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CONCISE ARTICLE

3-aryl-*N*-aminoylsulfonylphenyl-*1H*-pyrazole-5-carboxamides: a new class of selective Rac inhibitors

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Through a computational approach, five new compounds with potent and selective Rac inhibitory activity were identified. In particular, compound 4 was shown to selectively inhibit Rac activity in a concentration-dependent manner by affecting the GEF-dependent GDP-GTP exchange. This compound was more potent than the original inhibitors previously identified.

Introduction

In the last decade, the small GTPase protein Rac has gained increasing attention for its role on cardiovascular diseases. Transgenic mice expressing a constitutively activated Rac1 mutant in the myocardium developed either a lethal cardiac dilated phenotype or a transient cardiac hypertrophy that resolved with age.[1] In line with this evidence, cardiomyocyte knock-out specific mice for Rac1 are resistant to cardiac hypertrophy induced by angiotensin infusion.[2] The role of Rac on cardiac function has been associated with its regulatory function on the NADPH activity and thus generation of reactive oxygen species.[2] Moreover, endothelial Rac1 haploinsufficient mice showed decreased expression and activity of eNOS which correlated with the decrease in endothelium-dependent vasorelaxation, and caused mild hypertension.[3] This evidence suggests a protective role of Rac on endothelial cells and more in general on vascular diseases. Various agonists and extracellular matrix appears to be required for a proper Rac1-mediated modulation of endothelial barrier property, through the involvement of reactive oxygen species and actin cytoskeleton.[4-6] However, Rac directly regulates many other cellular events associated with the development of atherosclerotic plaque, including smooth muscle cell (SMC) migration[7] and proliferation,[8] and leukocyte-endothelial cell interaction.[9] Thus, the final outcome of the role of Rac on cardiovascular diseases is still controversial. Nevertheless, pathway-based genome-wide association analysis of coronary heart disease (CHD) has identified Rac1 as one of the biologically important gene in CHD, thus indicating that Rac represents a possible new pharmacological target for cardiovascular diseases.[10]

In 2004 it was described a new approach for the identification of selective Rac inhibitors that specifically interfere with the protein-protein interaction between Rac and the guanine nucleotide exchange factor (GEF).[11] More recently, we took advantage from this evidence to identify new Rac inhibitors by a pharmacophore virtual screening approach followed by molecular docking calculations.[12] These new inhibitors were shown to be selective on Rac, without affecting RhoA and Cdc42 activities, and to interfere with the binding between the GEF Tiam1 and its substrate Rac, thus blocking the exchange between GDP and GTP and Rac activation.[12] In particular, the two *N*-(sulfamoylaryl)arylamide derivatives **1** and **2** (Fig. 1) resulted the most potent. In the present study, to explore the potentiality of these hits, a selection of commercially available *N*-(sulfamoylaryl)arylamides variously substituted at the two regions shown in Fig. 2, was identified by virtual screening and tested for their Rac inhibitory potency.

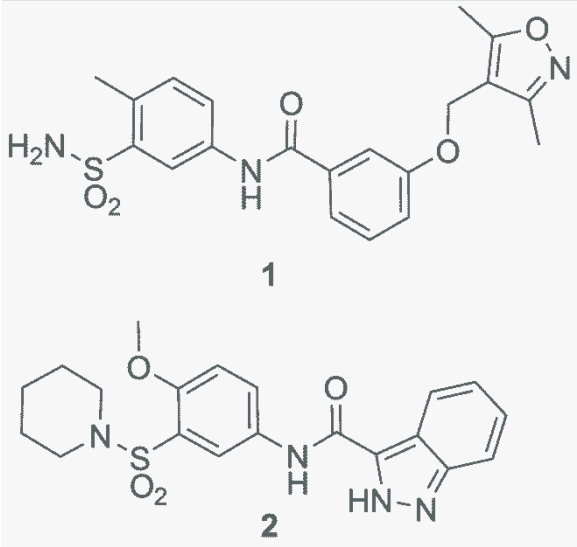


Fig. 1 Previously reported hit compounds **1** and **2**.

Results and Discussion

Virtual screening. Starting from the hit compounds **1** and **2** (Fig. 1),[12] a similarity search was performed directly on the ZINC DB website.[13] The clustering and search algorithms implemented in ZINC are based on a search engine provided by ChemAxon were both the query and molecules in the DB are represented by fingerprints, and the similarity degree is expressed as a Tanimoto coefficient (TC).

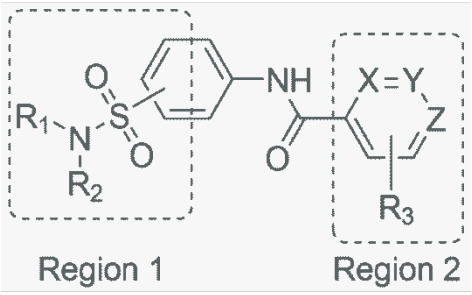


Fig. 2 Selected regions of interest for chemical modification.

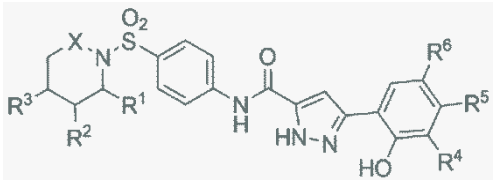
All the molecules with a TC value of ≥ 0.85 with respect to either compounds **1** (53 derivatives) and **2** (117 derivatives) were selected for molecular docking evaluation. As the receptor, the previously derived 3D model[12] was used and a consensus docking strategy using two different software, MOE[14] and Autodock4 (AD4),[15] and a total of three scoring functions (London dG and Affinity, implemented in MOE, and the default AD4 scoring function) was adopted as it proved to be a rather successful screening strategy.[12] Energies obtained by MOE and AD4 virtual screenings were compared and only those compounds for which the computed docking energies resulted above defined thresholds (affinity < -4.5, London dG < -7.0, AD4 binding energy < -6.5 kcal/mol) were selected. Within this selection, a total of 57 commercially available *N*-(sulfamoylaryl)arylamides with differences in regions 1 and 2 (Fig. 2) were selected and acquired for biochemical testing.

Identification of new selective Rac inhibitors by in vitro and cell-based assays. The pharmacological inhibition of the identified compounds on Rac activity was studied in cultured human SMCs by determining the amount of Rac-GTP by G-LISA assay.[12]

To induce Rac activation, human SMCs were starved for 48 h in DMEM containing 0.4 % of FCS and then stimulated for 2 min with 20 ng/mL platelet derived growth factor (PDGF)-BB. The effect of tested compounds was assessed by a 4 h pre-incubation of human SMC before stimulation. In a first series of G-LISA assays, a final non-toxic concentration of the 57 selected compounds was utilized, (between 10 and 200 μM) as determined by MTT cytotoxicity assay (data not shown). Among the 57 tested compounds, 23 efficiently reduced the intracellular content of Rac-GTP from a minimum of 24.8 % to a maximum of 63.7 % (Table S1, ESI). Importantly, five compounds were shown to be more effective than the reference compound **1** and fifteen were more effective than compound **2**, indicating a potential improvement of the pharmacological inhibition of Rac by the newly identified chemical entities.

A second series of analysis was performed at 25 μM concentration in order to select the most potent compounds. Compounds **3**, **4**, **5**, **11** and **21** showed the most potent inhibitory action on Rac protein and consistently reduced, by more than 50%, the intracellular amount of Rac-GTP (Table 1 and Table S2, ESI). A dose dependent effect on Rac activity showed that compounds **5** and **4** have the lowest IC_{50} values, 4.4 μM and 8.7 μM, respectively (Table 2, Fig. S1, ESI).

Table 1 Effect of selected compounds on Rac1 GTP levels.



cmpd	R^1	R^2	R^3	R^4	R^5	R^6	X	% inhibition at 25 μM
21	H	CH_3	H	CH_3	CH_3	H	$-CH_2-$	68.3±6.3 ***
3	H	CH_3	H	H	H	H	$-CH_2-$	67.4±1.5 ***
11	H	CH_3	H	H	CH_3	H	$-CH_2-$	65.3±9.0 ***
4	H	H	H	H	H	H	$-CH_2-$	61.1±3.0 ***
5	H	H	H	CH_3	CH_3	H	$-CH_2-$	59.3±1.2***
6	H	H	CH_3	H	H	H	$-CH_2-$	51.9±3.1**
10	H	H	CH_3	CH_3	CH_3	H	$-CH_2-$	46.5±17.7 ^{NS}
20	H	H	CH_3	CH_3	H	CH_3	$-CH_2-$	45.5±24.2 *
14	H	H	H	CH_3	CH_3	H	$-CH_2-CH_2-$	40.8±21.4*
16	H	H	H	H	CH_3	H	$-CH_2-CH_2-$	26.1±3.8 ***
24	H	H	CH_3	H	CH_3	CH_3	$-CH_2-$	21.9±0.2 ^{NS}
15	H	H	H	H	H	H	$-CH_2-CH_2-$	NE

NE: Not effective; NS: Not significant

*p<0.05; **p<0.01; ***p<0.001 Student's T-test.

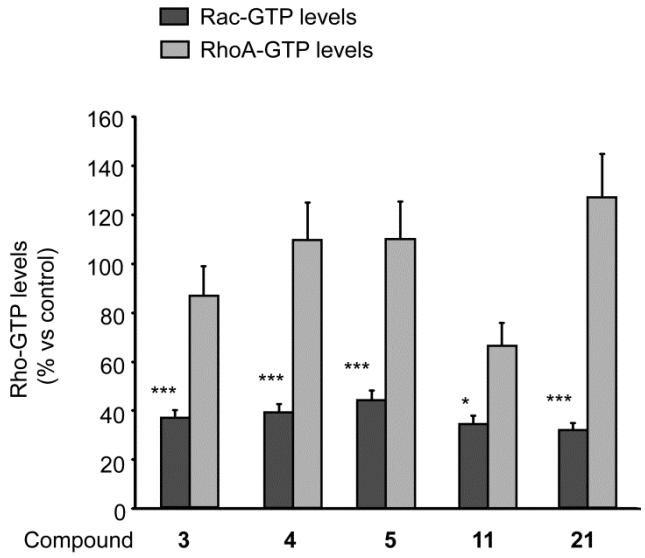


Fig. 3 Determination of the selectivity of the new Rac inhibitors. Cells were seeded at a density of 2 x 10^5/35 mm petri dish and incubated with DMEM supplemented with 10% FCS; 24h later the medium was changed to one containing 0.4% FCS, and the cultures were incubated for 48 h. At this time, the compounds were added to the cultured media at the final concentration of 25 μM and after 4 h Rac and RhoA activation was induced by PDGF-BB (10 ng/ml) for 2 min. Total protein extracts and G-LISA assays were then performed.

Table 2 Effect of selected compounds on Rac1-GTP levels and Cell Adhesion with calculated IC_{50} values.

Cmpd	Rac1-GTP levels IC_{50} (μM)	Cell adhesion IC_{50} (μM)
3	16.4±2.1	5.3±0.9
4	8.7±2.4	8.7±2.7
5	4.4±3.1	21.3±1.8
11	19.2±2.8	27.9±2.4
21	29.1±5.1	24.2±4.2

In order to determine the selectivity of action of the tested compounds on Rac, the G-LISA assay specific for RhoA was performed. As shown in Fig. 3, compounds **3**, **4**, **5**, and **21** strongly reduced the intracellular levels of Rac-GTP without significantly affecting the activity of RhoA, while compound **11** appears to be less Rac-selective. It should be noted that compound **3** has been tested as either a racemate or single enantiomers, but analogue results were obtained probably due to the scarce steric hindrance of the methyl group at position 3 of the piperidine ring (data not shown).

Time-dependent effect of compound **4** clearly demonstrated that that its inhibitory effect on Rac is selective and very rapid with a significant reduction of Rac-GTP after 1 h of incubation (Fig. S2, ESI).

To further investigate the basic molecular mechanism of Rac inhibition, we examined the effect of compound **4** on the exchange activity of Tiam 1 and TrioN, Rac-specific GEFs [16, 17] and Vav2, a GEF active on Rac1, RhoA, and Cdc42.[18] The treatment with compound **4** strongly reduced the Rac1-GTP levels induced by SMCs expressing either Tiam1, TrioN, or Vav2. These results indicate that compound **4** interferes with the GEF-mediated Rac activation (Fig. S3, ESI).

To delineate the anti-Rac activity of the selected compounds in cultured cells, the effect on Rac1-dependent cell adhesion was evaluated. Rac activity has been previously shown to be required for cell adhesion,[19] and its downregulation by siRNA directed to Rac1 strongly reduced the capability of cells to adhere to the petri dish (Fig. S4, ESI). The effect of the six selected new Rac inhibitors on a cell adhesion was then determined. SMCs were incubated for 4 h with increasing concentrations of compounds (10-50 μM), harvested by trypsinization, and then cell adhesion was determined. All the compounds were capable to significantly affect cell adhesion in a concentration dependent manner (Table 2, Fig. S5, ESI). In particular, compounds **4**, **11** and **21** showed very similar IC_{50} values on the cell adhesion and the inhibitory action on Rac, demonstrating the effective inhibition of a Rac-mediated cellular event by these compounds (Table 2). Finally, compound **4** was shown to inhibit lamellipodia formation in human SMCs induced by PDGF-BB (Fig. S6, ESI), a cellular response strikingly related to Rac1 activity.[20]

Structure activity relationships and binding mode analysis. Concerning Structure Activity Relationships, the 3,5-substituted pyrazole moiety generally wield a more potent activity compared to the phenyl moiety, especially if combined with an 2-hydroxyphenyl substituent.

Taking as a reference the compound **4** as the lesser substituted and one of the more potent molecules, we can see that the modification of the sulfon-piperidinamide moiety with the sulfon-azepanamide analog causes a total loss of activity, like in compound **15**, that can be partially restored by adding one or two methyl substituent on the phenol ring like in compound **16** and compound **14**. The rationale behind the phenol substitution is not trivial, in fact, compound **23** is inactive. By taking again compound **4** as a reference, the methylation of the piperidine ring substantially change the molecule activity. Indeed, modification at position 4 lead to a significant decrease in activity, as observed for compound **6**, compound **10**, compound **20** and compound **24**. The substitution of the pyrazole moiety with its benzocondensate (the previously reported compound **2**)[12] also leads to lower efficacy. The methylation at position 3 of the piperidine ring cause instead an increment in activity, as observed for compound **1**, and, in this case, the methylation of the phenol ring leads to the most effective molecule compound **21**.

A binding mode prediction for the most active compounds **3**, **4**, **5**, **11** and **21** has been obtained through the QM Polarized Ligand Docking workflow, present in the Schrodinger 2011 software suite,[21] followed by force field minimization of each pose, maintaining the receptor fixed, performed with the MOE software.

The best poses, selected after visual inspection, are generally recurrent for the same chemical family of compound (Fig. 4). The post-docking energy minimization using the MMFF94x force field reduces the diversity of the poses but only in few cases the RMSD between the minimized and non-minimized pose exceeds 2 Å (the 8% of the total poses), indicating that the QM polarized docking protocol is already efficient on his own.

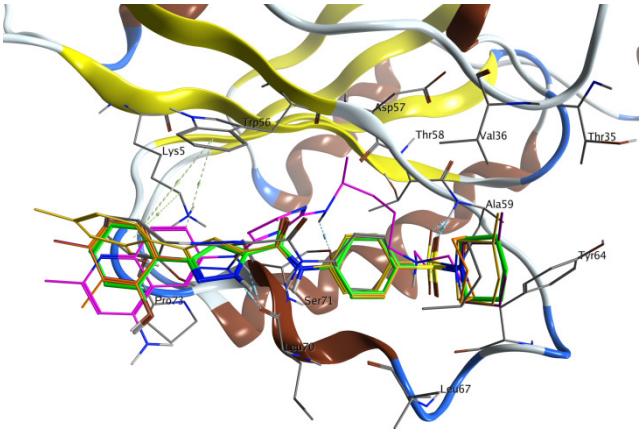


Fig. 4 Proposed binding mode for compounds **3**, **4**, **5**, **11** and **21** (carbon colored in grey, green, orange, ochra and purple, respectively). Compound NSC23766 (carbon colored in magenta), whose binding mode has been resolved by X-ray crystallography, is also reported as a reference.

Table 3. Most relevant binding contacts for compounds **3-5**, **11** and **21**

		ligand-receptor interactions
cmpd	residue	type of interaction
3	Lys5	CH/π (3-aryl···H_2CNH_3)
	Ala59	H-bond (SO_2···HN)
4	Trp56	CH/π (3-aryl C6-H···indole)
	Ala59	H-bond (SO_2···HN)
5	Trp56	CH/π (3-aryl C6-H···indole)
	Ala59	H-bond (SO_2···HN)
11	Ala59	H-bond (SO_2···HN)
	Leu70	H-bond (pyrazole 1H···O=C)
21	Trp56	CH/π (3-aryl C6-H···indole)
	Ala59	H-bond (SO_2···HN)

Basing on the obtained poses, we can assume that the sulfonamide Rac inhibitors, herein reported, present an analogue binding mode (Fig. 4), which can be compared to the one described for the reference compound NSC23766.[11, 22] As reported in Table 3 and graphically depicted in Fig. S7, ESI, the important interaction with TRP56 is maintained for compounds **4**, **5** and **21** by the aryl group trough a CH/π bond, while the aryl group of compound **3** interacts with the Lys5 side chain, which itself interacts with the indole moiety of Trp56. An additional interaction, peculiar for the class of compounds herein reported, is established within the SO_2 moiety and Ala59 NH. For compound **11**, an H-bond can also be observed between the pyrazole NH and the Leu70 carbonyl group..Hydrophobic interactions between the piperidine group and the pocket formed by Val36, Ala59, Tyr64 and Leu67 (although not reported in Table 3) could also be observed for all compound trough a visual inspection of the complexes.

Conclusions

Starting from previously identified hits,[12] by using a virtual screening approach we identified a series of 3-aryl-*N*-aminoylsulfonylphenyl-*1H*-pyrazole-*5*-carboxamides as potent and selective Rac inhibitors.

In particular, compounds **4** and **5** selectively inhibited Rac activity with IC_{50} values lower than the previously reported compounds **1** and **2** (8.7 μM and 4.4 μM compared to 12.2 μM and 24.1 μM, respectively).[12] The effect of the new compounds was then studied on cell adhesion, a cell-based assay directly linked to Rac activity. These set of experiments were considered more indicative of the effect of the new compounds on Rac functionality than the determination of the intracellular levels of Rac-GTP levels. The results of this analysis confirmed that all the compounds inhibited the cell adhesion with IC_{50} values similar to those observed by G-LISA assay. This observation demonstrated a direct relationship between the levels of Rac-GTP and its action on cell adhesion. In particular, compound **4** was the second most potent compound and inhibited the cell adhesion with an IC_{50} of 8.7 μM equal to that observed by G-LISA assay. Compound **4** completely inhibited lamellipodia formation in response to PDGF, a Rac1-dependent cellular response, further confirming its inhibitory action on Rac functionality.

Other small molecules with Rac1 inhibitory activity has been described after the firstly discovered compound NSC23766, with some differences in their mechanisms of action. Compounds **1** and **2** appear to act, similarly to NSC23766, by interfering with the Rac1-Tiam1 interaction. The same effect was shown for NSC23766 which also interferes with the binding Rac1-TrioN, while the effect on Vav2, another GEF capable to activates Rac, was not assessed.[11] Compound **4** described in the present report, was shown to affect the Rac activity induced by three different GEFs, Tiam1, TrioN, or Vav2, and thus with a similar mechanism of action as demonstrated for compounds **1**, **2** and NSC23766. A more specific inhibitory action was then observed for the compound ITX3 which interferes with Trio without affecting the activation of Rac and RhoG by both Tiam1 and Vav2.[23] Differently, compound EHop-016, another previously described potent Rac inhibitor, affects Vav2-mediated GDP-GTP exchange of Rac and shows a ten-fold lower affinity for Tiam1 and probably no effect on Trio.[24] While Vav2 is a GEF active on Rac1, RhoA, and Cdc42, compound EHop-016 was shown to interfere only with Rac-Vav2 interaction, a selective effect potentially responsible for the observed increased of RhoA activation in cancer cells for the higher availability of Vav2.[24] "Interestingly, compound **4** has shown a trend of induction of RhoA activity, although not statistically significant, suggesting a similar compensatory effect than that observed for compound EHop-016." A third chemical entity, compound EHT 1864, has been discovered to selectively inhibit Rac without interfering with the binding between Rac and GEF.[25] Although the mechanism of action has not yet been resolved, form the obtained data it has been suggested a possible allosteric mechanism determining a loss of bind with nucleotide.

In conclusion, through a computational approach we identified a new class of Rac inhibitors that potently and selectively reduced the intracellular levels of Rac GTP and its activity, demonstrated by effects on the lamellipodia formation and cell adhesion. These effects appear to be related to the inhibition of GEF-mediated GTP-GDP exchange on Rac, since compound **4** affected Rac activation induced by either Tiam1, TrioN or Vav2, implying a selective interference of Rac1-GEF interaction similar to that

described for compound **1** and **2** and NSC23766. This compound appears to be suitable for investigating its effect on atherosclerotic plaque formation in in-vivo experimental models.

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Notes and references

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