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Electronic supplementary information

Chemistry

General procedures

All reagents and solvents were purchased by Sigma-Aldrich and were used without further purification, unless indicated otherwise. Thin-layer chromatography (TLC) was performed on Silica Gel 60 F254 plates (Merck) with UV detection, or using a developing solution of conc. H_2SO_4/EtOH/H_2O (5:45:45), followed by heating at 180 °C. Flash column chromatography was performed on silica gel 230-400 mesh (Merck).. Mass spectra were recorded on ESI-MS triple quadrupole (model API2000 QTrap™, Applied Biosystems). High resolution mass spectra were recorded on QSTAR Elite® LC/MS/MS system (Applied Biosystems) that is a hybrid quadrupole/TOF instrument, equipped with the Analyst® QS 2.0 software. ^{1}H and ^{13}C spectra were recorded on a Varian 400 MHz MERCURY instrument at 300 K unless otherwise stated. Chemical shift are reported in ppm downfield from TMS as internal standard.

Synthesis of compounds 3 and 4

Compound **3**: A solution of compound **1** (71 mg, 0.29 mmol) and *N*-acetyl-D-glucosamine (50 mg, 0.23 mmol) in a mixture of DMF/AcOH/buffer acetate pH 4.5 (1:1:1, 3 mL) was stirred at 50°C for 24 h. The solvent was evaporated and the crude was purified by flash chromatography (AcOEt:MeOH:H_2O 11:1:0.3) obtaining compound **3** (48 mg, 46%) as light green powder. ^{1}H-NMR (400 MHz, D_2O): δ (ppm) 7.35 – 7.25 (m, 3H, Hbenzyl), 7.17 – 7.08 (m, 2H, Hbenzyl), 6.82 (d, *J* = 8.1 Hz, 1H, Harom), 6.79 (d, *J* = 1.4 Hz, 1H, Harom), 6.73 (dd, *J* = 8.1, 1.6 Hz, 1H, Harom), 4.33, 4.27 (qAB,2H, CH_2O), 4.23 (d, *J* = 9.7 Hz, 1H, H-1), 4.00 (m, 1H, H-2), 3.95, 3.81 (qAB, 2H, CH_2N), 3.87 (dd, *J* = 12.3, 1.6 Hz, 1H, H-4), 3.71 (dd, *J* = 12.4, 5.3 Hz, 1H, H-3), 3.48 – 3.34 (m, 2H, H-5, H-6a), 3.30 (m, 1H, H-6b), 2.05 (s, 3H, $COCH_3$). ^{13}C-NMR (100 MHz, d_4-MeOH): δ (ppm) 173.58, 146.30, 146.07, 137.73, 131.16, 130.01, 129.44, 129.42, 122.98, 118.54, 116.18, 79.73, 78.13, 77.49, 71.61, 71.60, 62.90, 53.95, 23.24. HRMS (FT-ICR): calcd for $C_{22}H_{28}N_2O_8$: 448.4663; found 449.3925 $[M+H]^+$.

Compound **4**: A solution of compound **1** (87.3 mg, 0.36 mmol) and lactose (156 mg, 0.43 mmol) in a mixture of DMF/AcOH/buffer acetate pH 4.5 (1:1:1, 4 mL) was stirred at 50°C for 24 h. The solvent was evaporated and the crude was purified by flash chromatography (AcOEt:MeOH:H_2O 8:2:0.5 to 8:2:1) obtaining compound **4** (105 mg, 52%) as light brown powder. ^{1}H-NMR (400 MHz, D_2O): δ (ppm) 7.35 (m, 3H, Hbenzyl), 7.18 (m, 2H, Hbenzyl), 6.95 (d, *J* = 1.2 Hz, 1H, Harom), 6.90 (d, *J* = 8.0 Hz, 1H, Harom), 6.86 (dd, *J* = 8.3, 1.2 Hz, 1H, Harom), 4.57, 4.38 (qAB, 2H, CH_2O), 4.41 (d, *J* = 7.8 Hz, 1H, H-1'), 4.15 (d, *J* = 8.9 Hz, 1H, H-1), 4.09, 3.92(qAB, 2H, CH_2N), 3.93 (m, 1H), 3.84 – 3.39 (m, 11H). ^{13}C NMR (100 MHz, D_2O): δ (ppm) 143.86, 143.71, 135.46, 129.75, 129.10, 128.85, 128.73, 123.00, 118.20, 116.08, 103.01, 91.33, 78.16, 77.02, 76.15, 75.84, 75.46, 72.60, 71.05, 69.74, 68.68, 61.19, 60.26, 56.53. HRMS (FT-ICR): calcd for $C_{26}H_{35}NO_{13}$: 569.5550; found 570.6317 $[M+H]^+$, 592.3824 $[M+Na]^+$.

Biology: Protein purification and nucleotide exchange assays

Recombinant N-terminal His-tagged H-Ras protein was expressed in *Escherichia coli* M15 [pREP4] strain harboring a pQE^{TM}-derived plasmid (Qiagen) and purified using a Ni^{2+} affinity column. For NMR experiments ^{15}N enriched H-Ras (aa 1-166) was expressed and purified using a previously published protocol.[1] The N-terminal His-tagged hSos1 catalytic domain (aa 553-1024) was purified as previously described.[2] The N-terminal GST-tagged RasGRF1 catalytic domain (aa 976–1262) was purified as previously described.[3] The exchange reaction between GDP and fluorescently labeled MANT-GDP (Molecular Probes) in Ras was performed by adding directly into an UV-cuvette 0.25 μM His-tagged H-Ras-GDP, 1.25 μM MANT-GDP and different concentrations of each compound (0-500 μM), in a buffer composed of 40 mM HEPES (pH 7.5), 5 mM DTE, and 10 mM $MgCl_2$. After 300 seconds of incubation, 0.0625 μM GST-RasGRF1$_{cat}$ or His- hSos1$_{cat}$ (GEF) was added to catalyze the exchange reaction. Compounds were dissolved in DMSO at 30 mM concentration (stock solution) and then diluted to the desired final concentration in the buffer described above. The fluorescence measurements were done at 25°C using a Perkin-

Elmer LS45 luminescence spectrometer with an excitation wavelength of 366 nm and emission wavelength of 442 nm. Each reaction was monitored for at least 1500 sec, and the corresponding experimental curve was fitted to a non linear growth-sigmoidal Hill curve (n = 1) with OriginPro 8.0 software. The initial exchange rate of each reaction was determined computing the first derivative at time 0 of the fitted curve, with OriginPro 8.0 software. When plotted as a function of the compound concentration, the initial exchange rates yield dose-response curves that allow determination of IC_{50} values of the inhibitory efficacy on the GEF-mediated nucleotide exchange on Ras. The exchange rate of the control reaction (in the absence of synthetic compounds) was normalized to 100.

Cell culture manipulation and proliferation assays

Normal NIH3-T3 (from ATCC) and *K-RAS* transformed (NIH-RAS) murine fibroblasts, obtained and characterized as described in Bossù et al.[4] according to a procedure described by Pulciani et al.[5], were grown at 37°C in a humidified atmosphere containing 5% CO_2, in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% new born calf serum, 2 mM glutamine, 100 units/ml penicillin and 100 mg/ml streptomycin (Invitrogen). Cells were passaged using trypsin–ethylenediaminetetraacetic acid (EDTA) (Invitrogen) and maintained in culture for 96 h before experimental manipulation. For proliferation analysis, cells were plated into six-well flat-bottomed culture plates at the density of 3000 cells/cm^2. At 18 h after seeding, predetermined concentrations of compound of interest (or water) was added to the cell culture. Compounds for cell culture assays were dissolved in sterile water (SIGMA) at 30mM concentration (stock solution), then diluted to desired final concentration in culture medium. Cells were harvested at different time points (24, 48, and 72 h from compound addition) and counted by Coulter Counter. For analysis of MAPK activitation, murine fibroblasts were plated (6000 cells/cm^2) in 60-mm tissue culture dishes. At 18 h after seeding, predetermined concentration of cmp (or water) was added to the cell culture. After 24h from treatment, both plate-adherent and in suspension cells were harvested in lysis buffer from PathSscan Sandwich ELISA kit (Cell Signaling). The detection of endogenous levels of Phospho-p44/42 MAPK was performed according to

manufacturer's suggestions, and the results were normalized on total protein content.

NMR Experiments

^1H-^{15}N HSQC spectra were acquired on a 600 MHz Bruker Avance spectrometer at 25 °C. The starting protein concentration was 810 μM in Tris-Citrate buffer (50 mM Tris/Citrate pH 6.5, 50 mM NaCl, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 10 % D_2O). Stock solutions of synthetic compounds were prepared in Tris-Citrate buffer containing 2 % methanol. Compounds **1** and **4** were added to ^{15}N H-Ras-GDP (1-166) in the 1:1, 2:1, 3:1 and 4:1 compound:protein molar ratios. Compound **3** was added in the 0.5:1, 1:1, 2:1 and 4:1 compound:protein molar ratios. The NMR data were processed and analyzed using NMRPipe software.[6] NMR resonance assignments were obtained from the BMRB database (Accession number 10051).[7] The ^1H and ^{15}N chemical shift changes were normalized and combined as a geometric average of the total chemical shift change in ppm:

$$\Delta\delta_{HN} = \sqrt{\frac{(\Delta\delta^N H)^2 + (\Delta\delta N)^2 / 25}{2}}$$

Chemical shift changes that are 1 standard deviation above average were considered significant. Chemical shift changes in either the ^1H or ^{15}N dimension for various titration points were plotted versus drug concentration using Microsoft Excel software and the K_d values were calculated for compounds **1**, **2**, **3** and **4** using non-linear regression.

The saturation transfer difference spectra were recorded on an 800 MHz Bruker NMR spectrometer at 25 ºC using a previously described pulse sequence.[8] The saturation was performed in the methyl region. In all cases the concentration of compounds was 100 µM and the concentration of H-Ras-GDP was 1 µM in the Tris-Citrate buffer. Control experiments were performed in the absence of H-Ras-GDP and showed no saturation transfer difference.

References

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Determination of dissociation constants for compounds 1-4 binding to H-Ras-GDP

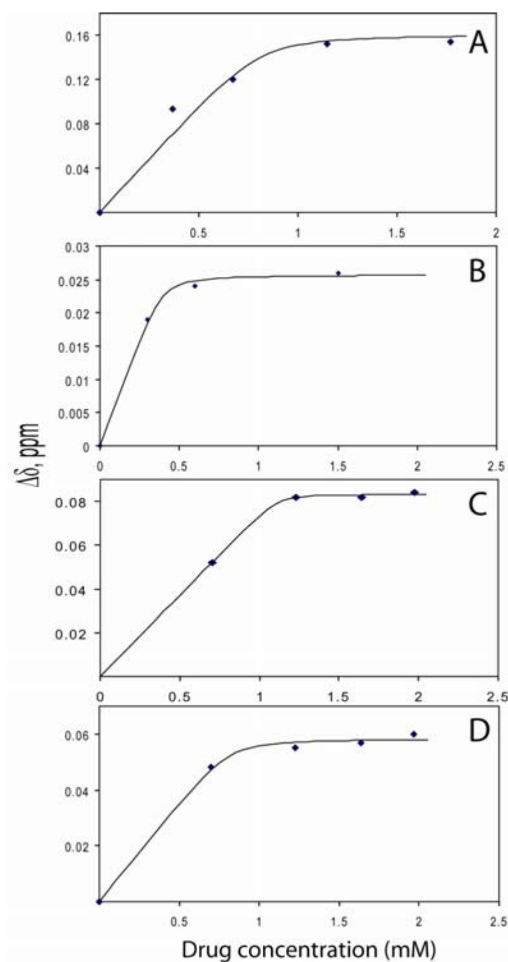

Figure S1. **Determination of H-Ras-GDP dissociation constants** for compound **1** (A), compound **2** (B), compound **3** (C) and compound **4** (D) using NMR titration. The chemical shift perturbations for residues Y71 (A), Y64 (B), R68 (C), and L23 (D) were fit using a "one set of binding sites" model to yield the dissociation constant. It was assumed that interaction with the predominant binding site was not affected by non-specific binding.

Dose-response inhibition of RasGRF1$_{cat}$-catalyzed nucleotide exchange of H-Ras by compounds 1-4

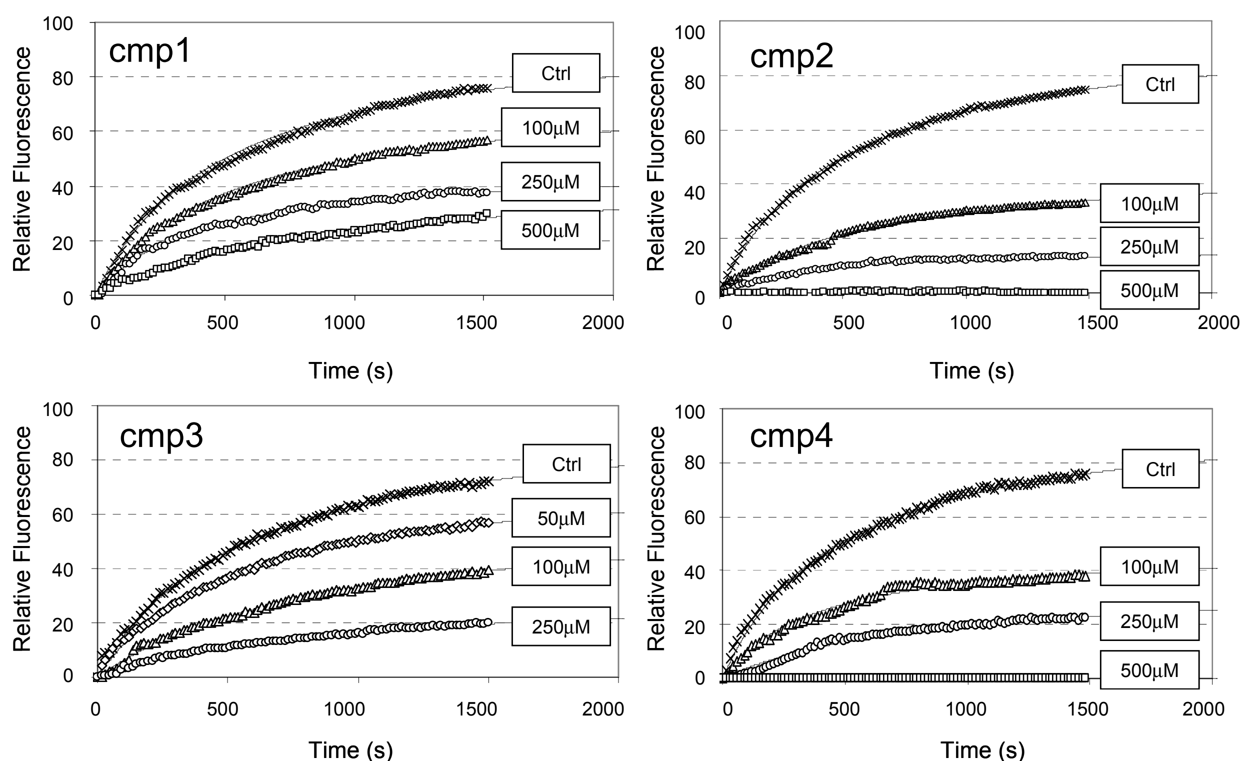

Figure S2 Effect of compounds 1-4 dissolved in DMSO-containing buffer on RasGRF1$_{cat}$-catalyzed nucleotide exchange of H-Ras. The exchange reaction on H-Ras was obtained adding to 0.25 µM H-RasGDP, 1.25 µM mantGDP, 0.0625 µM RasGRF1$_{cat}$ and different concentrations of compounds: 0 µM (crosses), 50 µM (open diamonds), 100 µM (open triangles), 250 µM (open circles), 500 µM (open squares). Fluorescence measurements were made with a Perkin-ElmerLS-45 luminescence spectrometer, monitoring the fluorescence each second for 1500 sec. For graphical reason in figure only a point every 15 has been plotted. Each experimental curve was fitted to a non linear 'growth-sigmoidal Hill' curve (n=1) with OriginPro 8.0 software, reported in graph as a thin line. In the graph the maximum value of relative fluorescence (100 on Y axis) represent the fully charged Ras status obtained as plateau of an exchange curve without compounds.

Dose-response inhibition of hSos1-catalyzed nucleotide exchange of H-Ras by compound 3

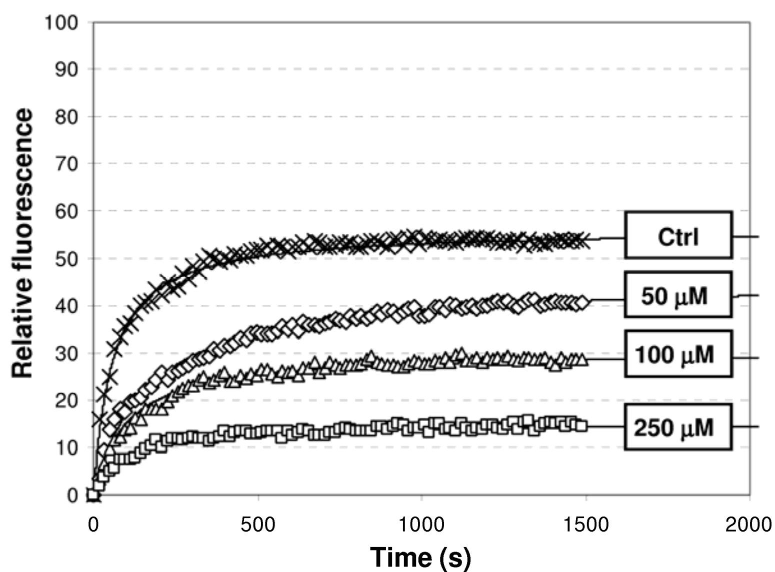

Figure S3 Effect of compound 3 dissolved in DMSO-containing buffer on hSos1$_{cat}$-catalyzed nucleotide exchange of H-Ras. The exchange reaction on H-Ras was obtained adding to 0.25 µM H-RasGDP, 1.25 µM mantGDP, 0.0625 µM hSos1$_{cat}$ and different concentrations of compound 3: 0 µM (crosses), 50 µM (open diamonds), 100 µM (open triangles), 250 µM (open squares). Fluorescence measurements were made with a Perkin-ElmerLS-45 luminescence spectrometer, monitoring the fluorescence each second for 1500 sec. In figure only a point every 15 has been plotted. Each experimental curve was fitted to a non linear 'growth-sigmoidal Hill' curve (n=1) with OriginPro 8.0 software, reported in graph as a thin line. In the graph the maximum value of relative fluorescence (100 on Y axis) represent the fully charged Ras status obtained as plateau of an exchange curve without compounds.