Supplementary Information

Discovery of high affinity non-covalent allosteric KRAS inhibitors that disrupt effector binding

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**Figure S1.** (Top) Multiseq structural alignment, using VMD (1), of the MD-derived conformer used for docking (blue) with 19 GDP- (5F2E, 4TQA, 4QO3, 4OBE, 4M1T, 4LYJ, 4LV6, 4EPY, 4EPR, 6ASE, 6ARK, 5WHD, 5WHA, 5W22, 5VBM, 5V9U, 5V9L, 5V6V, 5UQW) and 4 GTP- or GTP analogue-bound KRAS X-Ray structures (5UK9, 5USJ, 4DSO, 6BP1). KRAS structures co-crystallized with proteins or peptides were not considered; all others have been included. Nearly half (eight) of the GDP structures contain covalent ligands targeting KRAS\textsuperscript{G12C} in which pocket p1 between switch 2 and the \(\beta_1\)-\(\beta_3\) region is narrowed (orange). Based on the orientation of helix 2, three groups of conformations can be observed in the GDP structures, two of which also are sampled by the GTP structures. (Bottom) Displacement of helix 2 toward helix 3 and side chain reorganizations expanded pocket p1 (described by residues 5-7, 37-39, 50-56, 57-75 shown in surface representation in green; the expansion of a small hydrophobic pocket, originally occupied by benzamidine, during the MD simulation is highlighted in light green and circled in red).
Figure S2. In silico prediction and initial experimental characterization of putative KRAS inhibitors. (A) Chemical structure of eleven computationally predicted hits (compounds 1 through 11) selected for experimental testing in cell-based assays. (B) Western blots showing levels of phosphorylated ERK1/2 (p-ERK) in BHK cells ectopically expressing GFP-KRASG12D treated with vehicle (DMSO), a positive control U (the MEK inhibitor U0125) or compound at the indicated four concentrations. Five examples were shown illustrating cases where the ligand has no effect (compounds 1 and 2), increases p-ERK levels (compound 4) or decreases p-ERK levels (compounds 5 and 9). This assay was used as a primary screen to quickly assess the potential of a ligand to qualitatively affect the MAPK pathway; for the sake of efficiency, we did not measure total protein. (C) Representative Western blots (top: p-ERK (upper) and total GFP-KRASG12D (lower), the latter serving as loading control), and their quantification (bottom) for compounds 9 and 11 for which the Western analysis was repeated in an expanded concentration range. The reduction in p-ERK levels of compound 11-treated cells is not due to reduction in total ERK levels, as shown in Fig 3 of the main text. Data are averages over three independent experiments and error bars represent standard error. * = p < 0.02; ** = p < 0.005.
Figure S3. Microscale thermophoresis (MST) profiles monitoring the binding of compound 11 to the catalytic domain (residues 1-166) of \( \text{GDP}^{\text{KRAS}^{\text{WT}}} \) (black), \( \text{GDP}^{\text{KRAS}^{\text{G12D}}} \) (green), \( \text{GDP}^{\text{NRAS}^{\text{WT}}} \) (light orange), \( \text{GDP}^{\text{NRAS}^{\text{WT}}} \) (orange), \( \text{GDP}^{\text{HRAS}^{\text{WT}}} \) (light blue), \( \text{GDP}^{\text{HRAS}^{\text{WT}}} \) (pink), and Rap1b (cyan), as well as the full-length (residues 1-188; non-lipidated) \( \text{GTP}^{\text{KRAS}^{\text{WT188}}} \) (grey). MST profile of \( \text{GDP}^{\text{KRAS}^{\text{WT}}} \) (black) shows the onset of potential binding at > 20 µM compound 11 (\( \text{GDP}^{\text{HRAS}^{\text{WT}}} \) exhibits a somewhat similar trend but the data is noisy). Note the weaker affinity for the full-length wild type protein relative to the isolated catalytic domain (estimated \( K_D \) of 1.9 ± 0.4 versus ~0.3 (Fig 1 main text)). This suggests that the conformation of the full-length construct in solution is likely different from the isolated catalytic domain or cellular full-length KRAS (e.g., the p1 pocket could be partially occluded by the HVR in solution, which in the cell would have been engaged by the plasma membrane). Therefore, unless explicitly stated otherwise, we refer to the isolated catalytic domain throughout this manuscript when describing biophysical measurements.
Figure S4. Alignment of the MD-derived KRAS conformer used for docking (blue) with 9 GDP- (1XJ0, 1ZVQ, 2CE2, 2CLD, 2QUZ, 3LO5, 4L9S, 4Q21, 5VBE) and 27 GTP- or GTP-analogue-bound HRAS X-ray structures (1ZW6, 2CL6, 2CL7, 2EVW, 3I3S, 3K8Y, 3K9N, 3KKM, 3KKN, 3RRY, 3TGP, 4DLR, 4DLS, 4DLT, 4EFN, 4XVQ, 5B2Z, 5B30, 5VNZ, 5WDP, 5WDQ, 5X9S, 6Q21). HRAS structures co-crystalized with proteins or peptides were not considered; all others have been included. As in KRAS, the orientation of helix 2 modulates the accessible space between the β1-β3 region and switch 2 but there is no obvious difference between the KRAS and HRAS structures at the global level. There aren’t as many experimental structures for NRAS.

Effect of compound 11 on intrinsic and GEF-dependent nucleotide release and exchange reactions Figure S5 shows time-dependent decreases and increases of fluorescence intensity as a labeled-nucleotide dissociates from and binds to KRAS^WT, respectively. Compound 11 slightly reduced the rates of both intrinsic and SOS-mediated nucleotide exchange reactions, as well as the SOS-dependent (but not intrinsic) release of labeled-GDP. In particular, 11 decreased the intrinsic rate of nucleotide exchange by ~10-fold at >10 µM (Fig S5, top-left), but it has no effect on intrinsic nucleotide release (Fig S5, bottom-left). The latter is consistent with our observation from MST that 11 does not bind to GDP^KRAS^WT with high affinity. Since GTP hydrolysis is unlikely to occur within the timescale of our experiments (2, 3), a plausible interpretation of the former would be compromised GTP loading. This is possible if, for example, the ligand binds to the nucleotide free ‘transition state’ conformation of KRAS and induces reorganization of active site residues. This is supported by the fact that 11’s effect on the rate of nucleotide exchange is significantly smaller (only a 1.1-fold decrease, Fig S5 top-right). SOS stabilizes nucleotide free RAS in an open active site conformation (4, 5), which allows for faster expulsion and rebinding of GTP or GDP.
Figure S5. Effects of compound 11 on intrinsic and SOS-mediated nucleotide exchange and release. (Top) Intrinsic (left) and SOS-mediated (right) rates for the nucleotide exchange reaction KRAS$^{\text{GDP}}$ + BGTP → KRAS$^{\text{BGTP}}$ + GDP in a mixture of 0.5 µM each of KRAS, BGTP, and SOS. (Bottom) Intrinsic (left) and SOS-mediated (right) rates of the nucleotide release reaction KRAS$^{\text{BGDP}}$ + GTP → KRAS$^{\text{GTP}}$ + BGDP. Concentrations of KRAS and SOS were 0.5 µM and that of GTP was 100 µM. Ligand concentrations: 0 (circle), 0.78 (square), 1.56 (triangle), 3.12 (inverted triangle), 6.25 (left-sided triangle), 12.5 (right-sided triangle) and 25 µM (diamond). Intensities were normalized with respect to the value at 120 s. Linear or single exponential fits, starting from 120 s, were superimposed as solid lines. Inset: Rates as a function of ligand concentration.

Plotting the measured rates as a function of the total ligand concentration yielded additional insights into the effect of 11 in the intrinsic and GEF-catalyzed enzymatic activity of KRAS. We obtained an estimated $EC_{50}$ of 3.1 ± 1.2 µM from the intrinsic nucleotide exchange assay, and a similar value of 5.3 ± 2.0 µM from the SOS-mediated GDP release assay. These values are about 10-times larger than the $K_D$ of 11 for $^{\text{GTP}}$KRAS$^{\text{WT}}$, suggesting a potentially weaker binding to the nucleotide free state assuming, as argued above, that the observed effects on enzymatic activity are at least in part a result of binding to nucleotide free KRAS. Intriguingly, we obtained $EC_{50} = 0.6 ± 0.1$ µM from the SOS-dependent nucleotide exchange measurements, a value very close to the $K_D$ of 11 for $^{\text{GTP}}$KRAS$^{\text{WT}}$. This may reflect binding to the GTP-bound KRAS at the allosteric site of SOS. We note that all of the effects we observed on reaction rates are much smaller than those in KRAS-Raf interaction. Nonetheless, they are statistically significant and dose-dependent, suggesting that compound 11 may modulate KRAS activation through multiple mechanisms, with the dominant effect being on effector binding.
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