinase effectors. We recently determined that the EHT 1864 small molecule can inhibit Rac function *in vivo*. In this study, we evaluated the biological and biochemical specificities and biochemical mechanism of action of EHT 1864. We determined that EHT 1864 specifically inhibited Rac1-dependent platelet-derived growth factor-induced lamellipodia formation. Furthermore, our biochemical analyses with recombinant Rac proteins found that EHT 1864 possesses high affinity binding to Rac1, as well as the related Rac1b, Rac2, and Rac3 isoforms, and this association promoted the loss of bound nucleotide, inhibiting both guanine nucleotide association and Tiam1 Rac guanine nucleotide exchange factor-stimulated exchange factor activity *in vitro*. EHT 1864 therefore places Rac in an inert and inactive state, preventing its engagement with downstream effectors. Finally, we evaluated the ability of EHT 1864 to block Rac-dependent growth transformation, and we determined that EHT 1864 potently blocked transformation caused by constitutively activated Rac1, as well as Rac-dependent transformation caused by Tiam1 or Ras. Taken together, our results suggest that EHT 1864 selectively inhibits Rac downstream signaling and transformation by a novel mechanism involving guanine nucleotide displacement.

Rho family proteins (20 human members) comprise a major branch of the Ras superfamily of small GTPases (1, 2). Like Ras, Rho GTPases function as GDP/GTP-regulated binary on-off switches. In resting, quiescent cells, Rho GTPases exist pre-

dominately in their inactive, GDP-bound state. Upon growth factor stimulation, Rho-specific guanine nucleotide exchange factors (RhoGEFs)⁴ are activated, and they stimulate the intrinsic guanine nucleotide exchange activity to promote formation of the active GTP-bound protein. Rho-GTP binds preferentially to downstream effector proteins. Rho-specific GTPase-activating proteins then stimulate the intrinsic GTP hydrolysis activity of Rho GTPases, returning the protein to its inactive state and terminating effector interaction.

RhoA, Rac1, and Cdc42 are the best studied and understood members of the Rho GTPase family (1, 2). Rho GTPases are regulators of a diverse variety of cellular processes that include regulation of cell proliferation, actin cytoskeleton reorganization, and gene expression (3). Like Ras and other members of the Ras superfamily (4, 5), the aberrant activity of Rho GTPases has been implicated in cancer and other human diseases (6–10). In particular, the Rac subfamily has also been linked to cellular transformation. Rac is essential for transformation caused by Ras and other oncogenes (11–16). The Rac1b splice variant of Rac1 is constitutively active and transforming and found overexpressed in breast and colon cancers (17–19). Mutation and overexpression of Rac3 was seen in human brain tumors, and RNA interference demonstrated a role for Rac1 and Rac3 in human glioblastoma invasion (20, 21). Finally, the Vav RacGEF is found hyperactivated in pancreatic cancers, and suppression of Vav1 expression impairs pancreatic tumor cell growth (22).

Due to their central nature in many transforming events, Rho GTPases are therefore attractive and validated targets for anti-cancer therapies. One approach has involved small molecule inhibitors of the prenyltransferases (23, 24). Farnesyltransferase and geranylgeranyltransferase I catalyze the covalent modification of Rho GTPases by isoprenoid lipids, which then promote the membrane association required for Rho GTPase interaction with effectors and biological activity. However, because these prenyltransferases are involved in the modification of a multitude of other proteins important in cell prolifer-

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⁴ The abbreviations used are: RhoGEF, Rho-specific guanine nucleotide exchange factor; RBD, Rho GTPase binding domain; GST, glutathione S-transferase; PDGF, platelet-derived growth factor; LPA, lysophosphatidic acid; PBS, phosphate-buffered saline; siRNA, small interference RNA; mant, N-methylanthraniloyl; GTPγS, guanosine 5'-3-O-(thio)triphosphate; HA, hemagglutinin; FRET, fluorescence resonance energy transfer; DH/PH, tandem Dbp and pleckstrin homology; GDI, guanine nucleotide dissociation inhibitor; WT, wild type; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PAK, p21-activated serine/threonine kinase; GMPPNP, 5'-guanylyl imidodiphosphate.

ation and survival (25), inhibitors of prenyltransferases are likely to possess significant off-target activities and may lack sufficient specificity to selectively block Rho GTPase function. Another approach has involved inhibitors of the protein kinase effectors of Rho GTPases, for example, the Y-27632 small molecule inhibitor of the Rho-associated serine/threonine protein kinase (ROCK), a key effector of RhoA. However, because Rho GTPases utilize a multitude of other downstream effectors, ROCK inhibitors will impair only a subset of RhoA functions (26).

Inhibitors that directly interact with Rho GTPases would be preferable and exhibit greater specificity. However, to date, there has been limited success in the identification of inhibitors that specifically interact with small GTPases. One example is the NSC23766 small molecule, which was identified as a cell-permeable compound that can bind directly to Rac1 and prevent its activation by Rac-specific RhoGEFs (27). However, this inhibitor did not block Rac activation by some GEFs (Vav1), and Rac activation by other mechanisms will also be insensitive to NSC23766. Recently, we identified EHT 1864 as a small molecule that inhibited Rac-dependent amyloid precursor protein processing by γ -secretase and decreased A β production *in vitro* and *in vivo* (28). Our preliminary characterization found that EHT 1864 inhibited a variety of downstream signaling activities activated by ectopic expression of constitutively-activated Rac1(G12V) and decreased Rac1 association with the isolated Rho GTPase binding domain (RBD) of the p21-activated serine/threonine kinase effector PAK1 *in vivo*. However, the Rho GTPase specificity and mechanism of action of EHT 1864 was not determined. We report here on the further investigation of EHT 1864. We show that EHT 1864 selectively blocks platelet growth factor-stimulated activation of Rac1-dependent lamellipodia formation and not ligand activation of RhoA- or Cdc42-mediated actin reorganization. Our biochemical analyses determined that EHT 1864 also interacts with other Rac isoforms, associates with Rac1 tightly, and causes the release of bound nucleotide, thus preventing effector interaction, both *in vitro* and *in vivo*. Finally, we determined that EHT 1864 potently inhibited other Rac-dependent transformation processes, Tiam1- and Ras-mediated growth transformation. Our work suggests that EHT 1864 may form the basis for a novel class of specific GTPase inhibitors that function by impairing downstream effector engagement and activation.

EXPERIMENTAL PROCEDURES

Rho-GTP Formation Pulldown Analyses—For analysis of the effect of EHT 1864 on the formation of the GTP-bound form of RhoA, Rac1, and Cdc42 *in vivo*, pulldown analyses were done as we have described previously (28). Briefly, U87-MG cells were grown to 80% confluence. The cells were then treated with EHT 1864 or with vehicle, lysed in a buffer containing 0.5% Triton X-100, and clarified, and the protein concentrations were normalized. The GTP-bound Rho GTPase levels in the lysates were measured using a glutathione *S*-transferase (GST) fusion protein containing the isolated RBD derived from the Rac and Cdc42 effector, the PAK1 serine/threonine kinase (GST-PAK-RBD, Cytoskeleton Inc., Denver, CO) for Rac1-GTP or Cdc42-GTP detection, or the RhoA effector, Rhotekin (GST-Rhotekin-RBD, provided by A. Hall, University College, London, UK). Antibodies specific for

Rac1, Cdc42, and RhoA (Tebu, Le Perray en Yvelines, France) were then used for immunoblot analyses for GTP-bound Rho GTPase and for total cell lysate protein levels.

Actin Reorganization Analyses—NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum (designated complete growth medium). For analysis of the effect of EHT 1864 treatment on extracellular signal-induced changes in actin organization, we treated NIH 3T3 cells with platelet-derived growth factor (PDGF), lyso-phosphatidic acid (LPA), or bradykinin (Sigma-Aldrich), to activate endogenous Rac1, RhoA, or Cdc42, respectively. NIH 3T3 cells were plated on glass coverslips at a density of 2×10^4 cells per well in 12-well plates. 24 h after cell seeding, cells were maintained in Dulbecco's modified Eagle's medium supplemented with 0.5% calf serum for 16 h in the presence or absence of 5 μ M EHT 1864 or EHT 8560 for the last 4 h. Cells were stimulated for 15 min with 5 ng/ml PDGF, 40 ng/ml LPA, or 100 ng/ml bradykinin, then fixed for confocal immunofluorescence analysis. Cells on coverslips treated as described above were rinsed in phosphate-buffered saline (PBS) before being fixed for 15 min at room temperature with 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100 in PBS for 5 min. Glass coverslips were rinsed three times in PBS and incubated with Alexa Fluor 568 conjugated-phalloidin (Invitrogen) for 30 min. After two final washes in PBS and one in water, coverslips were mounted on slides using Fluor-Save Reagent (Calbiochem). The mounting medium was allowed to dry overnight away from light, before analysis.

To verify that PDGF-induced lamellipodia formation was dependent on Rac1 function, we transiently transfected the cultures twice at a 24-h interval, with a final concentration of 50 nM short interfering RNA (siRNA) against Rac1 or with an irrelevant siRNA (siCONTROL Non-Targeting siRNA from Dharmacon), using the calcium phosphate method. 12 h following the second transfection, cells were serum-starved and then PDGF-stimulated as described above. The Rac1 siRNA sequence has been previously described (29). Western blot analyses with mouse anti-Rac1 monoclonal antibody (clone 23A8, Upstate) were done to determine the degree of Rac1 silencing and with mouse anti- β -actin monoclonal antibody (clone AC-15, Sigma-Aldrich) to verify equivalent loading of total protein.

Recombinant Protein Expression and Purification—The pGEX-Rac1, pGEX-Rac1(15A), pGEX-Rac1b, pPRO-EXHTb-Rac2, pGEX-Rac3, pGEX-Cdc42, pGEX-Cdc42(15A), and pPRO-EXHTb-Tiam1 DH/PH bacterial expression plasmids have all been described previously (19, 30–34). Rac proteins were prepared similarly as described elsewhere (30, 35, 36). Briefly, BL21(DE3) cells expressing Rac1, Rac1b, Rac2, and Cdc42, respectively, were incubated at 37 °C, before induction with 1 mM isopropyl 1-thio- β -D-galactopyranoside for 4 h at 37 °C (Rac3 required overnight induction at room temperature). GST proteins were either purified using a batch method (30-min incubation of bacterial cell lysate with 150 μ l of glutathione-Sepharose, followed by three washes in lysis buffer (20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM MgCl₂, supplemented with 10 μ M GDP only for GST-Rac1(15A) and GST-Cdc42(15A) purification), or by fast-protein liquid chromatography using a 1-ml GSTrap FF column (Amersham

Biosciences), and subsequent concentration using centrifugal concentrators (Vivascience). His₆-Rac2, Rac1-Δinsert-His₆ (37), and His₆-Tiam1 DH/PH were purified by fast-protein liquid chromatography using a 5-ml HiTrap Ni column (Amersham Biosciences), before dialysis into 20 mM Tris·HCl (pH 7.5), 50 mM NaCl, 1 mM MgCl₂, and subsequent concentration of protein.

Recombinant Protein Nucleotide Loading—To load GST-Sepharose-bound small GTPases, the bead-bound GTPases were incubated in exchange buffer (20 mM Tris·HCl, 50 mM NaCl, 500 μM fluorescent *N*-methylantraniloyl (mant) derivatives of guanine nucleotides (mant-GDP and mant-GMPPNP, Roche Applied Science), 20 mM (NH₄)₂SO₄), for 1 min at 37 °C, before three washes in cold equilibration buffer (20 mM Tris·HCl, 50 mM NaCl, 1 mM MgCl₂). Nucleotide-loaded protein was then eluted from the beads using elution buffer (20 mM Tris·HCl, 50 mM NaCl, 1 mM MgCl₂, 0.1 mM glutathione) for 10 min on ice. To load His₆-tagged proteins, the relevant protein was incubated in exchange buffer for 1 min at 37 °C, before being separated from excess nucleotide in a Zeba Desalting Spin Column (Pierce Biotechnology), prepared as per manufacturer's protocol.

Fluorescent Intensity Measurements—Off-rate measurements were performed in a SPEX Fluorolog-3 Research fluorometer, in a 1-ml quartz cuvette (with constant stirring), with a total of 300 μl of 20 mM Tris·HCl (pH 7.5), 50 mM NaCl, 1 mM MgCl₂, and 2 μM small GTPase·mant-GDP at 25 °C. Exchange was initiated by addition of inhibitor to a final concentration of 50 μM or EDTA to a final concentration of 10 mM. Changes in fluorescence was monitored at λ_{ex} = 290 nm and λ_{em} = 440 nm, and data were recorded by the supplied software. On-rate measurements were performed in a Gemini Spectromax 96-well plate reader, in a 96-well plate (BD Falcon microtest plate) with a total of 100 μl of 20 mM Tris·HCl (pH 7.5), 50 mM NaCl, 1 mM MgCl₂, 2 μM mant-GDP, 2 μM small GTPase at 25 °C, and 50 μM of inhibitor. Exchange was initiated by addition of EDTA to a final concentration of 8 mM. Changes in fluorescence were monitored 4 min after inhibitor addition, at λ_{ex} = 290 nm and λ_{em} = 440 nm. Data were recorded by the supplied SoftMax Pro software. Measurements of excitation and emission spectra were performed in the SPEX Fluorolog-3 Research fluorometer, in 300 μl of 20 mM Tris·HCl (pH 7.5), 50 mM NaCl, 1 mM MgCl₂, and 1 μM inhibitor at 25 °C, in the presence or absence of 10 μM Rac.

Fluorescent Anisotropy Measurements—Fluorescent anisotropy measurements were performed in the SPEX Fluorolog-3 Research fluorometer, set up in the polarized T format, in a 1-ml quartz cuvette (with constant stirring), with a total of 300 μl of 20 mM Tris·HCl (pH 7.5), 50 mM NaCl, 1 mM MgCl₂ or PAK-RBD:GTPase association analyses, GST-PAK-RBD was titrated into 2 μM small GTPase·mant-GMPPNP in the presence or absence of 50 μM inhibitor (λ_{ex} = 360 nm, λ_{em} = 440 nm). For inhibitor:GTPase binding analyses, aliquots of small GTPase solution (containing 1 μM inhibitor) were titrated into a solution of 1 μM inhibitor in the cuvette. Changes in fluorescence anisotropy were monitored at λ_{ex} = 360 nm, λ_{em} = 440 nm, 30 s after each addition. All data analysis and curve fitting

were performed using Microsoft Excel and QuantumSoft's ProFit for Mac OS X.

Affinity Precipitation of the RacGEF Tiam1—50% confluent 10-cm dish of 293T cells were transfected by using Lipofectamine™ 2000 (Invitrogen) with 10 μg of pcDNA 3.1 expression plasmid alone (vector) or encoding Myc epitope-tagged Tiam1(C1199), an N-terminal truncated and constitutively activated mutant of human Tiam1 (a gift from G. Bollag, Plexxikon Inc.), which has been characterized previously (38). 48 h after transfection, the cells were lysed with 50 mM Tris, pH 7.5, 10 mM MgCl₂, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 0.25% sodium deoxycholate, and a protease inhibitor mixture tablet (Invitrogen). The lysates were cleared by centrifugation at 12,000 rpm for 10 min at 4 °C, and 400 μg of total protein extracts was used per condition. Five micrograms of either bacterially expressed GST, GST-Rac1(15A), or GST-Cdc42(15A), immobilized on glutathione-Sepharose™ 4B beads (Amersham Biosciences) and pre-treated with different doses of EHT 1864 for 30 min at 4 °C, was added to each condition. EHT 1864 was still present during the incubation with the lysates. The samples were rotated at 4 °C for 1 h, and then the beads were washed once with lysis buffer and twice with cold wash buffer (50 mM Tris, pH 7.5, 10 mM MgCl₂, 150 mM NaCl). Affinity-precipitated proteins were eluted in protein sample buffer and analyzed by SDS-PAGE and Western blotting with a monoclonal anti-Myc antibody 1/1000 (clone 9E10, Sigma-Aldrich).

In Vitro Binding of RhoGDIs to Rac1—A 50% confluent 10-cm dish of 293T cells was transfected by using Lipofectamine™ 2000 (Invitrogen) with 5 μg of pcDNA 3.1 expression plasmid alone (vector) or encoding RhoGDI1 (GDIα/RhoGDI) or RhoGDI2 (GDIβ/Ly/D4GDI) proteins (Guthrie cDNA Resource Center). 48 h after transfection, the cells were lysed with 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM dithiothreitol, 5 mM MgCl₂, 0.5% Triton X-100, and a protease inhibitor mixture tablet (Invitrogen). The lysates were cleared by centrifugation at 12,000 rpm for 10 min at 4 °C, and 350 μg of total protein extracts was used per condition. 10 μg of either bacterially expressed GST or GST-Rac1(WT), immobilized on glutathione-Sepharose™ 4B beads (Amersham Biosciences), was then used per condition. GST-Rac1 was first loaded with nucleotide for 10 min at 37 °C in a loading buffer containing 20 mM Tris, pH 7.5, 5 mM EDTA, 2 mM dithiothreitol, 5 mg/ml bovine serum albumin, and 2 mM GTPγS or GDP (150 μl of loading buffer per 10 μg of recombinant protein). The loading reaction was stopped by addition of MgCl₂ to 15 mM on ice. GDP- or GTPγS-loaded GST-Rac1 was treated first with different doses of EHT 1864 for 30 min at 4 °C and then added to the lysates, and the samples were rotated at 4 °C for 1 h in the presence or absence of EHT 1864. The beads were washed three times with lysis buffer, and the affinity-precipitated proteins were eluted in protein sample buffer and analyzed by SDS-PAGE and Western blotting with a rabbit polyclonal anti-RhoGDI1 antibody 1/5000 (A-20, Santa Cruz Biotechnology, Santa Cruz, CA) or a goat polyclonal anti-RhoGDI2 antibody 1/5000 (C-20, Santa Cruz Biotechnology), respectively.

Transformation Analyses—For primary focus formation assays, NIH 3T3 fibroblasts were plated at a density of 10⁵ cells/well of a 6-well plate. One day after seeding, cells were tran-

siently transfected with 50 ng of the pCGN-hygro expression plasmid alone (vector) or encoding H-Ras(61L). Cells were allowed to reach confluency and were maintained on complete growth medium supplemented with 5 μ M EHT 1864 or vehicle until focus formation occurred. The appearance of H-Ras(61L)-induced foci of transformed cells was quantitated after 14 days.

For establishment of NIH 3T3 fibroblasts stably transfected and expressing H-Ras(61L), Rac1 WT, Rac1(61L), or Tiam1(C1199), cells were plated at a density of 6×10^5 cells per 10-cm dish 1 day before transfection. 4 μ g of the pCGN-hygro empty vector (used as a control) or encoding HA-tagged H-Ras(61L), Rac1 WT or Rac1(61L) (39) were transfected into cells using LipofectamineTM 2000 (Roche Applied Science) according to the manufacturer's instructions. The same procedure was used with the pcDNA3.1-neo empty vector (control) or encoding Myc-tagged constitutively activated Tiam1(C1199). Two days after transfection, the transfected cells were replated in growth medium supplemented with either 200 μ g/ml hygromycin B (Roche Applied Science) or 400 μ g/ml G418 (Amersham Biosciences), respectively. After 10 days of selection, several hundred drug resistance colonies were then pooled together to establish mass populations of stably transfected cells.

NIH 3T3 cells stably expressing Rac1 WT, Rac1(61L), or Tiam1(C1199) proteins were used for secondary focus formation assays. Cells stably transfected with the empty pCGN-hygro or pcDNA3.1-neo plasmids were used as controls. For each stable cell line, 5×10^5 fibroblasts were plated into each well of 6-well plates. Each condition was performed in duplicate. The cultures were fed every other day with fresh complete growth medium alone or supplemented with 5 μ M of the compound EHT 1864. Activated Rac1- or Tiam1-induced transformed foci were visible after 3–4 weeks and were subsequently quantified under an inverted phase-contrast microscope at 4 \times magnification. Cells were then fixed in 10% acetic acid, 10% methanol and stained with 0.4% crystal violet dye.

To determine the effects of EHT 1864 treatment on the rate of cell proliferation, NIH 3T3 cells stably expressing oncogenic Ras were plated in 96-well plates at an initial density of 2×10^3 cells/well. The cells were cultured for up to 4 days in complete growth medium, either alone, or supplemented with 5 μ M EHT 1864. Cell growth was then assessed using the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) to a formazan product (40). Briefly, the MTT reagent (from a 5 mg/ml solution diluted in PBS) was added to the wells at a final concentration of 0.5 mg/ml, and the cells were further incubated for 4 h at 37 $^{\circ}$ C. The medium was then removed, and the reaction was terminated by adding 100 μ l/well Me₂SO. The absorbance was read at 570 nm using a microplate reader.

RESULTS

EHT 1864 Specifically Inhibits PDGF-induced Lamellipodia Formation—Our previous pull-down analyses showed that EHT 1864 reduced the association of endogenous Rac1 but not RhoA with the isolated GTP-dependent RBDs of PAK1 or Rhotekin, respectively (28). Rac1 and Cdc42 share overlapping downstream effectors that include PAK1. Because EHT 1864 impairs

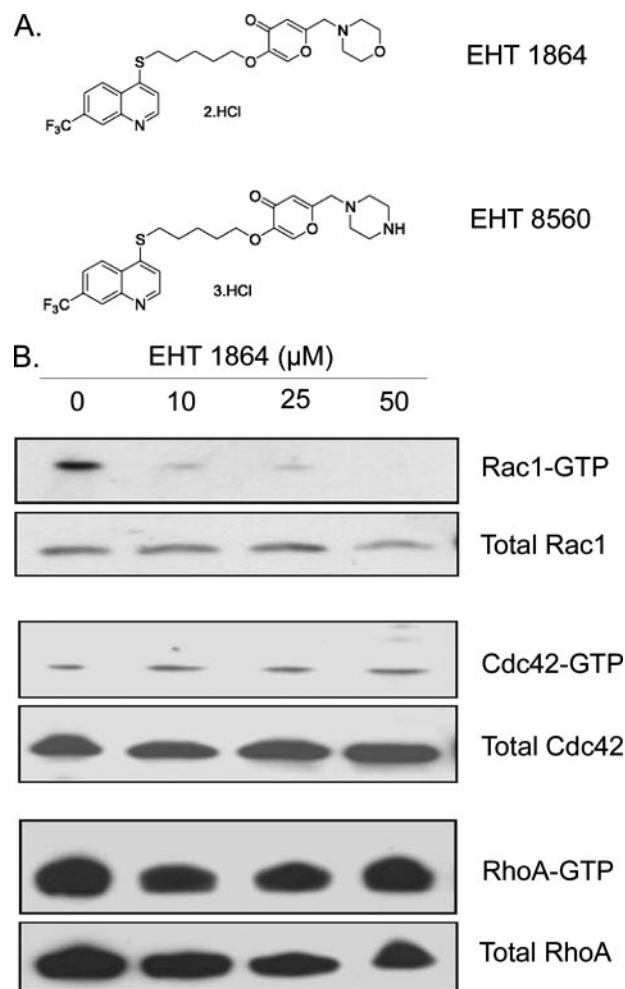


FIGURE 1. Structure and activity of EHT compounds. A, structures of EHT compounds used in this study: EHT 1864 and EHT 8560. EHT 8560 did not reduce the levels of RhoA-GTP, Rac1-GTP, or Cdc42-GTP when evaluated in pull-down assays with GST-Rhotekin-RBD or GST-PAK-RBD (data not shown) and was included as a negative control. B, EHT 1864 selectively reduces detection of Rac1-GTP. U87-MG cells were treated for 5 min with various doses of EHT 1864. Cell lysates with equivalent total protein were then used for pull-down analyses with GST-PAK-RBD (Rac1 and Cdc42) or GST-Rhotekin-RBD (RhoA), resolved by SDS-PAGE, followed by Western blot analyses with antibody specific for each Rho GTPase. Data shown are representative of seven (Rac1), nine (Cdc42), or three (RhoA) independent experiments.

Rac1 interaction with PAK-RBD, whether EHT 1864 could also inhibit PAK-RBD association with the related Cdc42 small GTPase remained a possibility. We therefore determined if EHT 1864 could also impair Cdc42 effector interaction. The structure of EHT 1864 and its related negative control compound (EHT 8560) used in this study are shown in Fig. 1A. As shown in Fig. 1B, EHT 1864 treatment dose-dependently inhibited association of endogenous Rac1 with PAK-RBD but did not reduce endogenous Cdc42 interaction with PAK-RBD, demonstrating the specificity of the compound toward Rac1 activity. It is noteworthy that loss of Rac1 association with PAK-RBD was not due simply to preferential loss of Rac1 expression, because EHT 1864 did not reduce the steady-state levels of endogenous Rac1.

To further delineate the specificity of EHT 1864 within cells and to evaluate the ability of EHT 1864 to inhibit Rac1-mediated cellular activities, we examined the effect of EHT 1864 on

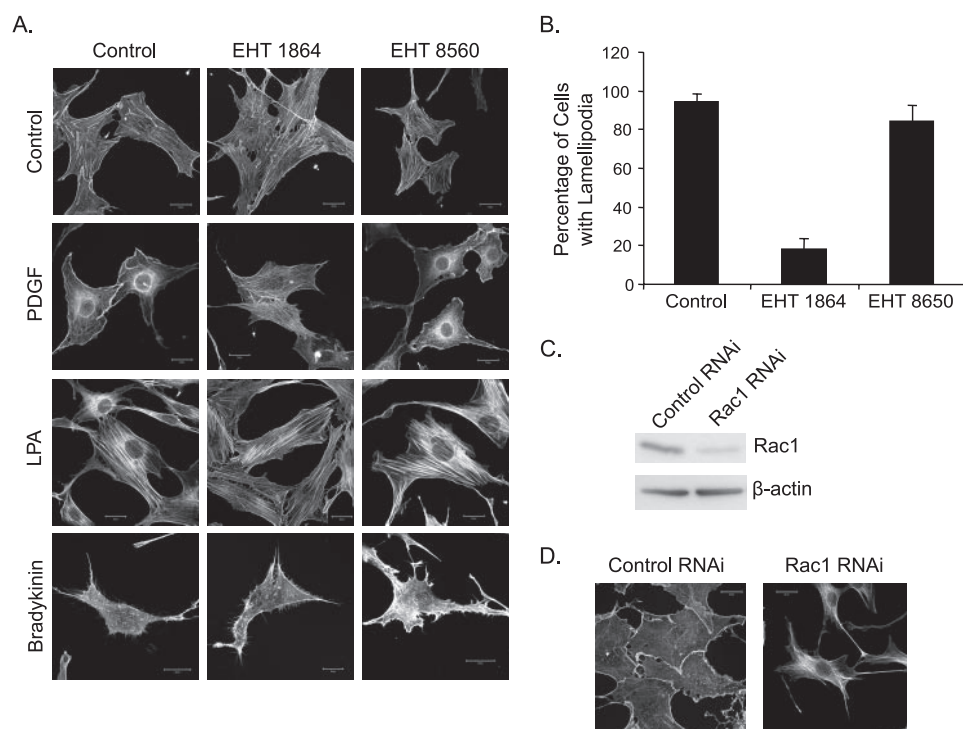


FIGURE 2. EHT 1864 selectively inhibits Rac-induced lamellipodia formation. *A*, EHT 1864 blocks PDGF- and not LPA- or bradykinin-induced actin reorganization. NIH 3T3 cells were incubated for 16 h in serum-free growth medium, either alone, or supplemented with 5 μ M EHT 1864 or EHT 8560 for the last 4 h. The cultures were then stimulated for 15 min with PDGF, LPA, or bradykinin and then fixed, and actin filaments were visualized with Alexa-phalloidin. Scale bar, 20 μ m. Results shown are representative of three independent experiments. *B*, quantitation of data shown in panel *A*: graphic representation of the percentage of PDGF-stimulated cells with lamellipodia, in the presence or absence of EHT 1864 or 8560, quantified on 100 cells for each condition. Results shown are the mean of three independent experiments. Error bars indicate the \pm S.E. *C*, PDGF-induced lamellipodia formation is Rac1-dependent. NIH 3T3 cells were transiently transfected twice with a Rac1 siRNA or an irrelevant siRNA used as a negative control. 12 h after the second transfection, cells were serum-starved for 16 h, then stimulated with PDGF for 15 min. Total protein extracts (20 μ g) from treated cells were resolved by SDS-PAGE and analyzed by Western blotting using anti-Rac1 monoclonal antibody and anti- β -actin antibody to verify equivalent total cell lysate protein loading. *D*, actin filament staining, performed as described above. Results shown are representative of two independent experiments.

Rac1-dependent cytoskeleton rearrangements and compared it to RhoA- and Cdc42-induced changes in actin cytoskeleton reorganization. It is known that PDGF activates Rac1 and induces Rac-mediated membrane lamellipodia formation in Swiss 3T3 fibroblasts (41), whereas LPA is responsible for RhoA activation, leading to RhoA-mediated formation of actin stress fibers and focal adhesion assembly (42), and bradykinin activates Cdc42, which in turn induces actin microspike and filopodia formation (43). To evaluate whether EHT 1864 could specifically block PDGF-induced lamellipodia formation, we treated serum-starved NIH 3T3 mouse fibroblasts with EHT 1864 and then stimulated the cells for 15 min with PDGF, LPA, or bradykinin. As shown in Fig. 2*A*, PDGF, LPA, and bradykinin efficiently induce membrane ruffling and lamellipodia formation, actin stress fibers, and filopodia, respectively. However, in the presence of EHT 1864, while LPA and bradykinin still induced their respective changes in actin cytoskeletal organization, PDGF-induced lamellipodia formation was blocked completely. We observed that EHT 1864-treated cells showed an \sim 80% reduction in PDGF stimulation of lamellipodia (Fig. 2*B*). In contrast, the structurally related, but inactive (for inhibition of Rac1 association with PAK-RBD) compound EHT 8560 did not affect lamellipodia formation in PDGF-stimulated cells

(Fig. 2, *A* and *B*). These data demonstrate that EHT 1864 is a potent and specific inhibitor of PDGF-induced lamellipodia formation.

Finally, to eliminate possible cell context differences in signaling, we wanted to verify that PDGF-induced lamellipodia in our strain of NIH 3T3 cells was dependent on Rac function. Although PDGF stimulation of lamellipodia has been shown to be Rac-dependent in Swiss 3T3 and other cell types, in light of our previous observations of significant NIH 3T3 strain variation in cellular responses to Rho GTPase activity (44), we felt it was important to perform this control experiment. We efficiently suppressed endogenous Rac1 expression by interfering RNA in NIH 3T3 cells (Fig. 2*C*) and stimulated cells with PDGF. We found that silencing Rac1 expression strongly impaired lamellipodia formation induced by PDGF (Fig. 2*D*), hence mimicking the lamellipodia-inhibitory effect of EHT 1864 observed in control cells expressing Rac1. Taken together, these data demonstrate that EHT 1864 specifically blocks extracellular stimulus induction of Rac1-mediated lamellipodia formation.

EHT 1864 Inhibits Rac-PAK Complex Formation—Our analyses *in vivo*

suggested that EHT 1864 decreased Rac1 interaction with PAK-RBD. To further evaluate the mechanism of action of EHT 1864, we examined the ability of EHT 1864 to regulate this interaction with purified recombinant proteins *in vitro*. For these analyses, we evaluated the effect of EHT 1864 on the formation of a Rac1-PAK-RBD or Cdc42-PAK-RBD complex, as measured by fluorescence anisotropy. We titrated GST-PAK-RBD into a solution containing 1 μ M Rac^{mant}-GMPPNP or 1 μ M Cdc42^{mant}-GMPPNP, in the presence or absence of EHT 1864. We found that in the absence of EHT 1864, for both Rac and Cdc42, fluorescence anisotropy increased (Fig. 3, *A* and *B*). This increase reflects an increase in overall complex size and provides a measure of the formation of a complex between the GTPase and PAK-RBD. In the presence of 50 μ M EHT 1864, a concentration that causes essentially a 100% loss of Rac-PAK-RBD interaction (Fig. 1*B*) (28), we observed that the complex formation between Cdc42 and PAK-RBD was unaffected (Fig. 3*A*), whereas the complex formation between Rac1 and PAK-RBD was completely inhibited (Fig. 3*B*). The lower relative values of anisotropy also suggest that nucleotide was lost from the Rac1 protein, into solution. These data suggest that EHT 1864 inhibited complex formation between Rac1 and PAK-RBD and

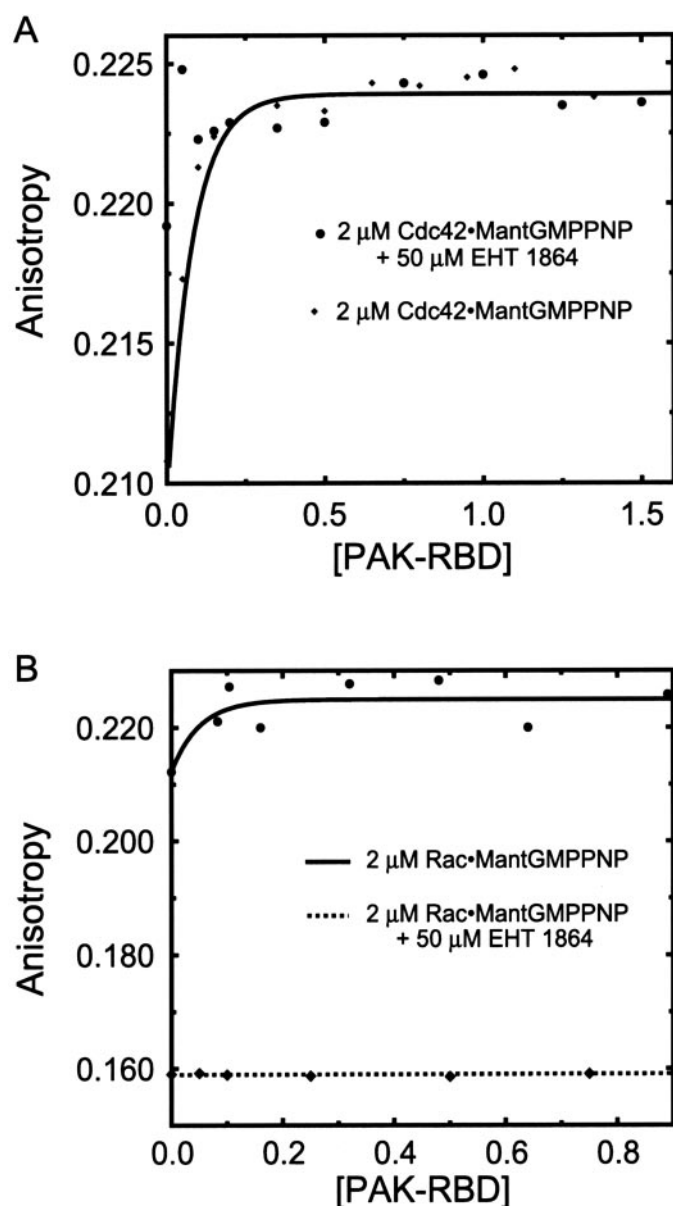


FIGURE 3. EHT 1864 inhibits Rac1 but not Cdc42 complex formation with PAK-RBD *in vitro*. Fluorescence anisotropy analyses were done to determine the ability of EHT 1864 to affect the binding of PAK-RBD to (A) Cdc42 or (B) Rac1. Each recombinant GTPase was preloaded with the non-hydrolysable and fluorescent mant-GMPPNP (final concentration, 2 μ M). The changes in fluorescence anisotropy ($\lambda_{\text{ex}} = 360$ nm $\lambda_{\text{em}} = 440$ nm) were measured upon titration of the PAK-RBD domain in the presence or absence of EHT 1864. The data were fitted to a hyperbolic binding curve, where appropriate. Data shown are representative of two independent experiments.

that EHT 1864 causes the release of nucleotide from Rac1, but not Cdc42.

EHT 1864 Associates Tightly with Rac1—The highly conjugated structure of the EHT 1864 and the inactive EHT 8560 (Fig. 1A) compounds suggests that they may be fluorescent. We observed that both EHT 1864 and EHT 8560 fluoresced strongly at the optimal excitation and emission wavelengths of $\lambda_{\text{ex}} = 360$ nm and $\lambda_{\text{em}} = 410$ nm (Fig. 4A). Upon addition of 10 μ M Rac1 to 1 μ M EHT 1864, there was a 33% increase in fluorescent intensity (Fig. 4B). This change in fluorescence allowed us to measure the strength of association between Rac1 and the

EHT compounds, using increases in fluorescence anisotropy of the EHT fluorophore. We titrated Rac-GDP against 1 μ M EHT 1864 or EHT 8560 and monitored the increases in fluorescence anisotropy ($\lambda_{\text{ex}} = 360$ nm, $\lambda_{\text{em}} = 410$ nm). The increases in anisotropy were then fitted to a hyperbolic binding curve (Fig. 4C). We observed that EHT 1864 bound tightly to Rac1, with a $K_D = 40$ nM. In contrast, as expected from its inability to block Rac1 association with the PAK-RBD, we observed no increases in anisotropy with Rac1 and EHT 8560. These results suggest that EHT 1864 shows high affinity binding to Rac1.

EHT 1864 Interferes with the Process of Nucleotide Exchange—The observed decrease in the fluorescence anisotropy value on addition of EHT 1864 to Rac1-mant-GMPPNP (Fig. 3B) suggested that this compound caused a release of Rac1-bound nucleotide into solution. To examine the effect of EHT 1864 on the state of bound nucleotide, we preloaded Rac1 with mant-GDP, and rapidly added EHT 1864 (50 μ M), EHT 8560 (50 μ M), or the Mg^{2+} -chelating agent EDTA (10 mM). EDTA treatment stimulates release of the bound nucleotide by Mg^{2+} chelation. We monitored the changes in fluorescence resonance energy transfer (FRET) between the mant group of the mant-GDP and the conserved tryptophan 56 of Rac1 ($\lambda_{\text{ex}} = 290$ nm, $\lambda_{\text{em}} = 440$ nm). These changes reflect the bound state of the nucleotide. On addition of EHT 1864 or EDTA, we observed a rapid and large decrease in fluorescence, reflecting the loss of FRET between the Trp and mant group (Fig. 5A). These data were fitted to a single exponential, with $k_{\text{obs}} = 0.038$ s $^{-1}$ and 0.14 s $^{-1}$, respectively. This decrease suggested that, like EDTA treatment, EHT 1864 stimulated the rapid release of mant-GDP into solution. In contrast, upon the addition of EHT 8560 we saw very little change in the levels of FRET, which is consistent with the lack of EHT 8560 binding to Rac and, therefore, its lack of activity toward the protein.

Because EHT 1864 induced nucleotide release, we asked whether Rac-bound EHT 1864 would be able to inhibit the re-association of nucleotide with Rac1, when induced by either EDTA or by a Rac-specific GEF, such as Tiam1. We incubated Rac1 in the presence of EHT 1864 and the fluorescent nucleotide analogue mant-GDP, and then initiated the exchange reaction with either EDTA or the Tiam1 catalytic fragment (spanning the tandem Dbl and pleckstrin homology (DH/PH) domains required for RhoGEF catalytic activity (33)). We observed that EHT 1864 caused a concentration-dependent inhibition of the association of mant-GDP with Rac1 that was stimulated by treatment with EDTA (Fig. 5B) or stimulation with Tiam1 (Fig. 5C). These data, together with the nucleotide release observed with EHT 1864, demonstrate that EHT 1864 interferes with the process of nucleotide exchange on Rac1.

EHT 1864 Does Not Affect RacGEF or RhoGDI Binding to Rac1 *In Vitro*—Our results showed that EHT 1864 dose-dependently inhibited the association of nucleotide with Rac1 stimulated by Tiam1. Because GEFs show preferential binding to the nucleotide-free state of their target GTPases (45), we analyzed whether EHT 1864 could modulate Rac1-Tiam1 interaction. For these *in vitro* analyses, we used a mutant version of Rac1 that harbors a mutation analogous to the 15A mutation that renders Ras deficient in GDP and GTP binding (46) and binds specifically to RhoGEFs that activate Rac1 (34).

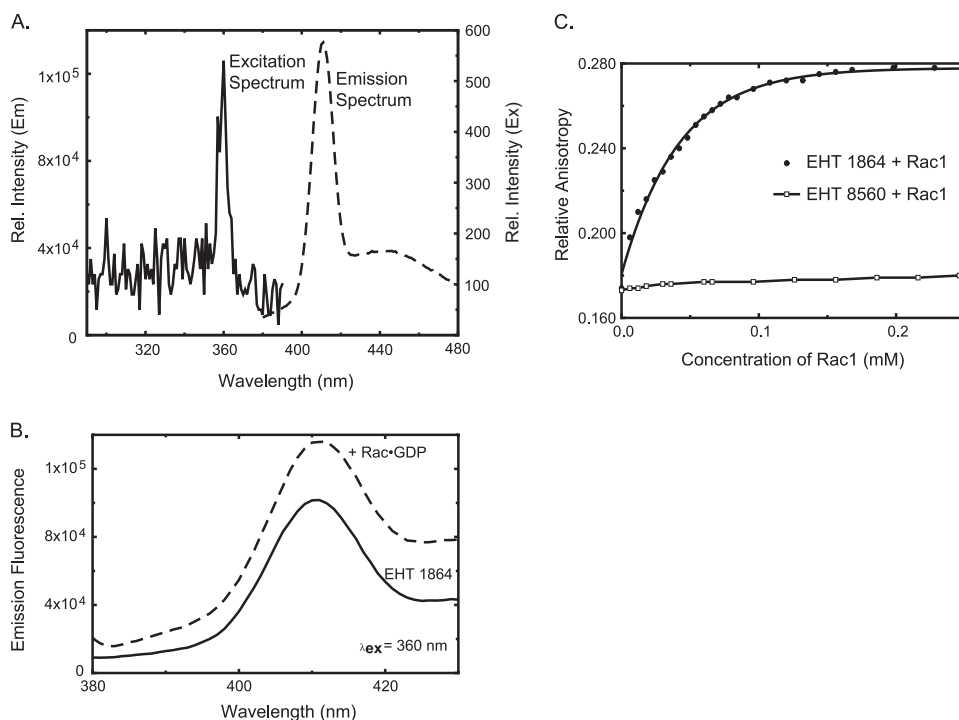


FIGURE 4. EHT 1864 binds tightly to Rac1. A, EHT 1864 is fluorescent. EHT 1864, at a final concentration of 1 μM , was incubated in 20 mM Tris HCl (pH 7.5), 50 mM NaCl, and 1 mM MgCl_2 , and an excitation spectrum (emission collected at 410 nm) and emission spectrum (excited at 360 nm) were recorded. B, Rac1 alteration of EHT 1864 fluorescence. 1 μM EHT 1864 was incubated in 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 1 mM MgCl_2 . Rac-GDP was added to a final concentration of 10 μM , and the change in fluorescent emission spectra ($\lambda_{\text{ex}} = 360$ nm) was recorded. C, EHT 1864 but not EHT 8560 binds to Rac1. Rac-GDP was titrated into a 1 μM solution of either EHT 1864 or EHT 8560 in 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM MgCl_2 . The changes in fluorescence anisotropy were measured at each addition ($\lambda_{\text{ex}} = 360$, $\lambda_{\text{em}} = 410$). The data for EHT 1864 were fitted to a hyperbolic binding curve.

GST fusion protein of the Rac1(15A) mutant was then tested for its ability to interact with Tiam1 in the presence or absence of EHT 1864. A constitutively activated mutant of Tiam1, Tiam1(C1199), was transiently overexpressed in 293T cells (Fig. 6A) and tested for its ability to bind to and be affinity-precipitated by the nucleotide-free Rac1(15A) mutant protein immobilized on glutathione-Sepharose beads. As shown in Fig. 6B, Tiam1(C1199) specifically interacts with Rac1(15A), and treatment with EHT 1864 did not affect this interaction. The same results were also observed in NIH 3T3 cells (data not shown). These data clearly demonstrate that EHT 1864 does not interfere with RacGEF association with Rac1.

In parallel, we investigated whether EHT 1864 could impair guanine nucleotide dissociation inhibitor (RhoGDI) binding to Rac1. Both RhoGDI1 (also called RhoGDI α /RhoGDI) and RhoGDI2 (RhoGDI β /Ly/D4GDI) associate with Rac1 (47, 48), and RhoGDIs have been described to interact preferentially with the GDP-bound form of Rac1 and to a lesser extent with the GTP-bound state (49, 50). RhoGDI1 and RhoGDI2 proteins were then transiently expressed in 293T cells and evaluated, in the presence or absence of EHT1864, for their ability to bind to and be affinity-precipitated by a GST-Rac1 WT recombinant protein immobilized on glutathione-Sepharose beads and pre-loaded with either GDP or GTP. Our results showed that both RhoGDI1 and RhoGDI2 interacted specifically with GST-Rac1 fusion protein, with a higher affinity for the GDP-bound state of the GTPase, and this *in vitro* association was not altered by EHT

1864 (Fig. 7, A and B). We observed the same results when the analyses were done in NIH 3T3 cells (data not shown).

EHT 1864 Shows Specificity for Rac Subfamily Members—Our pull-down and actin reorganization analyses *in vivo* suggest that EHT 1864 specifically interacted with Rac1 and not Cdc42 or RhoA. The Rac branch of the Rho family is composed of three closely related proteins, Rac1, Rac2, and Rac3, that share significant sequence (85%) and biochemical identity (51). Additionally, an alternative splice variant of Rac1, Rac1b, has been found to be expressed in human cancer cells (17, 18). Rac1b contains a 19-residue insert sequence upstream of the switch 2 region of Rac1, which perturbs interaction with regulator proteins and effectors and displays biological properties of a constitutively activated, transforming protein (19). Therefore, we determined whether EHT 1864 could also act on Rac1b, Rac2, and Rac3.

Using the changes in fluorescence anisotropy of the inhibitor as a measure of its binding to Rac family

proteins, we titrated recombinant Rac-GDP family proteins into a 1 μM solution of inhibitor and measured the increases in anisotropy ($\lambda_{\text{ex}} = 360$ nm, $\lambda_{\text{em}} = 410$ nm) (Fig. 8A). We observed that EHT 1864 bound to Rac1b and Rac2 with very similar affinities as we saw with Rac1 (50 nM and 60 nM, respectively). Rac3 bound with a lower affinity of ~ 250 nM. Fig. 8A also confirmed that EHT 1864 does not bind to Cdc42. We next examined the ability of EHT 1864 to stimulate nucleotide release from Rac2 and Rac3 proteins. We pre-loaded the Rac proteins with mant-GDP and observed the loss of FRET between the conserved tryptophan group in Rac2 or Rac3, and the mant group attached to the nucleotide, which reflects the release of the mant nucleotide into solution. We observed that EHT 1864 did induce nucleotide loss from Rac2 and Rac3 (Fig. 8B) with k_{obs} of 0.023 s^{-1} and 0.0011 s^{-1} , rates slower than that of Rac1 ($k_{\text{obs}} = 0.038 \text{ s}^{-1}$). These data suggest that EHT 1864 is most active on Rac1, both in its binding affinity and stimulation of nucleotide release. EHT 1864 also has significant activity on Rac2, but much less on Rac3.

EHT 1864 Blocks Cell Transformation Induced by Constitutively Activated Rac1 and by the Rac-dependent Tiam1 and Ras Transforming Proteins—Our analyses above indicate that EHT 1864 directly antagonizes Rac function. Therefore, unlike NSC23766, which did not block the activity of constitutively activated Rac1(61L) (27), we anticipated that EHT 1864 would effectively block Rac1(61L) function. It has been established previously that Rac1(61L) can promote cellular transformation

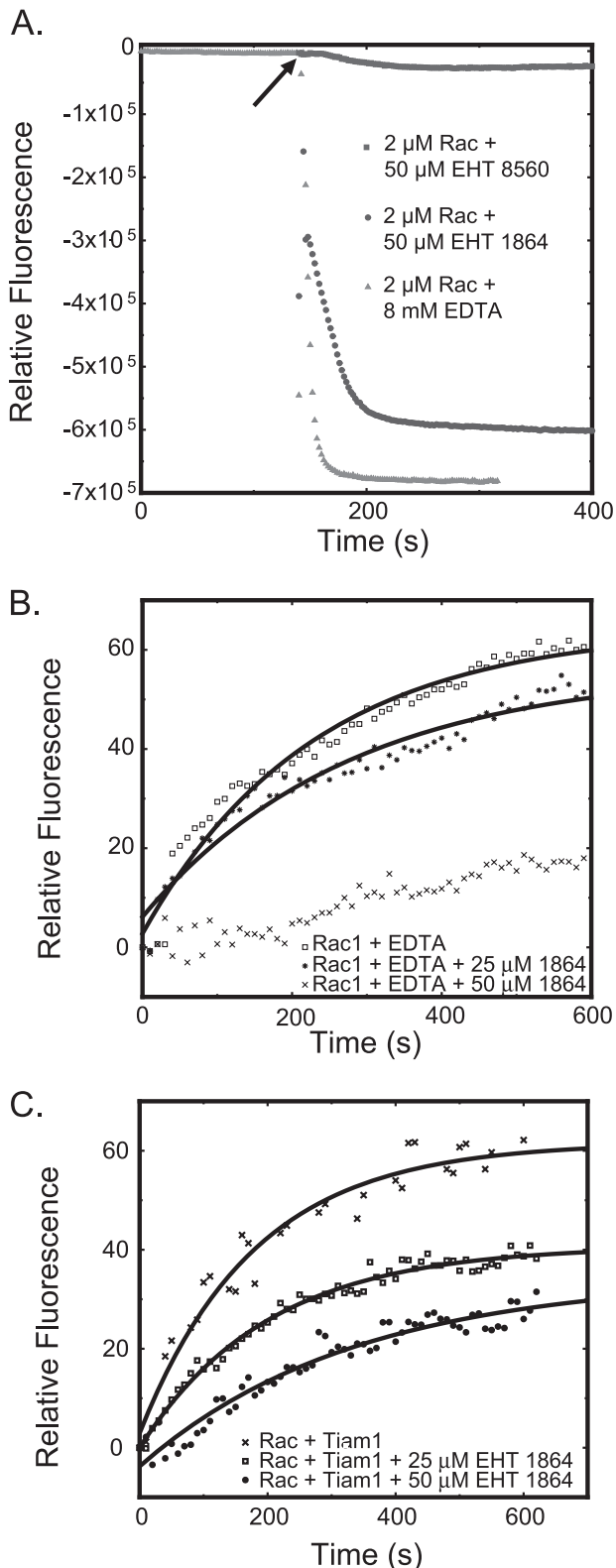


FIGURE 5. EHT 1864 interferes with the process of nucleotide exchange on Rac1. *A*, EHT 1864 stimulates release of prebound nucleotide. 2 μ M Rac-mant-GDP was incubated in a solution of 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM MgCl₂. After 140 s, EHT 1864 was rapidly added to a final concentration of 50 μ M. Changes in FRET, between tryptophan 56 of Rac1 and the mant group of the nucleotide analogue, were observed (λ_{ex} = 290 nm, λ_{em} = 440 nm). *B*, EHT 1864 impairs EDTA-induced nucleotide exchange with Rac1. 2 μ M Rac-GDP was incubated in a solution of 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM MgCl₂, 2 μ M mant-GDP and either 50 μ M or 25 μ M EHT 1864 for

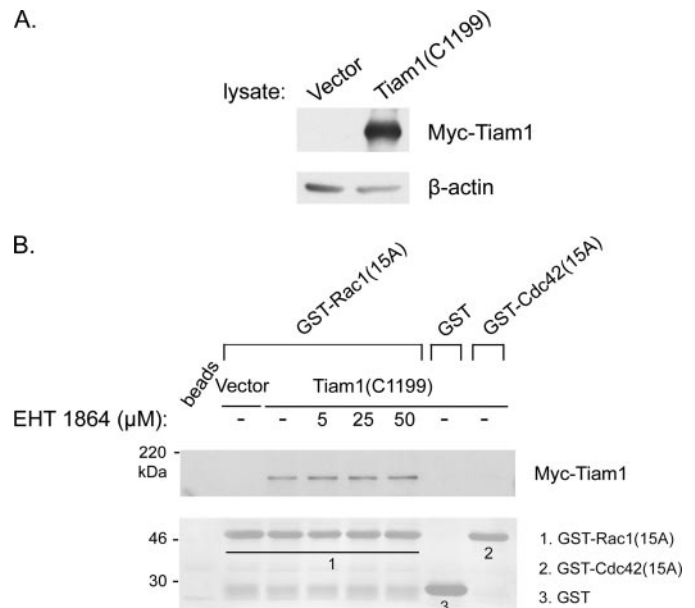


FIGURE 6. EHT 1864 does not impair Tiam1 binding to Rac1 *in vitro*. *A*, ectopic expression of Tiam1. 293T cells were transiently transfected with 10 μ g of pcDNA3.1 expression plasmid alone (vector) or encoding a Myc-tagged, N-terminally truncated and constitutively activated mutant of Tiam1, Tiam1(C1199). 48 h later, transfected cells were lysed, and the expression of Myc-tagged Tiam1(C1199) was detected by blot analysis using a monoclonal anti-Myc antibody. Blot analysis for β -actin was also done to verify equivalent total protein loading. *B*, EHT 1864 does not impair Tiam1 association with nucleotide-free Rac1. Control (Vector) or Myc-Tiam1-expressing lysates were incubated with GST-Rac1(15A) immobilized on glutathione-Sepharose beads in the presence of different doses of EHT 1864. Following washing, bound Tiam1(C1199) was detected by Western blotting with anti-Myc antibody (upper panel). GST and GST-Cdc42(15A) proteins served as negative controls. Amido Black staining (lower panel) was done to verify equivalent amounts of recombinant proteins used in the assay. Results shown are representative of three independent experiments.

when expressed in NIH 3T3 fibroblasts and other cell types, causing for example loss of density-dependent inhibition of growth (12, 39, 52). We performed secondary focus formation assays using wild-type NIH 3T3 cells and cells stably expressing either wild-type Rac1 or the constitutive active mutant Rac1(61L) (Fig. 9A). As shown in Fig. 9B, activated Rac1(61L), but not WT, caused focus-forming activity, and treatment of cells with EHT 1864 significantly abolished cellular transformation induced by Rac1(61L). EHT 1864 caused an 80% inhibition of focus-forming activity when compared with untreated cells expressing Rac1(61L) (Fig. 9C), indicating that EHT 1864 acts as a specific inhibitory reagent to reverse cell transformation attributed to Rac1 activation. Because Rac1(61L) is activated by a loss of Rho-specific GTPase-activating protein responsiveness, rather than RhoGEF activation, and because this mutant fails to bind RhoGDI (53), these results are also consistent with

4 min. Exchange was then initiated by addition of EDTA to a final concentration of 5 mM. Increases in FRET between tryptophan 56 and the mant group were monitored by observing the changes in fluorescence (λ_{ex} = 290 nm, λ_{em} = 440 nm). *C*, EHT 1864 alters Tiam1-induced nucleotide association with Rac1. 2 μ M Rac-GDP was incubated in a solution of 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM MgCl₂, 2 μ M mant-GDP and either 50 μ M or 25 μ M EHT 1864 for 4 min. Exchange was then initiated by addition of recombinant His₆-Tiam1 DH/PH (32), a Rac-specific RhoGEF, to a final concentration of 0.5 μ M. Increases in FRET between tryptophan 56 and the mant group were monitored by observing the changes in fluorescence (λ_{ex} = 290 nm, λ_{em} = 440 nm).

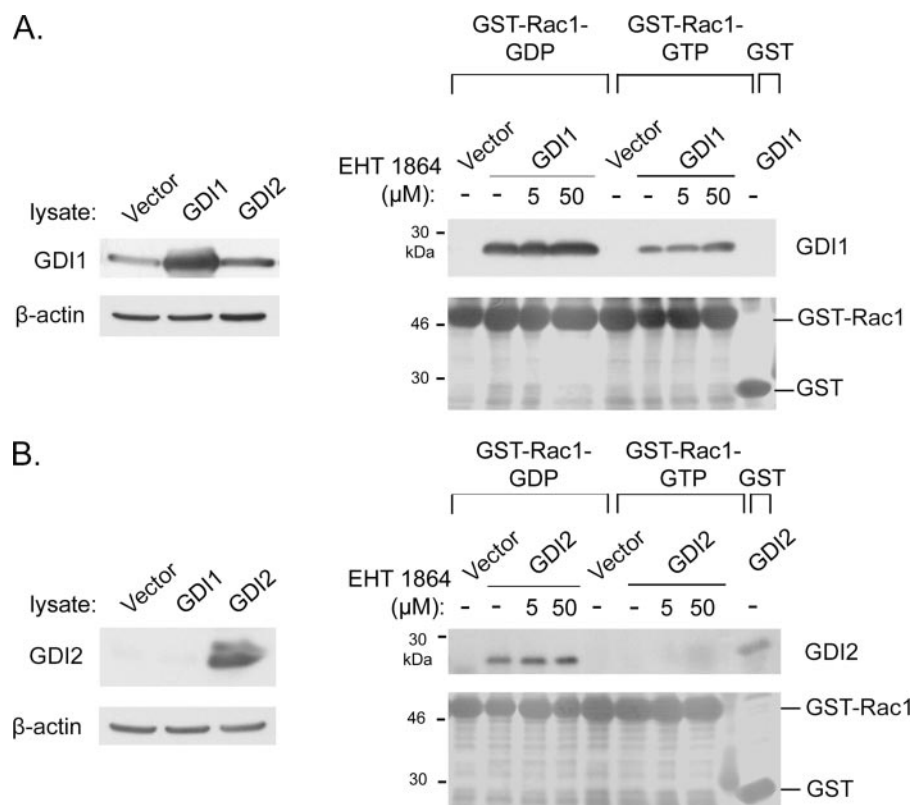


FIGURE 7. EHT 1864 does not affect RhoGDIs binding to Rac1 *in vitro*. EHT 1864 does not reduce Rac1 association with RhoGDI1 (A) or RhoGDI2 (B) *in vitro*. 293T cells were transiently transfected with 5 μ g of pcDNA3.1 expression plasmid alone (Vector) or encoding RhoGDI1 or RhoGDI2 proteins. 48 h later, transfected cells were lysed, and the expression of RhoGDI1 and RhoGDI2 was detected by blot analysis using a rabbit polyclonal anti-RhoGDI1 antibody or a goat polyclonal anti-RhoGDI2 antibody, respectively (left panels). Blot analysis for β -actin was also done to verify equivalent total protein loading. Lysates were incubated in the presence of different doses of EHT 1864 with GST-Rac1 immobilized on glutathione-Sepharose beads and loaded with either GDP or GTP. Following washing, bound RhoGDI1 and RhoGDI2 were detected by Western blotting with anti-RhoGDI1 and anti-RhoGDI2 antibodies, respectively (right panels). GST protein served as a negative control. Amido Black staining was done to verify equivalent loading for the recombinant proteins used in the assay. Results shown are representative of three independent experiments.

our data above that EHT 1864 inhibitory activity does not involve inhibition of RhoGEF or RhoGDI interaction. EHT 1864 also blocked the focus-forming activity of activated Tiam1(C1199) (Fig. 9, B and C), which causes Rac-dependent transformation of NIH 3T3 cells (52). Because we found that EHT 1864 did not block Tiam1 interaction with Rac1, this inhibition is presumably at the level of activated Rac1.

Previous studies found that Ras causes Rac activation, and inhibition of Rac activation by the Rac1(17N) dominant negative blocked Ras-mediated growth transformation on NIH 3T3 cells (11, 12). Rac1(17N) forms non-productive complexes with RacGEFs and prevents Rac activation. Recently, NSC23766 was identified as a small molecule inhibitor of RacGEF-mediated activation of Rac and of Ras-mediated growth transformation (27). Thus, inhibition of RacGEF activation of Rac is an effective approach for blocking Ras-transforming activity. Based on our observations that EHT 1864 impaired cellular transformation induced by constitutively activated mutants of Tiam1 and Rac1, we determined whether EHT 1864 could also block Ras transformation. We performed primary focus formation assays using NIH 3T3 fibroblasts transiently transfected with a constitutively active Ras mutant, H-Ras(61L), and cultured in the

presence or absence of EHT 1864. As shown in Fig. 10 (A and B), EHT 1864 treatment causes complete inhibition of activated H-Ras(61L)-induced focus formation. Similar results were observed in a secondary focus formation assay using NIH 3T3 cells stably expressing H-Ras(61L) (data not shown). We also evaluated the effect of EHT 1864 on the growth rate of NIH 3T3 cells stably expressing H-Ras(61L) protein (Fig. 10C). Whereas expression of H-Ras(61L) causes an enhanced rate of cell growth compared with control cells, treatment of cells with EHT 1864 strongly impaired oncogenic Ras-induced cell proliferation to a level similar to that of untransformed NIH 3T3 cells (Fig. 10D). In contrast, there was only modest reduction in the growth rate of untransformed cells. Taken together, these data demonstrate that the compound EHT 1864 strongly impacts on Ras-induced cellular transformation and suggest that inhibition of Rac effector interaction is also an effective strategy for blocking Ras-mediated transformation.

DISCUSSION

Rho family small GTPases are critically involved in many signaling pathways within cells, controlling a variety of different cellular responses (3). Their aberrant regulation or expression is found in a variety of disease states, such as cancers and neurological disorders, and therefore Rho GTPases are validated targets for novel therapeutic approaches (6–9, 10). However, to date, the development of effective small molecule inhibitors that directly target Rho family GTPases has been very limited. Currently, most progress has been made utilizing inhibitors that impair prenyltransferases that catalyze the lipid modifications critical for GTPase function or block protein kinases that function downstream of small GTPases (23, 24). We previously reported the identification and characterization of EHT 1864, and we found that this compound blocked activated Rac-mediated signaling, Rac association with PAK-RBD, and blocked the Rac-dependent processing of the amyloid precursor protein (28). In the present study, we further evaluated the specificity of EHT 1864 for endogenous Rac1 function, characterized its ability to interact with Rac isoforms, the biochemical mechanism by which it blocks Rac1 function, and assessed the ability of this inhibitor to block another Rac-dependent biological function, Ras-mediated growth transformation. Our results suggest that EHT 1864 is

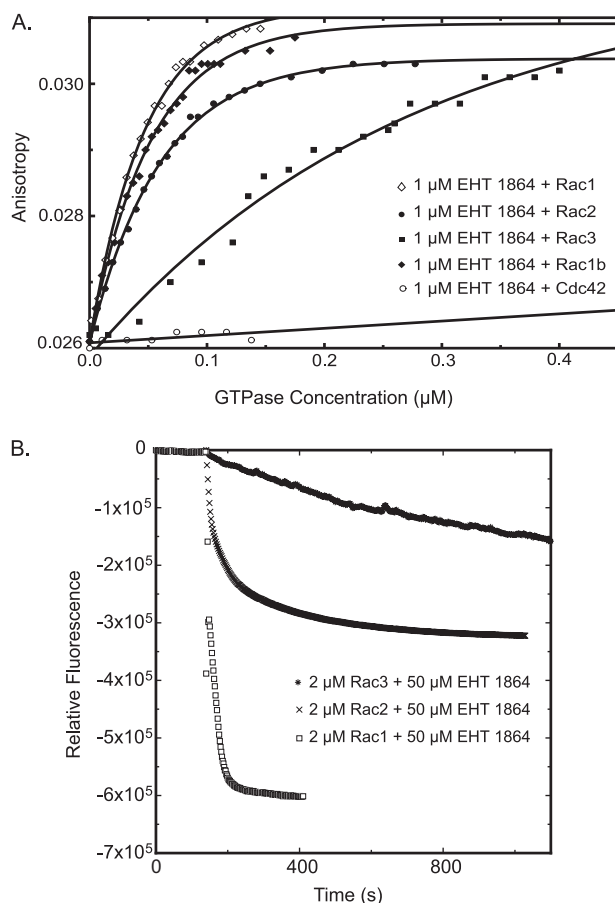


FIGURE 8. EHT 1864 demonstrates activity on other Rac isoforms. Fluorescence anisotropy analyses shows EHT 1864 association with Rac1, Rac1b, Rac2, and Rac 3 but not Cdc42. A, recombinant Rac-GDP proteins were titrated into a solution of 1 μM EHT 1864, 20 mM Tris-HCl, 50 mM NaCl, 1 mM MgCl₂, and the changes in fluorescence anisotropy were measured ($\lambda_{\text{ex}} = 360 \text{ nm}$, $\lambda_{\text{em}} = 440 \text{ nm}$) after 2-min equilibration. The data were fitted to a hyperbolic binding curve. B, 2 μM Rac2-mant-GDP, Rac3-mant-GDP, or Rac1-mant-GDP were incubated in 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM MgCl₂, and after 140 s, EHT 1864 was rapidly added to a final concentration of 50 μM . The changes in FRET between the tryptophan 56 and the mant group were observed, by measuring the changes in fluorescence ($\lambda_{\text{ex}} = 290 \text{ nm}$, $\lambda_{\text{em}} = 440 \text{ nm}$). The data were best fitted to a single exponential.

selective for Rac isoforms, binds to Rac with a high affinity, disrupts guanine nucleotide association, causing a loss of interaction with its effectors, and can effectively block Ras-mediated transformation. Thus, EHT 1864 may define a novel small molecule inhibitor scaffold that may be further developed for directly antagonizing the function of Ras superfamily small GTPases.

Our previous observations suggested that EHT 1864 prevented Rac1 but not RhoA interaction with downstream effectors. To further delineate the Rho GTPase specificity of EHT 1864, we determined that EHT 1864 selectively blocked extracellular stimuli-induced activation of endogenous Rac1 and not of RhoA or Cdc42. This inhibition was associated with a decreased ability of PAK-RBD to pulldown-activated Rac-GTP but not Cdc42-GTP from cells. Because Cdc42-GTP also binds to PAK-RBD, these results argue that EHT 1864 interacts with Rac itself, rather than with PAK-RBD. This conclusion is also consistent with our previous observation that EHT 1864 blocked the ability of ectopically expressed activated

Rac1(G12V) to stimulate transcription from Jun- and NF- κ B-responsive promoters, signaling pathways that utilize distinct effectors (39).

One possible mechanism for EHT 1864 inhibition of Rac association with effectors may involve binding to the switch I (residues 30–38) and/or switch II (60–76) sequences that are involved in Rac effector binding. Missense mutations in the switch regions impair effector binding. Alternatively, EHT 1864 may cause a reduction in the GTP-bound state that shows higher affinity for effector binding due to its interference in the nucleotide exchange process and therefore the GTP-bound:GDP-bound equilibrium within the cell. Our analyses with recombinant Rac1 protein *in vitro* determined that EHT 1864 binds tightly to Rac1 (with nanomolar affinity), reducing its affinity for nucleotide, through induction of nucleotide release and inhibition of nucleotide binding, thus preventing effector binding both *in vitro* and *in vivo* to Rac. Because all Rac1 effector interactions are GTP-dependent, EHT 1864 is expected to block Rac1 interaction with all effectors. Thus, EHT 1864 will be expected to be more effective in blocking Rac1 functions, when compared with inhibitors of a specific subset of Rac1 effectors (e.g. inhibitors of PAK kinases). Additionally, we also show that EHT 1864 binds closely related Rac isoforms, Rac2 and Rac1b, and with a lower affinity, Rac3. The activity against Rac1b is important in light of the fact that this splice variant is constitutively activated and transforming and preferentially expressed in breast and colon cancers (17–19).

We observed that EHT 1864 caused a loss of nucleotide from Rac1 *in vitro*. However, the mechanism for this activity is not resolved, and structural determination of a co-complex of EHT 1864 with Rac1 will provide critical clues for this mechanism. The absence of impact of EHT 1864 on the binding of the Rac-specific RhoGEF, Tiam1, to Rac1, together with the ability of EHT 1864 to inhibit cellular transformation induced by constitutive activated Rac1(61L) mutant, which is GEF-independent, argue that EHT 1864, unlike NSC23766, acts neither on RacGEF interaction nor on GEF-induced Rac1 activation, and instead, acts directly on the small GTPase itself. Loss of bound nucleotide may result from direct displacement of the nucleotide by EHT 1864 (by lowering the overall affinity (K_D) of the nucleotide for the GTPase?), direct displacement of Mg²⁺ or loss of its coordination (36) by EHT 1864, or potentially through an allosteric mechanism caused by EHT 1864 binding at another site on the small GTPase. Our preliminary observations⁵ indicate that nucleotide release is more rapid when Rac is associated with the triphosphate nucleotide, suggesting the involvement of Mg²⁺ as a key step in the release mechanism (36). Further biochemical analyses, such as the use of NMR to identify Rac1 residues whose coordinates are altered by EHT 1864 binding, crystallization of the inhibitor in complex with Rac1 for a full structural determination, and direct measurement of inhibitor affinity in the presence or absence of nucleotide or Mg²⁺ via SPR (such as Biacore), will be required to completely elucidate this mechanism.

⁵ A. Shutes, unpublished observations.

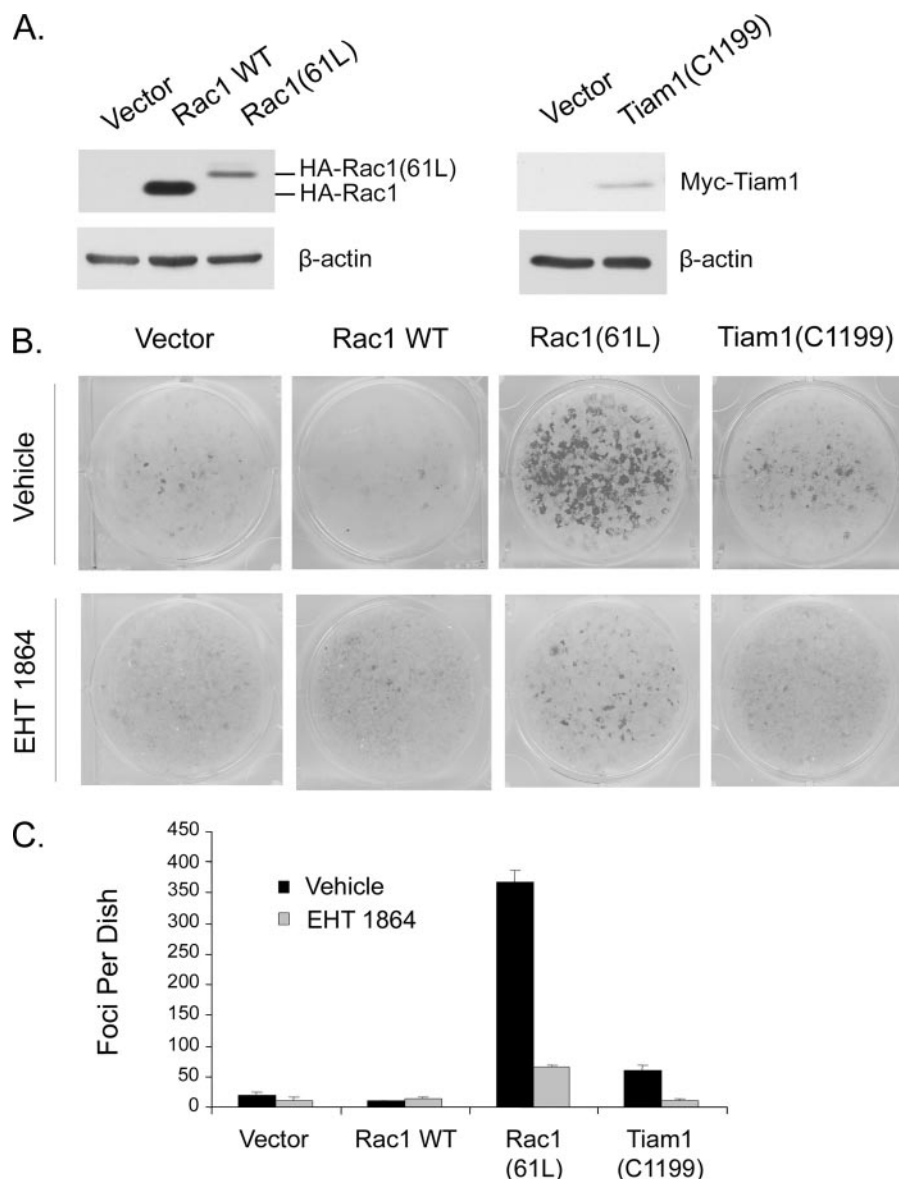


FIGURE 9. EHT 1864 specifically reverses cell transformation induced by constitutively activated mutants of Rac1 and Tiam1. A, NIH 3T3 fibroblasts were stably transfected with vectors encoding Rac1 WT or the constitutive active mutants Rac1(61L) or Tiam1(C1199). Transfection with the empty expression plasmid alone (*Vector*) served as a negative control. The expression levels of HA epitope-tagged Rac1 WT and Rac1(61L) proteins were detected by Western blotting using an anti-HA antibody. Myc-tagged Tiam1(C1199) protein expression level was detected using an anti-Myc antibody. The amounts of β -actin are shown as a loading control. B, EHT 1864 reverses focus formation induced by Rac1(61L) and Tiam1(C1199). NIH 3T3 cells stably expressing the proteins indicated were plated and allowed to reach confluency. Cells were cultured in complete growth medium, either alone or supplemented with 5 μ M EHT 1864. The appearance of foci of cells was observed by bright field 3–4 weeks after plating. Cells were then fixed and stained with crystal violet. C, quantitation of data shown in panel B. The number of foci of transformed cells was analyzed by phase-contrast microscopy. Data shown are representative of two independent experiments, each performed in duplicate.

Binding of EHT 1864 to Rac1 is tight, with a $K_D = 40$ nM. This compares to the K_D of GTP association with small GTPases of ~ 20 – 30 nM in the presence of Mg^{2+} (54–56). This tight association may explain the potency of EHT 1864 as observed previously (28). The K_D of the interaction between EHT 1864 and Rac1b and Rac2 was also comparable to that of Rac1 (50 nM and 60 nM, respectively), yet the K_D of interaction with Rac3 is 5-fold higher (230 nM). Rac1b, an alternative splice variant of Rac1, contains a 19-amino acid insert immediately behind the

switch II region (residues 60–76) of Rac1. This has been shown to be sufficient to disrupt effector interaction itself (19, 30, 57, 58). That EHT 1864 is able to associate tightly with Rac1b suggests that the extra 19 amino acids do not significantly interfere with its binding site. Of the three Rac proteins (1, 2, and 3), Rac1 and Rac2 are the most divergent, yet it is to these two that EHT 1864 associates most tightly. There are only five positions in their amino acid sequences where Rac1 and Rac2 are similar to each other, yet different to Rac3: Ile-111, Lys-128, Lys-147, Arg-187, and Lys-186. Of these, only Ile-111, in the Rho insert region, is located on a side of the protein facing the nucleotide binding pocket. Yet RhoA, which is unable to associate with EHT 1864, also has an Ile at the equivalent position. This may suggest further complex residue-drug interaction, or potentially an allosteric mechanism of drug action. Due to the intricate nature of protein/drug interfaces, the binding site of EHT 1864 may ultimately be best understood by a complete structural elucidation.

Previous studies demonstrated that Ras causes activation of Rac and that Rac function is critical for Ras-mediated growth transformation. In these studies, Rac function was blocked by use of a dominant negative Rac1(17N) mutant (11, 12), which blocks RacGEF activation of Rac (59), or by NSC23766, a cell-permeable small molecule that can bind directly to Rac1 and prevent its activation by Rac-specific RhoGEFs (27). In contrast to these approaches for blocking Rac function, our studies suggest that EHT 1864 disrupts formation of activated Rac1-GTP, thereby disrupting Rac1 interaction with its downstream effectors. As

anticipated from this mode of action, we also found that EHT 1864 treatment can effectively block the ability of mutationally activated Ras to cause growth transformation. Thus, EHT 1864 may define a novel approach for the development of anti-Ras inhibitors for cancer treatment. The 20-fold greater potency seen with EHT 1864, when compared with the 100 μ M concentration required for NSC23766 to block Rac activity *in vivo*, will facilitate our ability to evaluate the activity of EHT 1864 in mouse tumor models.

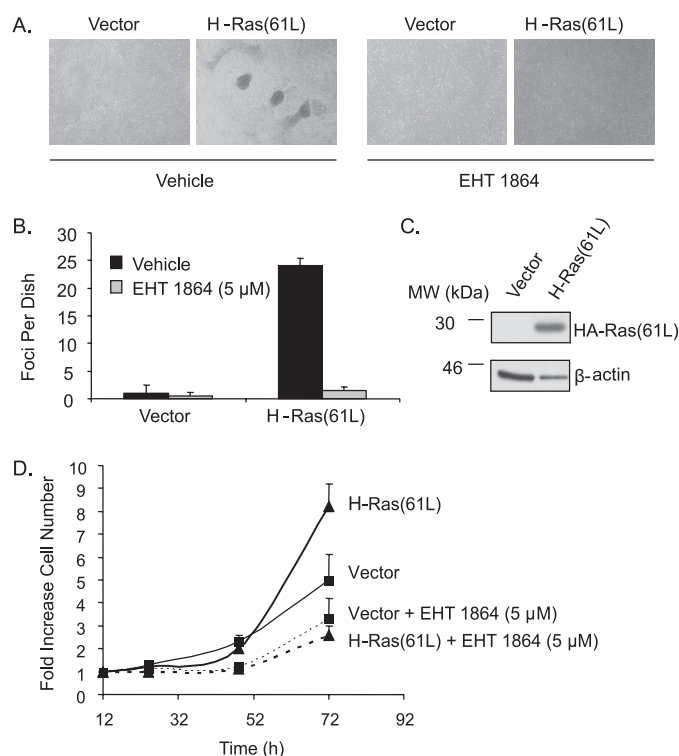


FIGURE 10. EHT 1864 inhibits oncogenic Ras-mediated growth transformation. A, focus formation induced by oncogenic Ras is blocked by treatment with EHT 1864. NIH 3T3 fibroblasts were transfected with 50 ng of pCGN-hygro expression plasmid alone (Vector) or encoding the constitutive active H-Ras(61L) mutant and were allowed to reach confluency. Cells were cultured in complete growth medium, either alone, or supplemented with 5 μM EHT 1864. The appearance of H-Ras(61L) foci of transformed cells was assessed 14 days after plating by using a bright field. B, quantitation of data shown in panel A. The number of foci was analyzed by phase-contrast microscopy. Data shown are representative of two independent experiments, with each performed in duplicate. C, EHT 1864 inhibits Ras-induced cell growth of NIH 3T3 fibroblasts. NIH 3T3 fibroblasts were stably transfected with pCGN-hygro expression plasmid alone (Vector) or encoding constitutively activated H-Ras(61L). The expression level of the HA epitope-tagged H-Ras(61L) protein was determined by Western blotting using an anti-HA antibody. The amounts of β-actin are shown as a loading control. D, NIH 3T3 cells, stably transfected as described above, were plated in 96-well plates at an initial density of 2×10^3 cells/well. The cells were cultured for the indicated number of days in complete growth medium, either alone, or supplemented with 5 μM EHT 1864, and cell proliferation was then assessed by an MTT assay. Each condition was performed in eight replicates, and the data shown are representative of two independent experiments.

The small molecule NSC23766 is the only Rac activation inhibitor described to date (27). When compared with this compound, the different mode of action of EHT 1864 displays crucial advantages. First, NSC23766 was effective against Rac1 activation by some, but not other, RhoGEFs that activate Rac1. NSC23766 blocked Rac activation by the Rac-specific GEFs Tiam1 and TrioN but was inactive against Vav1, a more promiscuous GEF that activates Rac1, and additionally RhoA and Cdc42 (27). EHT 1864 should be active against Rac activated by all RacGEFs. The importance of this property is emphasized by a recent study that found that Vav1 overexpression caused Rac-specific and -dependent growth transformation of pancreatic carcinoma cells (60). Hence, EHT 1864, and not NSC23766, is expected to be an effective inhibitor of Vav1-driven pancreatic cancers, and our future studies will evaluate this possibility. Second, EHT 1864, and not NSC23766, is effective in blocking

transformation by the GAP-deficient, constitutively activated Rac1(61L) mutant (our study and Ref. 27). Hence, EHT 1864, and not NSC23766, is expected to be effective in blocking Rac activation by RhoGEF-independent mechanisms. For example, we found that EHT 1864 can bind the constitutively activated and transforming Rac1b variant overexpressed in breast and colon cancers. Thus, EHT 1864, and not NSC23766, is anticipated to be effective against tumor cells that express this Rac1 splice variant. Additionally, Rho GTPases may become activated in cancers (e.g. DLC-1) or in mental retardation (e.g. oligophrenin) by the loss of Rho-specific GTPase-activating protein function, and EHT 1864, but not NSC23766, will be effective against Rac activation by the loss of Rho-specific GTPase-activating protein activity (61). Finally, because Ras is mutationally activated in human cancers, the mechanism of action of EHT 1864, and not NSC23766, may identify an approach to develop related inhibitors specific for constitutively activated Ras. In summary, EHT 1864 is not simply another version of NSC23766 and identifies a novel pharmacologic approach for Rac inhibition.

In summary, we have determined that EHT 1864 is a Rac-specific inhibitor that directly binds and impairs the ability of this small GTPase to engage critical downstream effectors required for growth transformation. Despite the considerable body of evidence implicating the aberrant function of Ras and Rho family small GTPases in cancer and other human diseases, there has been a striking lack of success in the pharmacologic development of inhibitors that directly target small GTPases. The identification of ATP-competitive inhibitors has been a highly successful approach for the development of clinically important protein kinase inhibitors. However, the considerably higher affinity of binding of guanine nucleotides to small GTPases has made GTP-competitive inhibitors an unrealistic approach for the development of inhibitors of small GTPases. The specificity and activity of EHT 1864 against the Rac proteins represents a significant step forward in the development of such drugs, and EHT 1864 may provide a template from which other small GTPase drugs may be rationally designed.

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