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

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Experimental Section

Computational methods.

Virtual screening. The consensus strategy, using two different docking software (MOE and Autodock4) used to select the tested compounds from the ZINC database[1] was previously described.[2]

Binding mode analysis. A database of the 23 selected molecules plus the previously reported compounds **1** and **2** was generated using the MOE 2010 software,[3] then it was prepared for docking using the Ligprep application of the Schrodinger Suite 2011.[4] Ligprep calculated the atomic partial charges with the MMFFs force field, generated all possible ionization states at pH 7±1 using the Epik engine, and lastly generated all possible tautomers. The so obtained database was in the end composed of 44 single molecular entity.
To perform an exhaustive docking on the above described database, the structure of Rac1 complexed with NSC23766, obtained as previously described,[2] was utilized as the receptor. The QM Polarized Ligand Docking workflow implemented in the Schrodinger software suite 2011 was then applied as follow: a potential grid for an empirical docking was initially generated using Glide, centred on the centroid of the co-crystalized ligand NSC23766 and restricted to a radius of 15 Å, with the other software options set as default. A first empirical docking using the Standard Precision (SP) settings was performed, and for each entry of the input database were generated 5 pose. Then the application Jaguar calculated the polarized partial charges of the ligands using the Accurate settings (uses the 6-31G*/LACVP* basis set, B3LYP density functional, and "Ultrafine" SCF accuracy level).[Schrodinger manual] After this the ligands with QM calculated atomic partial charges, were re-docked with the SP procedure generating a total of 10 poses per single initial entry, and then evaluated using the proprietary GlideScore. The output database consisted in a total of 440 poses that were then exported maintaining the QM charges and processed with MOE 2010. In particular, a force field minimization was performed for each pose, maintaining the receptor fixed, using the MMFF94x force field with the GB/VI solvation model and a 0.01 gradient with a maximum of 500 iteration. After this the database obtained was visually inspected and the most consistent poses selected for binding mode analysis.

Biochemical Assays.

Compounds. Compounds selected from the virtual screening procedure were acquired from either Enamine (http://www.enamine.net/) or InterBioScreen (http://www.ibscreen.com/) and were guaranteed pure at 95% by both NMR and LC-MS analyses. The purity of compounds **1-25** was also verified by HPLC analysis and resulted above 98% (stationary phase Ascentis SI Supelco. 3 µm. 4.6 x 150.0 mm; mobile phase. n-hexane/ethanol 70:30; flow rate 0.7mL/min; detection UV 254. 270 and 290 nm). Chemical compounds were dissolved in DMSO to 10 mM final concentration and stored at 4 °C.

Reagents and antibodies. DMEM, trypsin-EDTA, penicillin, streptomycin, non-essential amino acid solution, fetal calf serum (FCS), disposable culture flasks and petri dishes were purchased from Euroclone S.p.A. (Pero, Milan, Italy). Platelet derived growth factor-BB (PDGFBB) was purchased from tebu-bio s.r.l. (Magenta, Milan, Italy). For western blot analysis, the following antibodies were used: rabbit polyclonal anti-HA (Zymed Laboratories; Milan, Italy), mouse monoclonal anti-Rac1 (clone 23A8, Upstate), anti-mouse peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Lab; Cambridgeshire, UK). FITC-phalloidin was purchased from Sigma Aldrich (Milan, Italy).

Cell culture. Human SMCs (A 617 from human femoral artery) were grown in monolayers at 37 °C in a humidified atmosphere of 5% CO_2 in DMEM supplemented with 10% (v:v) fetal calf serum (FCS), 100 U/ml penicillin, 0.1 mg/ml streptomycin and non-essential amino acids.[2] The medium was changed every third day. SMCs were identified for growth behavior, morphology and using monoclonal antibody specific for α-actin, the actin isoform typical of SMC. For the experiments cells were seeded at a density of 2x105/35 mm petri dish and incubated with DMEM supplemented with 10% FCS; 24 h 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 and after 4 hours G-LISA assays or cell adhesion assay was performed.

G-LISA™ assay. The intracellular amount of Rac-GTP and RhoA-GTP was determined by using the G-LISA assay (Cytoskeleton. Inc Denver. CO. USA) according to according to manufacturer's instructions.[5]
MTT-assay. The determination of the conversion of MTT to formazan was determined by using a commercially available kit (Millipore, Billerica, MA, USA), according to the manufacturer's instructions.

Cell adhesion assay. Adhesion studies were performed as described previously.[6] Briefly, SMCs were harvested with Trypsin-EDTA after 4h incubation with increasing concentrations of tested compounds, centrifuged and resuspended in DMEM with 0.4% FCS and plated into 96 well/plates at 100,000 cells/well. Plates were incubated for 15 min at 37 °C in the incubator, and the assay was terminated by rinsing the plates with PBS and fixing the cells with 4% paraformaldehyde. Cells were stained with 0.5% toluidine blue for 1 min and rinsed in water. They were then solubilized by the addition of 1% SDS and quantitated in a microtiter plate reader at 595 nm.

Western blot analysis. Cells were lysed with a solution of 50 mM Tris pH 7.5, 150 mM NaCl, 0.5% Nonidet-P40, containing a protease and phosphatase inhibitor cocktails (Sigma Aldrich, Milan, Italy). Equal amount of total protein per sample were separated by SDS-PAGE under reducing conditions, transferred to Immobilon PVDF (GE Healthcare Little Chalfont, Buckinghamshire, UK) and subsequently immunoblotted with antibody anti Rac1 (Clone 23A8, Millipore) following appropriate secondary antibody, prior to visualization by enhanced chemiluminescence (LiteAblot Extend Long Lasting Chemiluminescent Substrate, EuroClone) [7].

Transfection of siRNA. The siRNAs directed to Rac1 was from Ambion life technologiesTM (Silencer Select Pre-designed siRNAs).[8] Transfections were performed using siLentFectTM Lipid Reagent. (BIO-RAD laboratories, Hercules, CA, USA) according to the manufacturer's protocol.[9] SMCs were seeded at a density of 2×10^5/Petri dish (35 mm) the day before the transfection in 10% FCS DMEM.

Cells were then transfected with 20 nM of siRNA for 24 h then the medium replaced with DMEM containing 0.4% FCS for an additional 24 h before performing the adhesion assay assay.

Transfection of plasmid encoding GEFs. Tiam1 vector was kindly provided by J. Collard (Cancer Institute, Amsterdam, The Netherlands); TrioN vector was from P. Fort (Centre de Recherche de Biochimie Macromoléculaire, Universités Montpellier I et II, France) and Vav2 vector from X. Bustelo (Centro de Investigación del Cáncer-Cancer Research Center, CSIC-University of Salamanca, Spain). Transfections were performed using jetPEI^{TM} transfection reagent (Polypus-transfection SA, Illkirch, France) according to the manufacturer's protocol. SMCs were seeded at a density of 2×10^5/Petri dish (35 mm) the day before the transfection in 10% FCS DMEM. Cells were then transfected for 24 h then the medium replaced with DMEM containing 0.4% FCS for an additional 24 h. Cells were then incubated with compound **2** and Rac-GTP levels determined by G-LISA assay after 4 h.

Cytoskeleton staining. Cells were fixed in 4% paraformaldehyde at room temperature for 10 minutes, permeabilized in 0.1% Triton X-100 in PBS for 5 minutes, and incubated with FITC-phalloidin (Sigma Aldrich, Milan, Italy). Cells were then washed four times with PBS and coverslipped with Mowiol mounting media. Cytoskeleton staining of cells was analyzed by fluorescent microscope (Axiovert M220 Zeiss Instr., Oberkochen, Germany).[10]

Statistical analysis. All data shown are representative of at least three replicate experiments. Data are expressed as mean ± SD. Statistical analyses were performed using the unpaired Student's T test. P values <0.05 were considered significant. The concentration of compounds required to inhibit 50% of Rac-GTP intracellular levels (IC_{50}) was calculated by nonlinear regression curve (SigmaPlot software; Systat Software, Inc., Point Richmond,CA).

Table S1. Effect of selected compounds on Rac-GTP levels in human SMCs.

Vendor code	Cmpd	% inhibition	Concentration (µM)
T6187337	**1**	37.0	50
T6187339	**2**	45.5	50
STOCK5S-48446	**3**	63.7	25
STOCK5S-26339	**4**	63.1	25
STOCK5S-37744	**5**	55.6	5
STOCK5S-58152	**6**	52.3	50
T5777243	**7**	48.9	100
T5801231	**8**	44.5	25
STOCK5S-52086	**9**	44.0	100
STOCK5S-31786	**10**	42.4	25
STOCK5S-50919	**11**	41.9	200
T5398886	**12**	40.9	25
T5699113	**13**	39.2	50
STOCK5S-48608	**14**	38.8	25
STOCK5S-37597	**15**	38.4	25
STOCK5S-28085	**16**	37.4	25
STOCK5S-28850	**17**	37.1	200
Z230622818	**18**	32.5	100
T5548728	**19**	31.6	50
STOCK5S-46255	**20**	30.0	100
STOCK5S-51425	**21**	28.9	10
T5811511	**22**	28.6	200
STOCK5S-26482	**23**	27.6	50
STOCK5S-51349	**24**	26.1	10
STOCK5S-53628	**25**	24.8	10
STOCK5S-40823	**26**	24.0	100
T5655077	**27**	23.3	200
STOCK5S-59917	**28**	22.6	10
T5363791	**29**	22.5	50
T5229659	**30**	22.3	100
STOCK5S-53854	**31**	22.1	10
T5221363	**32**	21.4	200
T5541947	**33**	20.8	50
T5754748	**34**	20.6	100
Z248171216	**35**	20.4	50
Z247643606	**36**	20.0	100
T5699958	**37**	19.9	10
T5248801	**38**	18.1	50
T5405075	**39**	14.8	50
Z278446374	**40**	14.6	100
T5635153	**41**	12.9	25
Z279036138	**42**	11.8	50
STOCK5S-29378	**43**	11.1	10
Z279178018	**44**	10.8	10
T5777322	**45**	10.6	50
Z100617904	**46**	9.8	50
Z236003274	**47**	9.0	50
T5655074	**48**	8.5	100
T5544199	**49**	8.1	50
Z236003280	**50**	NE	10
STOCK5S-41183	**51**	NE	10
STOCK5S-33198	**52**	NE	10
T5573888	**53**	NE	100
STOCK5S-51704	**54**	NE	10
STOCK5S-28957	**55**	NE	100
STOCK5S-04006	**56**	NE	25
STOCK5S-37705	**57**	NE	50
STOCK5S-38792	**58**	NE	10
STOCK5S-56191	**59**	NE	10

NE: Non-effective.

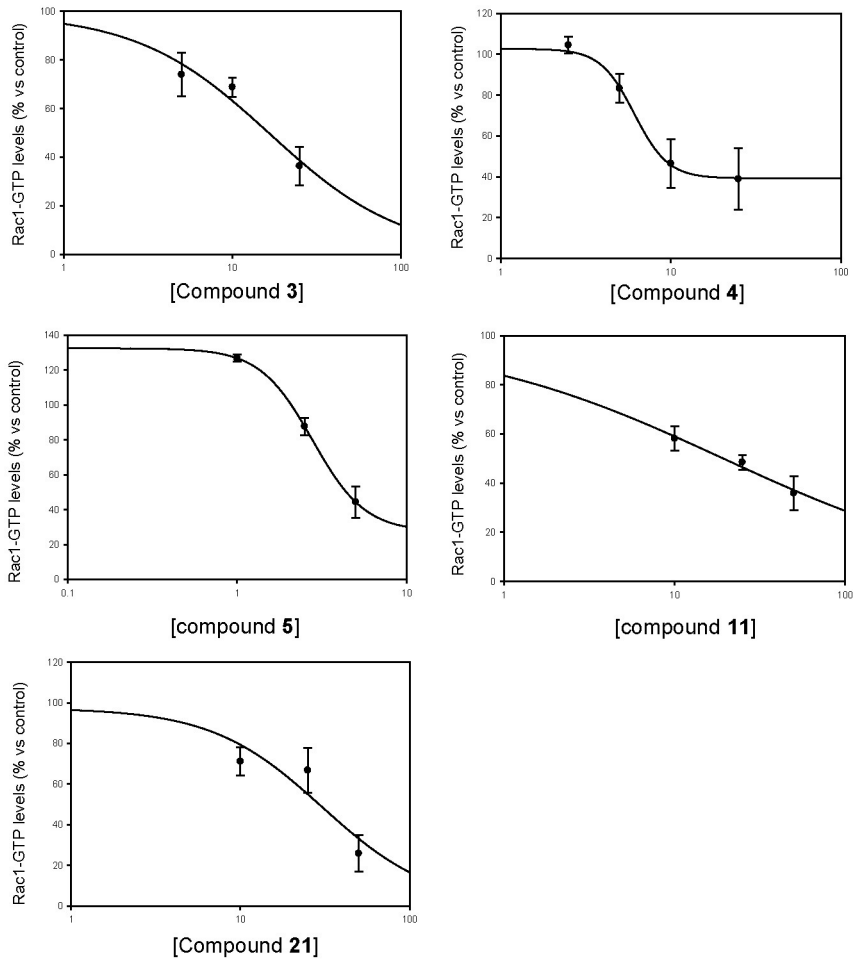

Figure S1. Dose-dependent Rac inhibitory effect of the new Rac inhibitors.
Cells were seeded at a density of $2x10^5$/35 mm petri dish and incubated with DMEM supplemented with 10% FCS; 24h later the medium was changed to one containing 0.4% FCS, and the cultures were incubated for 48h. At this time, the compounds were added to the cultured media at increasing concentrations and after 4h Rac activation was induced by PDGF-BB (10ng/ml) for 2min. Total protein extracts and G-LISA assays was then performed.

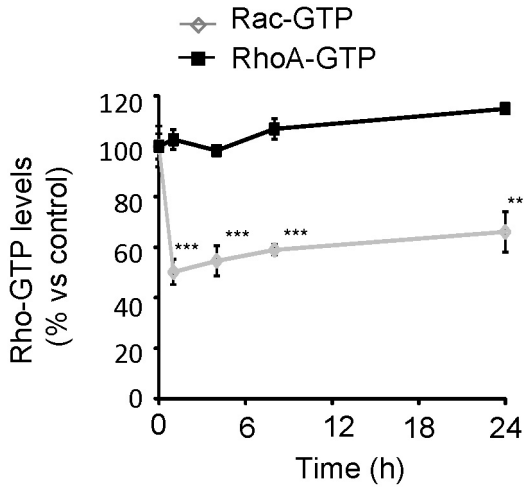

Figure S2. Time-dependent effect of compound **4** on Rac and RhoA activity.
Cells were seeded at a density of $2x10^5$/35 mm petri dish and incubated with DMEM supplemented with 10% FCS; 24h later the medium was changed to one containing 0.4% FCS, and the cultures were incubated for 48h. At this time, compound **4** (25 µM) was added to the cultured media. Total cell lysates were then prepared after 0 (control) 1, 4, 8 and 24 h and the levels of GTP-bound forms of Rac and RhoA determined by G-LISA assays. ***p<0.001; **p<0.01 vs Control

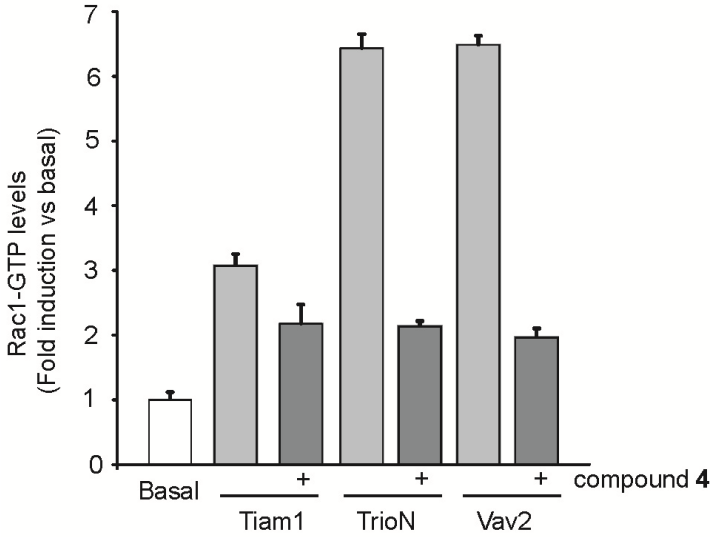

Figure S3. Effect of compound **4** on Rac1 activity induced by Tiam1, TrioN or Vav2. SMCs were transiently transfected with plasmids encoding for either Tiam1, TrioN or Vav2 and incubated for 4h with compound **42** (25µM) in DMEM containing 0.4% FCS. At the end of the incubation Rac1-GTP levels were determined by G-LISA assay.

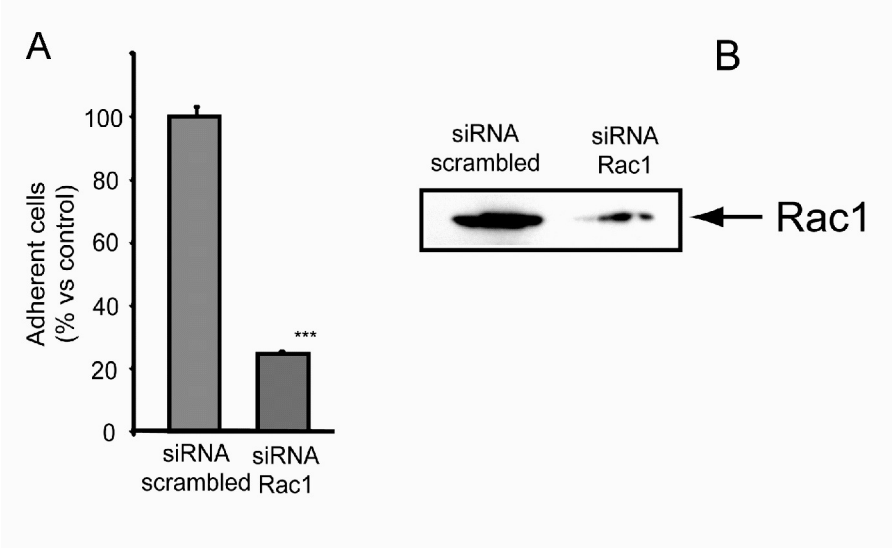

Figure S4. Role of Rac1 on SMC adhesion.
A and B) Cells were seeded at a density of $2x10^5$/35 mm petri dish and incubated with DMEM supplemented with 10% FCS; 24 h later the cells were transfected with siRNA against Rac1 or nonsilencing control siRNA scrambled. A) After 24h SMCs were starved with medium containing 0.4% FCS for an additional 24h. At this time, the cells were harvested with Trypsin-EDTA and cell adhesion on cultured petri dish evaluated. B) A parallel set of petri dishes were utilized for the determination of Rac1 expression by western blot analysis with a monoclonal antibody anti Rac1 (Clone 23A8, Millipore).

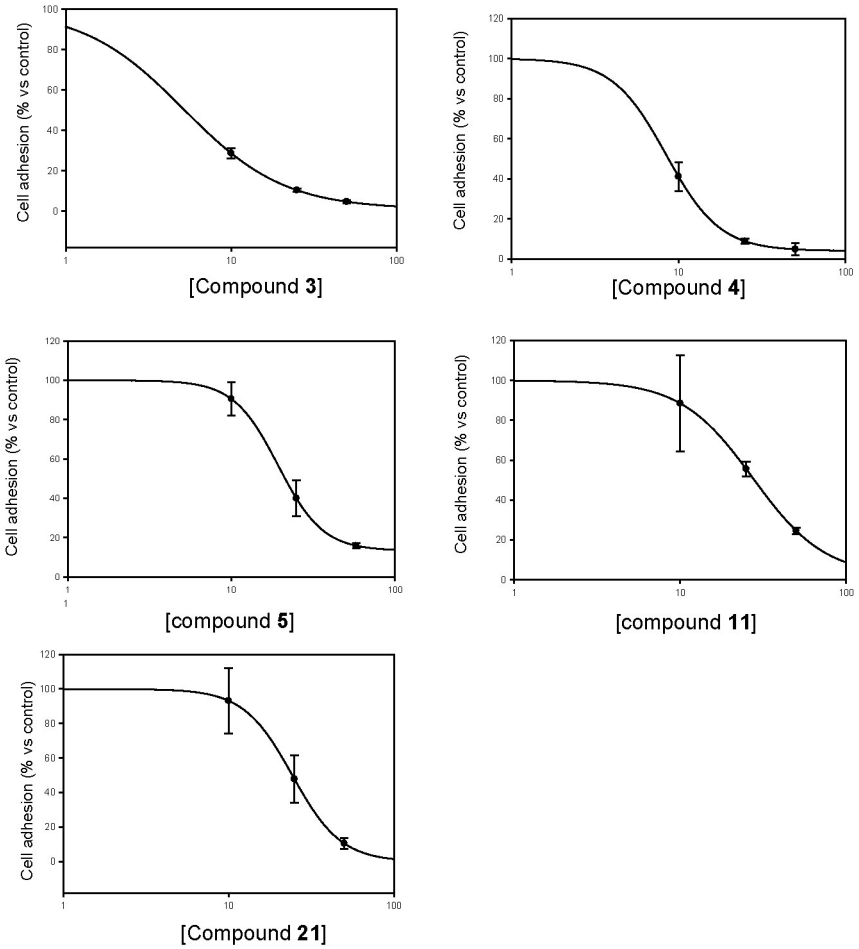

Figure S5. Effect of compound **3**, **4**, **5**, **11** and **21** on cell adhesion.
Cells were seeded at a density of $2x10^5$/35 mm petri dish and incubated with DMEM supplemented with 10% FCS; 24h later the medium was changed to one containing 0.4% FCS, and the cultures were incubated for 48h. At this time, the compounds were added at indicated final concentrations to the cultured media and after 4h harvested with Trypsin-EDTA and cell adhesion on cultured petri dish evaluated.

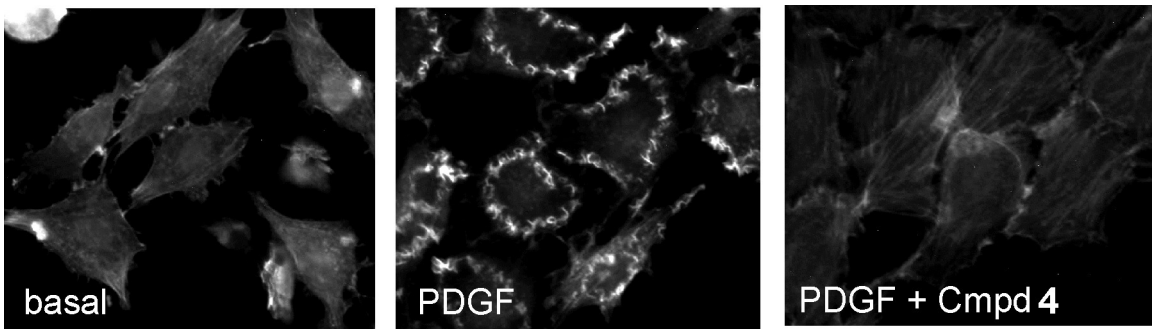

Figure S6. Effect of compound **4** on lamellipodia formation in human SMCs.
Cells were seeded at a density of $2x10^4/cm^2$ in a 4 well tray incubated with DMEM supplemented with 10% FCS; 24h later the medium was changed to one containing 0.4% FCS, and the cultures were incubated for 48h. At this time, the compound **4** was added to the cultured media at the final concentration of 10μM and after 4h SMCs were stimulated by adding PDGF-BB (10 ng/ml) for 10min. Cells were then fixed in 4% paraformaldehyde and labeled with FITC-phalloidin (Sigma Aldrich, Milan, Italy). Cytoskeleton staining of cells was analyzed by fluorescent microscope (Axiovert M220 Zeiss Instr., Oberkochen, Germany).

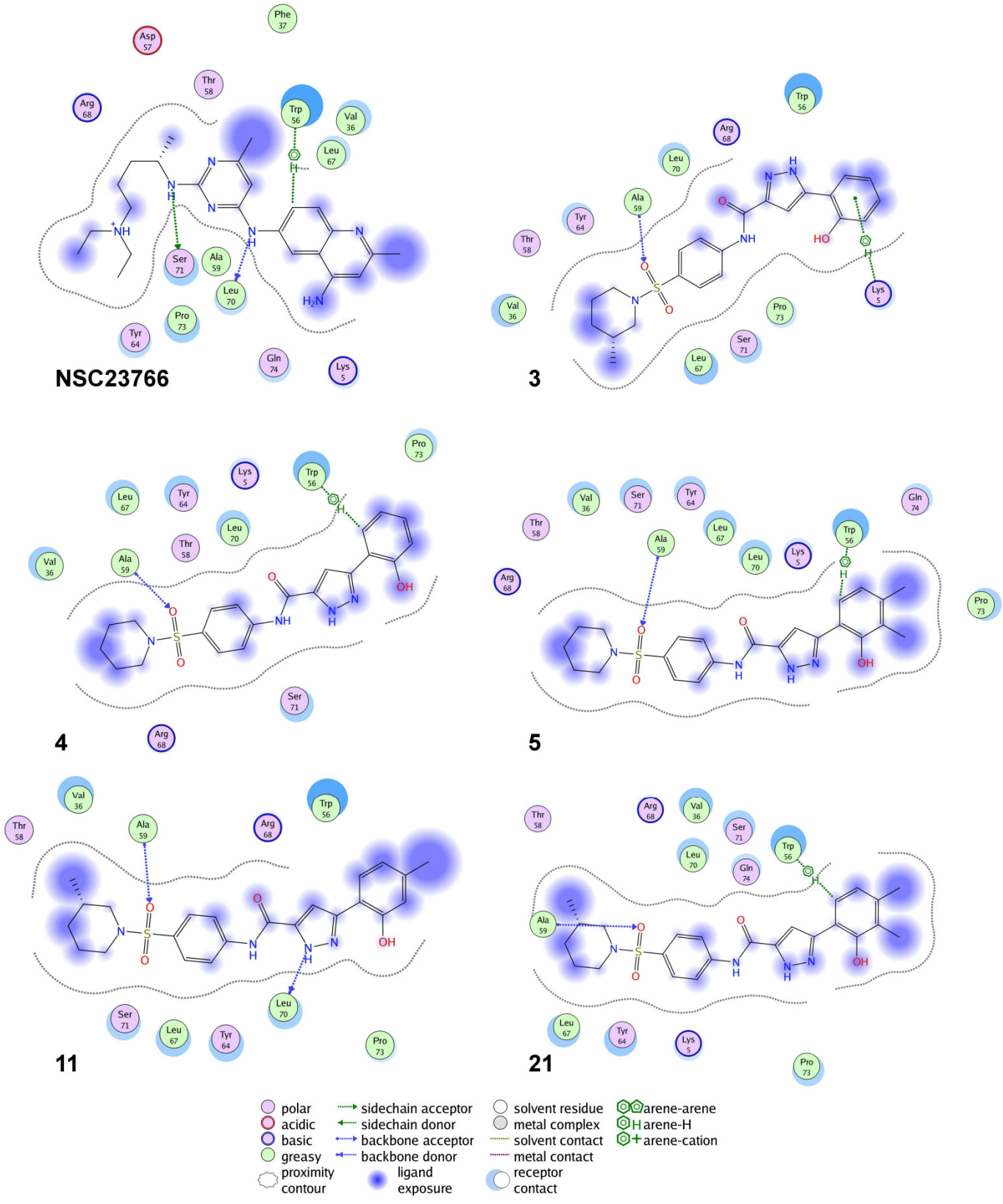

Figure S7. Most relevant binding contacts for compounds **3-5**, **11** and **21**. Binding contacts observed for NSC23766 are also reported as a reference.

References

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