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Structure–activity relationship of isoform selective inhibitors of Rac1/1b GTPase nucleotide binding

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ABSTRACT

The synthesis of a series of berberine, phenantridine and isoquinoline derivatives was realized to explore their Rho GTPase nucleotide inhibitory activity. The compounds were evaluated in a nucleotide binding competition assay against Rac1, Rac1b, Cdc42 and in a cellular Rac GTPase activation assay. The insertion of 19 AA in the splice variant Rac1b is shown to be sufficient to introduce a conformational difference that allows compounds **4**, **21**, **22**, and **26** to exhibit selective inhibition of Rac 1b over Rac1.

Alternative splicing is a fundamental process, which is responsible for much of the tremendous transcriptome diversity that is derived from a finite number of genomically encoded genes. The alterations in mRNA structure that result can lead to profound effects on protein structure, function and ligand specificity. When deregulated, alternative splicing can also be a driving force for the onset and progression of disease.[1a]

Among genes that exhibit alternative splicing, Rac1 belongs to the Rho family of small GTP binding proteins and regulates diverse cellular processes, including actin cytoskeleton organization, membrane trafficking, proliferation, and gene expression.[1b] Like Ras and other members of the Ras superfamily, the GTP binding state of Rac1 is tightly controlled by guanine nucleotide exchange factors (GEF) and GTPase-activating proteins (GAP). Rho-GDP dissociation inhibitor (Rho-GDI) exerts additional regulation of Rho-GTPases by sequestering the GDP-bound forms in the cytoplasm, preventing their activation (Fig. 1).

Rac1 overexpression and altered function of Rac1-specific regulators or effectors have been found in various diseases that include several cancers[2a,b] and Alzheimer's disease.[2c] Rac1 can promote cellular transformation and is required for Ras-induced transformation.[3] Rac1 also induces cell survival and, by modulating the actin cytoskeleton, regulates cell adhesion as well as motility and invasion, activities required for tumor metastasis.[4] Alternative splicing variant Rac1b was identified and found to be overexpressed in colorectal and breast tumors.[5] Rac1b has transforming

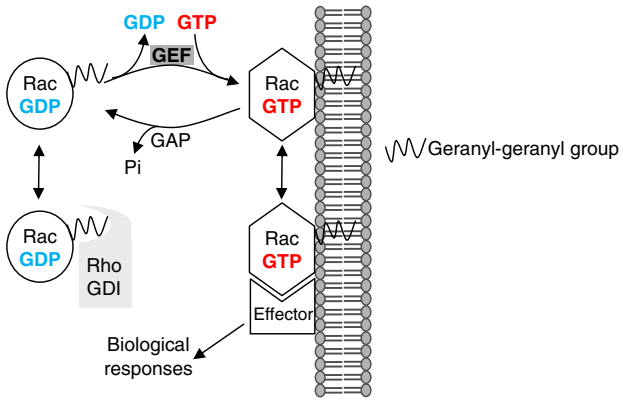

Figure 1. Regulation of Rac activity. Rho-GTPases cycle between an active GTP-bound and an inactive GDP-bound state. Rho-GDI (Rho-guanine nucleotide dissociation inhibitor) sequesters GDP-bound Rac GTPase in the cytoplasm and inhibits the spontaneous GDP–GTP exchange activity. Guanine nucleotide exchange factors (GEFs) catalyse GDP release and GTP binding. The GTP-bound GTPase is then able to interact with effectors to initiate downstream responses. GTPase-activating proteins (GAPs) stimulate the low GTPase activity to promote the conversion of the GTP-bound to the GDP-bound form, which terminates signal transduction. P_i: inorganic phosphate.

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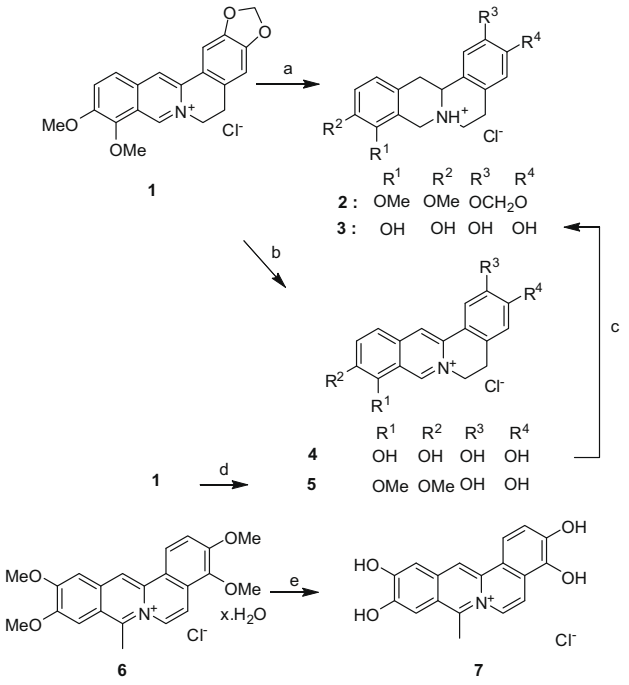


Scheme 1. Reagents and conditions: (a) $NaBH_4$ (4 equiv), MeOH, rt, 3 h, 89%; (b) BBr_3 (8 equiv), dry CH_2Cl_2, reflux, 63%; (c) $NaBH_4$ (4 equiv), MeOH, rt, 3 h, 71%; (d) BCl_3 (4 equiv) dry CH_2Cl_2, reflux, 52%; (e) (i) BBr_3 (6 equiv), dry CH_2Cl_2, rt, 48 h; (ii) 2 N HCl, 99%.

characteristics when exogenously expressed in cultured cells[6a], and its expression promotes cell cycle progression and resistance to apoptosis.[6b]

In addition, Rac1b induces epithelial–mesenchymal transition in mouse mammary carcinoma cells[7] and facilitates tumour progression by activating the Wnt signaling pathway and decreasing the adhesive properties of colorectal cancer cells.[8a] These observations provide a rationale for the use of Rac1 and Rac1b nucleotide binding inhibitors for the treatment of cancers and Alzheimer's disease[2c] with an alternative mode of action for small molecule GTPase regulation that does not proceed through a GEF[8b,c] or a GDI[8d] dependant mechanism.

In our a search for potent GTP binding inhibitors,[2c,9a] we initiated a medicinal chemistry program designed to study and improve the GTPase inhibitory activity of sanguinarine like alkaloid natural products[9b] that were shown to possess GTPase inhibitory activity.[9c]

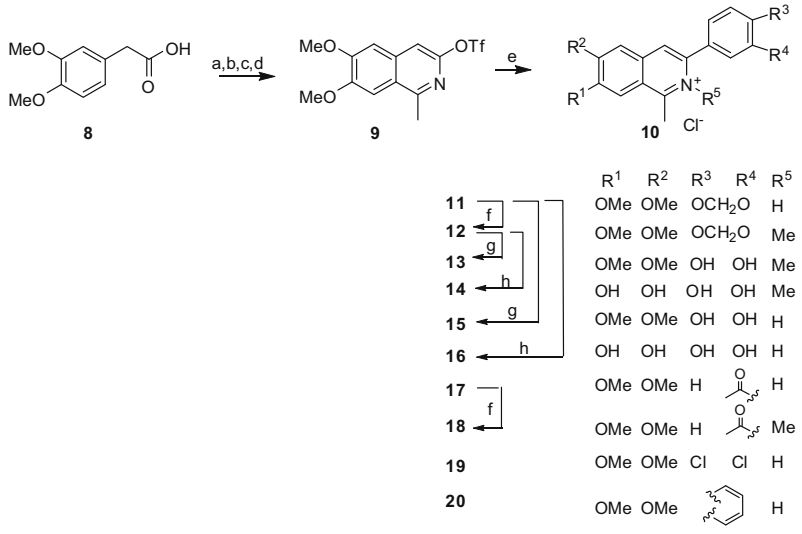
(±)-Canadine **2** was obtained after reduction of berberine **1**. The tetrahydroxy berberine analog **4** and demethylene berberine **5** were prepared from **1** by Lewis acid catalyzed ether deprotection. Subsequent treatment of **4** with $NaBH_4$ afforded tetrahydroxycanadine **3** (Scheme 1). The tetrahydroxyderivative **7** was obtained from coralyne chloride **6** using BBr_3.

We envisioned that phenantridine alkaloids (Formula IV) could be regarded as analogs with an alternative connection between the A and C rings of the berberine structure after rotation around their closest junction bond. Therefore, we sought to determine if analogs such as 3-aryl-isoquinolines (formula V) that can adopt the restricted conformations of formula I–III and formula IV were able to demonstrate higher inhibition in the nucleotide binding assay. In order to obtain 3-aryl-6,7-dimethoxy-isoquinolines(**11**–**20**) (Scheme 2), 3,4-dimethoxyphenylacetic acid **8** was esterified and acylated to form a benzopyrylium perchlorate salt which upon treatment with ammonia formed a 3-hydroxyisoquinoline[9d] that was triflated to form **9**. The triflate **9** was coupled to 3,4-methylenedioxyboronic acid using Suzuki conditions to form **11** followed by an alkylation using iodomethane to form **12**. Selective methylenedioxy deprotection was performed on **11** and **12** using BCl_3 to form respectively **15** and **13** while tetra hydroxyl derivative **14** and **16** were obtained from a complete aryl ether deprotection of **12** and **11**.

To obtain isoquinolines **17**, **19** and **20** the triflate **9** was coupled in presence of $Pd(dppf)Cl_2CH_2Cl_2$ with 3-acetylphenylboronic acid, 3,4-dichlorophenylboronic acid and 2-naphthaleneboronic acid respectively and **17** was N-methylated with MeI to obtain **18**.

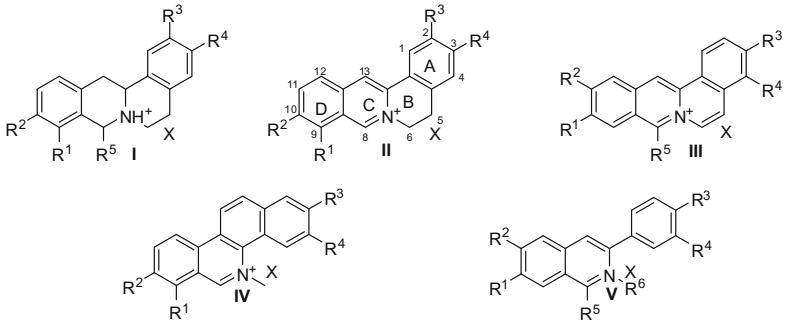
The compounds were evaluated using a nucleotide binding competition assay.[10] The nucleotide binding activity was monitored using the increase of the fluorescence intensity (FI) that accompanies the incorporation of Bodipy-GTP into the GTPase. All compounds were tested initially at 50 μM to identify the compounds to be selected for additional characterization.

In the protoberberine series, we observed a general increase in potency with the highest unsaturated compounds (Table 1.) We found that the reduction of berberine **1** to (±)-canadine **2** resulted



Scheme 2. Reagents and conditions: (a) H_2SO_4, MeOH, 91%, overnight; (b) Ac_2O, $HClO_4$ 0 °C to rt 74%; (c) NH_4OH, H_2O, 99%; (d) $PhN(SO_2CF_3)_2$, Et_3N, DMSO, 82%; (e) 2 N aq Na_2CO_3, $Pd(dppf)Cl_2CH_2Cl_2$, $ArB(OH)_2$, toluene, 80 °C, 42–62%; (f) MeI, CH_3CN, 90 °C, 62–64%, Amberlite Cl^-; (g) (i) BCl_3, CH_2Cl_2, −78 °C to rt; (ii) 2 N HCl; (h) (i) BBr_3, −78 °C to rt, CH_2Cl_2; (ii) 2 N HCl.

Table 1
Nucleotide binding inhibition assay



Compd	Formula	X	R^1	R^2	R^3	R^4	R^5	R^6	Rac1[a]	Rac1b[a]	cdc42[a]
2	I	Cl^-	OMe	OMe	$-OCH_2O-$		H	—	1 ± 3	0 ± 4	4 ± 6
3	I	Cl^-	OH	OH	OH	OH	H	—	70 ± 6	67 ± 9	ND
24	I	Cl^-	OMe	OMe	$-OCH_2O-$		Bz	—	23 ± 3	31 ± 4	9 ± 2
1	II	Cl^-	OMe	OMe	$-OCH_2O-$		H	—	14 ± 2	23±1	8 ± 2
25	II	Cl^-	OMe	OMe	OMe	OMe	H	—	15 ± 2	19 ± 3	8 ± 2
5	II	Cl^-	OMe	OMe	OH	OH	H	—	39 ± 2	54 ± 2	31 ± 9
4	II	Cl^-	OH	OH	OH	OH	H	—	>100	>100	>100
6	III	Cl^-	OMe	OMe	OMe	OMe	Me	—	35 ± 5	44 ± 5	23 ± 4
7	III	Cl^-	OH	OH	OH	OH	Me	—	63 ± 4	72 ± 4	70 ± 3
26	IV	Cl^-	$-OCH_2O-$		$-OCH_2O-$		—	—	63 ± 6	>100	63 ± 2
21	IV	Cl^-	OMe	OMe	$-OCH_2O-$		—	—	49 ± 7	95 ± 3	35 ± 10
22	IV	Cl^-	OMe	OMe	OH	OH	—	—	47 ± 5	61 ± 4	38 ± 2
23	IV	Cl^-	OH	OH	OH	OH	—	—	100	100	73 ± 4
11	V	Cl^-	OMe	OMe	$-OCH_2O-$		Me	H	13 ± 2	18 ± 2	14 ± 3
12	V	Cl^-	OMe	OMe	$-OCH_2O-$		Me	Me	0 ± 3	3 ± 2	0.3 ± 0.3
15	V	Cl^-	OMe	OMe	OH	OH	Me	H	25 ± 2	46 ± 5	15 ± 1
13	V	Cl^-	OMe	OMe	OH	OH	Me	Me	6 ± 2	16 ± 2	5 ± 2
17	V	Cl^-	OMe	OMe	H		Me	H	16 ± 3	26 ± 2	7 ± 2
18	V	Cl^-	OMe	OMe	H		Me	Me	20 ± 3	30 ± 4	7 ± 1
19	V	Cl^-	OMe	OMe	Cl	Cl	Me	H	9 ± 7	41 ± 9	5 ± 1
20	V	Cl^-	OMe	OMe			Me	H	46 ± 2	51 ± 6	29 ± 5
14	V	Cl^-	OH	OH	OH	OH	Me	Me	23 ± 1	37 ± 5	12 ± 1
16	V	Cl^-	OH	OH	OH	OH	Me	H	18	29	15 ± 3

[a] Values are expressed as % of Bodipy-GTP binding inhibition and are means of at least three independent experiments. Standard error of the mean (SEM) is given next to the ± sign.

in a significant loss of activity against all GTPases tested and that coralyne **6**, a compound with added unsaturations and a 3,4,10,11-tetramethoxy-8-methyl substitution pattern, exhibited a slightly higher inhibition than berberine ***1***. However, independently of the number of unsaturations in formula I, II and III, conversion of the methoxy or the methylenedioxy analog to tetrahydroxy substituted structures **3, 4** and **7** led to a general increase in nucleotide binding inhibition. The examination of the phenantridine analog chelerythrine and sanguinarine (**21** and **26**, formula IV) revealed that this class of alkaloids produced a much higher inhibitory activity in general when compared to the berberine series (formula I, II and III).

Compounds from the 3-aryl-isoquinoline series (formula V) were all found to be much less active than their structurally restricted analogues. Interestingly, in this series, compounds with a fused phenyl ring **20**, a 3′,4′-dihydroxy **15** or dichloro substitution on the 3-aryl moiety **19** exhibited an increased nucleotide binding inhibitory activity when compared to its 3′,4′-dimethoxy substituted 3-aryl analog **11**.

The nucleotide binding inhibition IC_{50} was calculated for the most active compounds and the inhibition of Rac GTPase activation was evaluated in Rac1b overexpressing HEK293 cells using a Rac specific antibody and the G-LISA™ assay[11] (Table 2).

For all three Rac1, Rac1b and Cdc42, the **GDP** IC_{50} values observed (2.7 ± 0.2, 1.8 ± 0.1, 1.4 ± 0.1 μM) in the nucleotide binding assay were similar for all three GTPases. However, we found significant differences in all other substances identified. The compounds displayed a general tendency to be more selective for Rac1b when compared to Rac1 and Cdc42. In particular **22, 4, 26** and **21** inhibitory activities are 2.5, 3, 13 and >14-fold higher towards Rac1b than Rac1.

In order to explore hypothesis that could explain the differences observed, we analyzed the amino acid sequences exposed to the GTP binding site in Rac1, Rac1b and Cdc42 and found a high degree of sequence homology (Fig. 2).

However, the alternative splicing that leads to the production of Rac1b is associated with a 19 AA insertion near the switch II domain. Even if the sequence variation is away from the binding site,

Table 2
IC_{50} values for nucleotide binding inhibition assay and % inhibition of GTPase activation at 50 μM

Compd	Rac1 IC_{50}[a] (μM)	Rac1b IC_{50}[a] (μM)	Cdc42 IC_{50}[a] (μM)	G-Lisa[b] (%)	*c*Log *P*[c]	*c*Log *D*[d] pH 7.4
GDP	2.7 ± 0.2	1.8 ± 0.1	1.4 ± 0.1	—	—	—
3	34 ± 5	68 ± 3	>100	<20	1.23	−0.12
4	8 ± 3	2.7 ± 0.4	6 ± 1	<20	−0.52	−0.55
5	63 ± 10	31 ± 4	>100	35 ± 2	0.49	0.48
7	38 ± 14	13 ± 4	26 ± 3	<20	2.97	0.45
15	104 ± 13	37 ± 6	>100	61 ± 6	3.58	3.34
20	58 ± 3	62 ± 18	>100	<20	5.53	5.51
21	>100	7 ± 1	>100	40 ± 10	1.86	1.86
22	54 ± 8	22 ± 8	73 ± 8	39	0.92	0.68
23	9.3 ± 0.6	8.6 ± 1.3	22 ± 2	<20	−0.09	−0.34
26	58 ± 4	4.6 ± 0.4	32 ± 1	50 ± 3	1.79	1.79

[a] Values are expressed in μM as IC_{50} of Bodipy-GTP binding inhibition and are means of at least three independent experiments.
[b] Standard error of the mean (SEM) is given next to the ± sign.
[c] Values calculated using Pallas 3.0 with Prolog *P* 6.0 Module.
[d] Values calculated using Pallas 3.0 with Prolog *D* Module at pH 7.4.

this insertion is sufficient to produce a large conformational difference in the switch I domain that moves residues 31 to 35 and generates a much larger nucleotide binding pocket in Rac1b.[12] When molecular docking experiments were performed using Molegro Virtual Docker[13] with **21** and Rac1b, we observed a binding mode that allows the ligand to bury the methylene dioxy group where the guanine group of GDP is located in the published nucleotide bond Rac1b structure[12] and exposes the dimethoxy substituents in the direction of the switch I domain (Fig. 3, Rac1b). In the binding pocket, the best pose suggests an hydrogen bonding interaction between both backbone amide hydrogens of Ala178 and Thr134 and one oxygen of the methylene dioxy group of **21**, a π–π edge face interaction between Phe28 and the phenantridine moiety and finally favorable interaction with the hydrophobic environment created by Leu179 the methylene groups of Lys135, Cys18 and Phe28 (Fig. 4). When the docking procedure was applied to **21** and Rac1[14] a totally different binding mode was observed. In this binding mode, the ligand cannot interact with the Rac1 amino acids exposed to the guanine group of GDP because the switch I domain is in a closed conformation that prohibits the ligand to access this binding pocket. Alternatively, the ligand could engage in a binding mode perpendicular to the one observed in Rac1b closer to the phosphate binding pocket (Fig. 3, Rac1). Since this binding mode does not take advantage of the deep guanine binding pocket, it is possible that this leads to an energetically unfavored state that confers the selectivity observed for **21**.

Using the cellular GTPase activation assay, we found that compounds **5**, **15**, **21** and **26** inhibit Rac activation (35–61%). However compounds **3**, **4**, **7**, **20** and **23** that are active in the nucleotide binding assay were found inactive in the cellular assay. Since cellular penetration is an additional requirement to inhibit Rac activation, it is expected that compounds with a poor cellular penetration profile are found inactive in a cellular assay. To further support this hypothesis, a *c*Log *P* and *c*Log *D* analysis revealed that compounds **3**, **4**, and **23** that are inactive in the cellular assay also exhibit negative *c*Log *D* values indicating a strong tendency to for these molecules to partition preferentially in water (Table 2). However, the *c*Log *D* data can't provide a rationale for compound **7** and **20** since they were both found to have positive *c*Log *D* (0.45 and 5.53).

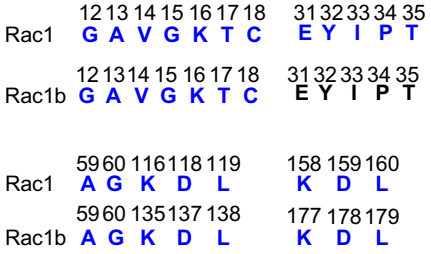


Figure 2. Residues in blue are located 5 Å away from the nucleotide in the Rac1[14] and Rac1b[12] X-ray co-crystal structure.

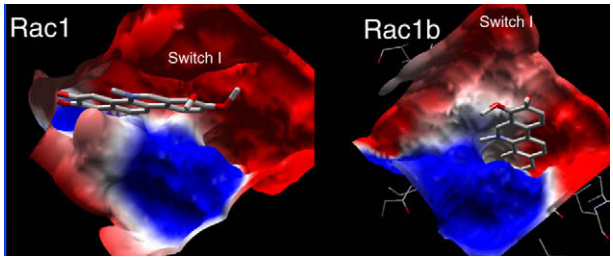


Figure 3. Docking results obtained with chelerytrine **21** in Rac1 and Rac1b using Molegro Virtual Docker[13] represented using the electrostatic protein surface in the binding site. The surfaces of Rac1 and Rac1b are colored by electrostatic potential with red and blue for negative and positive potential, respectively.

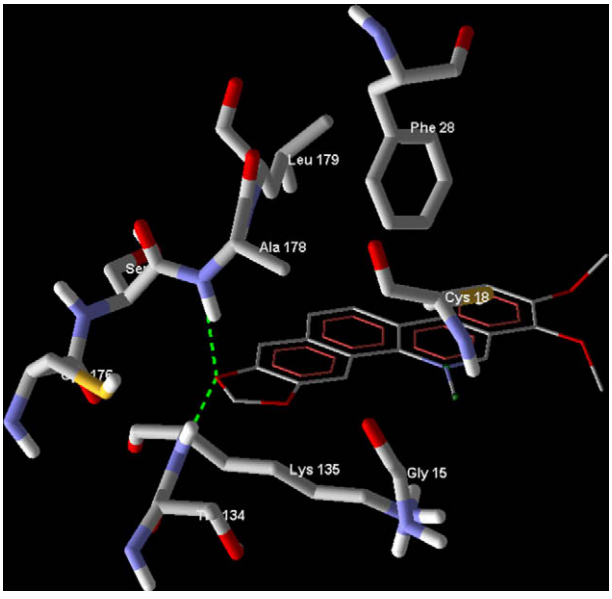


Figure 4. Orientation of the first ranked pose of **21** in Rac1b using MolegroVirtual Docker[13] (displayed following the atom type color codes and thin stick). For the sake of clarity, only a few amino acid residues of Rac1b GTP binding site are represented with the hydrogen bonds highlighted with a green dashed line.

Nevertheless, we can't exclude that it is possible that in some case the ligand binding maintain the GTPase in an active conformation.

The results obtained demonstrate that the isoquinoline **15** and phenantridine derivatives **21**, **22**, **26** are competitive inhibitors of Bodipy-GTP nucleotide binding, inhibit Rac activation in a cellular model and show selective inhibition of Rac1b over Rac1 and Cdc42.

Although compound **26** and **21** were shown to interact with other proteins,[15] Protein Kinase C[16] and oligonucleotide targets,[17] this study provides important structural features required for the development of isoform selective Rac1b nucleotide binding inhibitors.

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10. *Guanine nucleotide competition assay*: bacterially-expressed GST-Rac1, His6-Tiam1 (DH/PH domains), GST-Rac1b and GST-Cdc42 were prepared in 50 mM Tris pH 8.6, 50 mM NaCl, 1 mM $MgCl_2$ and 10% glycerol and cDNAs were kindly provided by Dr. Channing J. Der. The guanine competition assay was monitored as the increase in relative fluorescence of a fluorescent GTP analog, the BODIPY-GTP (BODIPY FL GTP; invitrogen), upon binding. The guanine nucleotide exchange factor Tiam1 was used to stimulate the binding of the nucleotide to Rac1, whereas no GEF was needed for Rac1b and Cdc42 (intrinsic activity). In our experiments, 2 μM of Rac1b, Cdc42 or Rac1 plus 1.3 μM of Tiam1 were incubated in buffer containing 20 mM Tris pH 8.6, 50 mM NaCl and 1 mM $MgCl_2$ for 10 min at 20 °C. After equilibrium, 2 μM BODIPY-GTP with or without 50 μM of compound to be tested were added. Fluorescence was measured using a Fluoroscan (Thermo Scientific; λ_{exc} 485 nm, λ_{em} 538 nm) before and after the addition of the fluorescent nucleotide and compound. Maximal RFU value obtained at the end of the recording (40 min after BODIPY-GTP addition) was used for comparison with appropriate control.
11. Rac1b activation assay: HEK293 cells overexpressing human Rac1b were plated in 100 mm diameter dishes and grown for 5 days in MEM medium supplemented with 10% FBS, L-glutamine and antibiotics. Cells were then treated with 50 μM of compounds or the solvent for 5 minutes, and the GTP loading of Rac1b was measured using a Rac Activation Assay Kit (Cytoskeleton) according to manufacturer's recommendations.
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