Small molecule stabilization of the KSR inactive state antagonizes oncogenic Ras signalling

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Deregulation of the Ras–mitogen activated protein kinase (MAPK) pathway is an early event in many different cancers and a key driver of resistance to targeted therapies¹. Sustained signalling through this pathway is caused most often by mutations in K-Ras, which biochemically favours the stabilization of active RAF signalling complexes². Kinase suppressor of Ras (KSR) is a MAPK scaffold³–⁵ that is subject to allosteric regulation through dimerization with RAF⁶,⁷. Direct targeting of KSR could have important therapeutic implications for cancer; however, testing this hypothesis has been difficult owing to a lack of small-molecule antagonists of KSR function. Guided by KSR mutations that selectively suppress oncogenic, but not wild-type, Ras signalling, we developed a class of compounds that stabilize a previously unrecognized inactive state of KSR. These compounds, exemplified by APS-2-79, modulate KSR-dependent MAPK signalling by antagonizing RAF heterodimerization as well as the conformational changes required for phosphorylation and activation of KSR-bound MEK (mitogen-activated protein kinase kinase). Furthermore, APS-2-79 increased the potency of several MEK inhibitors specifically within Ras-mutant cell lines by antagonizing release of negative feedback signalling, demonstrating the potential of targeting KSR to improve the efficacy of current MAPK inhibitors. These results reveal conformational switching in KSR as a druggable regulator of oncogenic Ras, and further suggest co-targeting of enzymatic and scaffolding activities within Ras–MAPK signalling complexes as a therapeutic strategy for overcoming Ras-driven cancers.

Ras is the most frequently mutated human oncogene. Yet, despite recent breakthroughs, therapeutic options to target Ras-dependent cancers remain limited¹. Studies conducted in several different model systems support the possibility of Ras-targeted interventions via KSR³–⁵,⁸–¹⁰. However, due to its status as a pseudokinase and role as a non-catalytic regulator of core signalling enzymes¹¹–¹⁲, pharmacological approaches that target KSR have been lacking. This is in contrast to current drug discovery and development efforts that have focused extensively on direct inhibitors of the Ras effector kinases RAF, MEK, and ERK¹³. To explore an alternative form of pharmacological modulation and identify Ras–MAPK antagonists via KSR, we focused on large forward genetic screens conducted in flies and worms that identified mutant Ras-selective suppressor alleles in KSR¹³–¹⁴. The studies in flies alone evaluated approximately 900,000 randomly mutated strains searching for genetic modifiers of a Ras(G12V)-dependent rough-eye phenotype¹⁵. We mapped the suppressor alleles onto the primary sequence of KSR (Extended Data Fig. 1a) and a recently determined X-ray crystal structure of the human KSR2 pseudokinase domain in complex with MEK1 and ATP, and noted a high concentration of suppressor mutations immediately adjacent to the KSR ATP-binding pocket (Fig. 1a). On the basis of this analysis, we hypothesized that the RAF and MEK interaction interfaces in KSR may be uncoupled through ligands that engage the KSR ATP-binding pocket. Specifically, we speculated that small molecules, which bias KSR towards a state similar to that revealed in the KSR2–MEK1–ATP crystal structure, might function as antagonists of KSR-dependent regulation of RAF and MEK.

To identify active-site-directed ligands of KSR, we screened a collection of 176 structurally diverse kinase inhibitors for direct competition of an activity-based probe (ATP biothiol) that specifically labels the ATP-binding pocket of purified KSR2–MEK1 complexes (Fig. 1b, c). From this analysis we identified APS-1–68-2 as a competitor of probe-labelling of KSR2–MEK1. This quinazoline–biphenyl ether compound has previously been described as both a Src and epidermal growth factor receptor (EGFR) family kinase inhibitor. Synthetic tailoring of APS-1–68-2 generated highly informative structure–activity relationships (Fig. 1d). For example, deletion of the terminal phenyl group (APS-1–82-1) or extension of the ether linker (APS-2-12) diminished KSR2–MEK1 probe competition. Notably, addition of a single methyl group at the internal phenyl generated a potent probe compound (APS-3-77) that was essentially inactive (IC₅₀ of KSR2 > 10,000 nM). To assess the biological function of these compounds as Ras–MAPK pathway antagonists, we developed a simplified cell-based reconstitution system to directly monitor KSR-driven MAPK signalling (Fig. 1e). This system, in which cellular MAPK signalling is dependent on KSR expression, was found to be sensitive to known Ras suppressor mutations in KSR (Fig. 1f). Likewise, APS-2-79 also suppressed KSR-stimulated MEK and ERK phosphorylation (Fig. 1g; lanes 1 versus 2). The suppression of MAPK signalling by APS-2-79 was dependent on direct targeting of KSR as an active site mutant (KSR(A690F)), which has previously been demonstrated to stimulate KSR-based MAPK outputs independent of ATP-binding¹⁶, significantly diminished the activity of APS-2-79 (Fig. 1g; lanes 5 versus 6, NS; lanes 2 versus 6, P < 0.005). Notably, the negative control for KSR-binding (analogue APS-3-77; see Extended Data Fig. 2b, c for comparative selectivity profiling) was inactive, whereas a positive-control RAF inhibitor, dabrafenib, was active irrespective of the KSR-mutational status (Fig. 1g). Therefore, on the basis of similarity in phenotype and also direct-binding activity, we identify APS-2-79 as a small-molecule mimic of KSR alleles that suppress oncogenic Ras mutations.

KSR-based activity of APS-2-79 as a MAPK antagonist was further evaluated using reconstitution assays. Dose-dependent phosphorylation of MEK on Ser218/Ser222 by RAF in vitro could be enhanced at least fivefold in the presence of KSR (Extended Data Fig. 3a–c). KSR-stimulated MEK phosphorylation by RAF was markedly reduced by the addition of APS-2-79, but not by APS-3-77 (Extended Data Fig. 3d, e). APS-2-79 was inactive when KSR was absent or when the KSR2(A690F) mutant was used for in vitro assays (Extended Data Fig. 3d, f, g), suggesting that the activity of APS-2-79 derives from direct targeting of KSR. Indeed, APS-2-79 lacked direct activity against the highly homologous active RAF family kinases, including recombinant

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BRAF and CRAF, or cellular BRAF(V600E) (Extended Data Figs 2, 3, 4a). Therefore, on the basis of reconstitution and selectivity assays, we conclude that APS-2-79 functions as an antagonist of MEK phosphorylation by RAF through direct binding of the KSR active site.

Notably, we found that a previously described ATP-competitive and active-state binder of KSR termed ASC24 (ref. 7), in contrast to APS-2-79, did not antagonize KSR-dependent MEK phosphorylation by RAF (Extended Data Fig. 3d, e), suggesting that inhibition of catalytic activity alone in KSR is insufficient to block MAPK signalling. Consistent with this notion, removal of putative KSR phosphorylation sites in MEK (Extended Data Fig. 7) is insufficient to block MAPK signalling in cells (Extended Data Fig. 3d, e). 

Previous studies established that genetic suppressors in KSR may impede RAF-induced conformational changes in KSR required for MEK activation or destabilize KSR–MEK and KSR–RAF complexes (ref. 7,17,18). To distinguish between such possible modes of action, we determined an X-ray crystal structure of the KSR2–MEK1 complex bound to APS-2-79 (Fig. 2a). In the APS-2-79-bound state, KSR2 binds MEK1 in a 1:1 fashion within a quaternary arrangement that is nearly identical to the ATP-bound state of KSR2–MEK1 complexes (Extended Data Fig. 5). Within both states, KSR2 and MEK1 bind via a face-to-face arrangement mediated largely through reciprocal helix αG and activation segment interactions, and KSR2 homodimerizes through the N-lobe along a crystallographic two-fold symmetry axis producing a hetero-tetramer of KSR2–MEK1 dimers. In the APS-2-79-bound state, only KSR2 was found to possess strong ATP-resistant label remaining (%), which are indicative of ATP-binding-pocket ATP biotin label remaining (%), which are indicative of ATP-binding-pocket.

Figure 1 | The small molecule APS-2-79 mimics KSR alleles that suppress oncogenic Ras mutations. a, Oncogenic Ras-suppressor mutations (red) localize to the ATP-binding pocket (yellow), as well as RAF– and MEK–interaction interfaces, in KSR. Shown is the putative structure of the RAF–KSR–MEK complex. b, An activity-based probe (ATPbiotin) specifically labels the ATP-binding pockets of purified KSR2–MEK1 complexes. 2 μM of ATPbiotin was incubated with KSR2–MEK1 in the presence of the indicated concentrations of free ATP. Biotin, RAF- and MEK–interaction interfaces, in KSR. Shown is the putative mutations (red) localize to the ATP-binding pocket (yellow), as well as suppress oncogenic Ras mutations. a Figure 1, b Co-expression of full-length KSR–Flag and MEK1–GFP

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backbone at Cys742 further mediates APS-2-79–KSR2 interactions. Notably, functionalization of the N1 with a methyl group (APS-3-6) greatly diminished KSR2–MEK1 activity, whereas replacement of the N3 with –CH (APS-2-16) was moderately tolerated (Extended Data Fig. 7). Therefore, on the basis of crystallographic analysis and also structure–activity relationships data from our analogue series, APS-2-79 binds directly to KSR2 within the KSR2–MEK1 complex.

In both the APS-2-79- and ATP-bound states of KSR2–MEK1, KSR2 directly engages the activation segment of MEK1, burying the Ser218–Ser222 region and presumably shielding this segment of MEK from direct engagement with the activation segment of MEK1, burying the Ser218–Ser222 region and presumably shielding this segment of MEK from direct engagement with the ATP-bound MEK1 suggested that APS-2-79 antagonizes RAF phosphorylation on MEK mediated by these two kinases. Indeed, APS-2-79 behaves as a KSR-dependent antagonist of RAF-mediated MEK phosphorylation by shifting the equilibrium of RAF–MEK complexes so as to populate the OFF state (Extended Data Fig. 5c).

Comparison of the ATP-bound and APS-2-79-bound states of KSR2–MEK1 suggested that APS-2-79 antagonizes RAF phosphorylation on MEK indirectly by impeding KSR–RAF heterodimers. As well as APS-2-79 binding, the dimer interface of KSR2, including residues Trp685 and His686, demonstrated perturbations relative to the ATP-bound state directly coordinates the β and γ phosphates (Fig. 2e). The two modes by which ATP and APS-2-79 affect KSR-based interactions on MEK appear mutually exclusive as both ligands induce conformations that would otherwise clash with one another (Fig. 2e). We interpret these structures to suggest that APS-2-79 stabilizes an inactive state of KSR2 characterized by reinforcement of negative regulatory interactions. Indeed, APS-2-79 behaves as a KSR-dependent antagonist of RAF-mediated MEK phosphorylation by shifting the equilibrium of RAF–MEK complexes so as to populate the OFF state (Extended Data Fig. 5c).

Figure 2 | Structural analysis of APS-2-79 bound to the KSR2–MEK1 complex. a. The KSR2–MEK1–APS-2-79 complex. Highlighted are two key phospho-regulatory residues in MEK1, Ser218 and Ser222. b. Magnified stereo view of interactions between KSR2 and APS-2-79. Fc – Fom map contoured at 3.5σ, generated with APS-2-79 omitted, is represented as a blue mesh. c. Schematic of the APS-2-79 binding site within KSR2. d. Magnified view of the KSR2 active site bound to APS-2-79, including the ‘induced lock’ (residues 1809–Q814; orange). The disordered P-loop is highlighted by a dashed line. e. Overlay between the ATP-bound (yellow) and APS-2-79-bound states of KSR2.

Figure 3 | APS-2-79 impedes higher order assembly of RAF–KSR–MEK complexes. a. Mapping of residues with a root-mean-square (r.m.s.) deviation of >2.0 Å between the ATP- and APS-2-79-bound states of KSR2–MEK1 (blue) highlights alterations at contact residues Trp685 and His686 within the putative KSR–RAF heterodimer interface. b–g. BRAF and BRAF mutants (F667E and/or R509H) were immobilized on sensor-heads and KSR2–MEK1 or MEK1 assembly was monitored using bio-layer interferometry. Association occurred from 0 to 660 s and dissociation was monitored thereafter up to 1500 s. APS-2-79 was added in the presence of KSR2–MEK1 at a concentration of 25 μM. Kd values represent the mean ± s.e.m. derived from global fitting of all 5 binding curves.
Figure 4 | APS-2-79 Enhances the efficacy of the clinical MEK inhibitor trametinib within cancer cell lines containing K-Ras mutations.

a. Dose–response curves of APS-2-79 and trametinib (MEKi) on viability of K-Ras-mutant (HCT-116, A549) and BRAF-mutant (A375, SK-MEL-239) cell lines. Bliss scores represent the mean calculated from two biological replicates of the depicted concentration matrices. Numbers listed within synergy matrices, which represent the percentage of growth inhibition relative to DMSO controls, are the mean of the replicates. Insets highlight dose–response of trametinib in the absence or presence of 1 μM APS-2-79 (points along each line represent the mean of two biological replicates).

b. Synergy, as determined by Bliss independence scores (see Methods), for combinations of APS-2-79 and APS-3-77 with trametinib. Error bars represent the mean ± s.d. of Bliss scores as determined in a and Extended Data Fig. 9, derived from either K-Ras-mutant and BRAF-mutant cancer cell lines (n = 5 for each). * * P < 0.005 by two-tailed unpaired t-testing.

c. Pathway analysis suggests that the increased potency of trametinib in the presence of APS-2-79 occurs through enhanced downregulation of Ras–MAPK signalling (as measured by phospho-ERK). HCT-116 and SK-MEL-239 cells were treated for 48 h with increasing concentrations of trametinib combined with DMSO. 250 nM, and 1 μM APS-2-79. IC50 values represent the mean ± s.d. (n = 2 biological replicates).

d. Model for synergy between the MEK inhibitor (MEKi) trametinib and the KSR inactive state binder (KSRi) APS-2-79. APS-2-79 enhances the efficacy of trametinib by antagonizing MEKi-induced Ras–MAPK signalling complexes.

To specifically monitor KSR2–BRAF dimerization relative to other possible interactions, we identified a mutation in BRAF(F667E) that eliminates binding to free MEK but not KSR2–MEK1 complexes (Fig. 3d, e). KSR2–MEK1 interacted in a 1:1 fashion with the BRAF(F667E) mutant with a Kd of 1.99 ± 0.09 μM; closely matching previously published BRAF–BRAF dimerization values. Notably, the addition of a secondary mutation, known to perturb KSR2–BRAF dimers (BRAF(F667E/R509H); Fig. 3f), completely abrogated any binding signal between KSR2–MEK1 and BRAF. In the presence of APS-2-79, the KSR2–BRAF(F667E) dimers did not associate (Fig. 3g), consistent with the prediction of the crystal structure suggesting that APS-2-79 may impede RAF–KSR dimers. In contrast, the control compound APS-3-77 did not impede KSR2–BRAF interactions (Extended Data Fig. 8c). Therefore, we conclude that BRAF can dimerize with KSR2–MEK1 complexes directly via KSR2, and this interaction is antagonized by APS-2-79.

Ras mutations occur in approximately 25% of all cancer patients and are highly associated with poor response to therapy. Significant progress has been made in targeting BRAF/V600E-mutant melanoma, however RAF and MEK inhibitors have failed to achieve significant clinical efficacy in Ras-mutant disease owing in part to mechanisms of inhibitor-induced transactivation and feedback, respectively. MEK-inhibitor feedback has been characterized by upstream Ras activation and induction of higher-order RAF–RAF and also RAF–KSR complexes. In an engineered cell system, we found that a Ras-suppressor allele (R718H) within KSR reduced MEK inhibitor-induced feedback (Extended Data Fig. 4c), suggesting the possibility that KSR heterodimerization may limit the efficacy of MEK inhibitors. Owing to the more pronounced role of KSR in Ras-mutant, as opposed to RAF-mutant signalling, we hypothesized that stabilization of the KSR-inactive state (KSRi) via APS-2-79 may potentiate the effect of MEK inhibitors by limiting feedback in Ras-mutant models. We therefore tested for synergy of APS-2-79 with MEK inhibitors in Ras-mutant cell lines, and used RAF-mutant cell lines as controls.
We found that APS-2-79 shifted the cell viability dose response to trametinib in Ras-mutant cell lines HCT-116 and A549, but not BRAF mutant cell lines SK-MEL-239 and A375 (Fig. 4a). Although the cellular effects of APS-2-79 alone were modest, combination analysis over full concentration matrices revealed that KSRi synergizes with trametinib, and other MEK inhibitors (Extended Data Fig. 9a), specifically in Kras mutant cell lines (Fig. 4b). APS-3-77, and additional control compounds (Extended Data Fig. 9b and 10), did not demonstrate Ras-mutant-specific synergy, supporting the hypothesis that the enhanced activity of trametinib when combined with APS-2-79 depends on co-modulation of KSR. To determine the possible mechanism for APS-2-79 and trametinib synergy, we examined MAPK signalling and found that APS-2-79 treatment caused a twofold enhancement in the IC50 of trametinib on ERK phosphorylation in the Ras-mutant HCT-116 cell line but not the RAF-mutant SK-MEL-239 cell line (Fig. 4c, Extended Data Fig. 4d). The data presented here provide proof-of-concept for the use of KSRi to overcome a key liability of a clinical MEK inhibitor in K-Ras mutant cells. Indeed, we posit stabilization of the KSRi as a mechanism to impede feedback activated Ras–MAPK signalling induced by MEK inhibition (Fig. 4d).

Here we have identified a unique conformation in KSR through the discovery of APS-2-79. This compound offers a foundation for the development of a new class of targeted therapies based on stabilization of the KSR inactive state. Future efforts will be directed towards improving the pharmacological properties of APS-2-79 to enable in vivo and clinical studies. In general, the stabilization of conformational states with small-molecule modulators may be an effective strategy to target other pseudokinases.26,27 Furthermore, the results presented here, using KSRi in combination with clinical MEK inhibitors, suggests a mechanism to improve the efficacy of inhibitors that target enzymatically active kinases through co-modulation of pseudokinase–active kinase signalling complexes.

Online Content Methods, along with any additional Extended Data display items and Online Content, are available in the online version of the paper; references unique to Online Content are available in the online version of the paper; references unique to Online Content are available in the online version of the paper; references unique to Online Content are available in the online version of the paper; references unique to Online Content are available in the online version of the paper; references unique to Online Content are available in the online version of the paper; references unique to Online Content are available in the online version of the paper; references unique to Online Content are available in the online version of the paper; references unique to Online Content are available in the online version of the paper; references unique to Online Content are available in the online version of the paper; references unique to Online Content are available in the online version of the paper; references unique to Online Content are available in the online version of the paper; references unique to Online Content are available in the online version of the paper.

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Author Contributions N.S.D. conducted biochemical, structural, and cell-line studies, A.P.S. synthesized compounds. A.C.D. supervised research. All authors analysed data.

Author Information Coordinates and structure factors have been deposited with the Protein Data Bank under accession code 5KRR. Reprints and permissions information is available at www.nature.com/reprints. The authors declare competing financial interests: details are available in the online version of the paper. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to A.C.D. (arvin.dar@mssm.edu).

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Extended Data Figure 1 | See next page for caption.
Extended Data Figure 1 | Projection of Ras(G12V) suppressor alleles onto the primary and tertiary structure of KSR. a, Schematic representation of KSR from *Drosophila*, *Caenorhabditis elegans*, and KSR1 or KSR2 from humans. Suppressor mutations within KSR identified from forward genetic screens are highlighted with red stars. Allele names and corresponding mutations are given. Two alleles in KSR found in the *Drosophila* screen are shown; one encoding for substitutions in a coil–coil SAM domain (CC-SAM) at the N terminus of *Drosophila* KSR (S548) and a second mutant in the predicted ATP-binding pocket of the KSR pseudokinase domain (S638). Eight distinct alleles were described in two separate studies conducted in *C. elegans*. The vast majority of the mutants localize to the pseudokinase domain of KSR and in particular ATP-contact residues (yellow). Residues highlighted in red and shown in the lower panel correspond to the human KSR2 residue equivalents of suppressor mutations found in *Drosophila* and *C. elegans* orthologues.

b, KSR is a scaffold for the Ras–MAPK signalling pathway. Phosphorylation of MEK1/2 at Ser218 and Ser222 by RAF, or ERK1/2 via phosphorylation at Thr202 and Tyr204 by MEK, are key events in signalling through the Ras–MAPK signalling pathway. c, Purification of the KSR2–MEK1 complex from insect cells. The KSR2 pseudokinase domain (KSR2(KD)) and MEK1 were co-expressed using the SF21 insect cell system. Lysis was performed by one freeze–thaw and sonication. Lysates were incubated with cobalt resin for 2 h and KSR2(KD)–MEK1 was eluted using a high-imidazole buffer. Eluate was then incubated with tomato etch virus (TEV) protease and λ-phosphatase overnight. The mixture was then applied to an ion-exchange column (Sp-HP) to separate stoichiometric KSR2–MEK1 complexes from free MEK1 and TEV. Fractions containing KSR2–MEK1 were applied to a gel-filtration column for final purification. 

d, Schematic of the ATP$_\text{biotin}$ probe-labelling assay on KSR2–MEK1 complexes and screen for inhibitors. e, ATP$_\text{biotin}$ directly labels KSR2 and MEK1 within purified complexes. Deconvoluted mass spectrum for KSR2–MEK1 complexes incubated with ATP$_\text{biotin}$. KSR2 and MEK1 spectra are included in the top and bottom panels, respectively. f, Graphical representation for ATP$_\text{biotin}$ probe-labelling of KSR2–MEK1 complexes in the presence of increasing free ATP as shown in Fig. 1b. Corresponding IC$_{50}$ values listed for both KSR2 and MEK1.
Extended Data Figure 2 | APS-2-79 and APS-3-77 are positive and negative binders of KSR2. a, Chemical structures of APS-2-79 and APS-3-77 with respective IC_{50} values (mean ± s.d.; n = 2 biological replicates) for KSR2. b, Representative western blot images of in vitro ATP_{biotin} competition assays using recombinant MAPK family member proteins. Probe-labelling of the indicated kinases were measured in the presence of increasing concentrations of APS-2-79, APS-3-77, or a positive control compound. For CRAF, BRAF, and BRAF(V600E), the positive control was dabrafenib; for MEK1, the ATP-competitive inhibitor termed Wyeth-2b (ref. 28); and for ERK, SCH722984 (ref. 29). The listed IC_{50} values include mean ± s.d. based on two biological replicates. c, APS-3-77 and APS-2-79 share partially overlapping kinome-wide inhibitory profiles. The graph shows the percentage of inhibition of APS-2-79 and APS-3-77 (both at 1 μM) against 246 kinases. The raw data for this graph is in Supplementary Table 1. d, Inset showing the 25 kinases most inhibited by APS-2-79 and APS-3-77. Kinases with near-equal sensitivity to these inhibitors as measured here include YES1, ERBB4, and FGR; variable sensitivity kinases include CSK, HCK, and MERTK.
Extended Data Figure 3 | See next page for caption.
Extended Data Figure 3 | APS-2-79 hinders RAF-mediated MEK phosphorylation in a KSR-dependent manner. a, Schematic of the RAF phosphorylation assay of free KSR2–MEK1 and MEK1. b, Phosphorylation of the indicated concentrations of MEK1 and the KSR2–MEK1 complex by BRAF (200 nM) in the presence of 1 mM ATP. Representative blots for phospho-MEK (top; as detected using a MEK1/2(pS218/pS222) antibody) and total MEK (tMEK; bottom) are shown. c, Plots of pMEK versus time (seconds) at various concentrations of MEK1 and the KSR2–MEK1 complex. Bands were quantified and the phospho-MEK signal normalized relative to lane 20 in both panels. Data points of two biological replicates are included along each line. The rate of MEK phosphorylation (Kobs; pMEK per second; far right) are represented in bar graphs and are derived from the linear phase of the plots in the left hand panels. Bars represent mean of two biological replicates; values for each replicate are shown as points. d, Rates of BRAF (left) and CRAF (right) phosphorylation of the indicated MEK complexes (KSR2–MEK1; KSR2(A690F)–MEK1; and free MEK). Bars represent mean of two biological replicates; values for each replicate are shown as points. e–g, APS-2-79 inhibits BRAF and CRAF phosphorylation of MEK in a KSR-dependent manner. Phosphorylation of 500-nM KSR2–MEK1 (e), or KSR2(A690F)–MEK1 (f), and MEK1 (g) by BRAF (200 nM) or CRAF (10 nM) in the presence of 1–mM ATP and the indicated inhibitors. Representative western blots of phospho-MEK (as detected using a MEK1/2pS218/pS222 antibody) are shown. Bars represent mean of two biological replicates; individual data points of each replicate are shown.

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Extended Data Figure 4 | See next page for caption.
Extended Data Figure 4 | APS-2-79 activity is not dependent on KSR phosphorylation sites in MEK or direct RAF inhibition. a, APS-2-79 does not affect BRAF(V600E)-induced MAPK activation in cells. 

BRAF(V600E)–Flag was expressed for 24 h in 293H cells. Cells were then treated for 2 h with DMSO or 5 μM of either APS-2-79, APS-3-77, or dabrafenib before collection and western blot analysis of phosphorylated MEK (MEK1/2(pSer218/pSer222)) and ERK (ERK1/2(pT202/pY204)).

b, Removal of putative KSR phosphorylation sites in MEK (MEK(AAAA); S18A, T23A, S24A, S72A; ref. 7) neither hinders KSR-dependent MAPK signalling, nor the activity of APS-2-79. Co-expression of full-length KSR–Flag and wild-type MEK1–GFP or MEK(AAAA)–GFP leads to enhanced MAPK signalling within 293H cells as visualized by immunoblotting for phosphorylated MEK (MEK1/2(pSer218/pSer222)) and ERK (ERK1/2(pT202/pY204)). APS-2-79 impedes KSR-stimulated MAPK signalling within cells through wild-type and MEK(AAAA) equally. Bars and error bars indicate pMEK and pERK intensity and standard deviations, respectively. Signals were normalized relative to lane 5. Error bars indicate the mean ± s.d. (n = 3 biological replicates). 

***P < 0.0005 by two-tailed unpaired t-testing. c, The dimer-deficient KSR(R718H) mutant, relative to wild-type KSR, is compromised in MEK-inhibitor-induced feedback. 293H cells were co-transfected with MEK–GFP and KSR–Flag or KSR(R718H)–Flag for 24 h and then treated with increasing concentrations of trametinib (range of 0.13 to 100 nM; threefold dilutions) for an additional 48 h. Cells were collected and analysed by western blot. d, Phospho-AMPK remains unchanged in HCT116 cells upon co-treatment with APS-2-79 and trametinib. HCT116 cells were treated with APS-2-79 and/or trametinib for 48 h. Phospho-AMPK (top), phospho-ERK(pERK), and total MEK (bottom) western blots are shown.

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Extended Data Figure 5 | Higher order assembly of the KSR2–MEK1 complex bound to ATP or APS-2-79. a, Assembly of the KSR2–MEK1 heterodimer bound to APS-2-79. A crystal-packing two-fold symmetry axis of the asymmetric unit containing a single KSR2–MEK1 complex produces the heterotetramer. KSR2 bound to APS-2-79 is coloured green, and MEK1 is coloured red. The activation segments of KSR2 and MEK1 are coloured orange and white, respectively. The ‘induced lock’ (residues 809 to 814) within KSR2 is highlighted as orange, red and blue spheres.
b, Assembly of the KSR2–MEK1 heterodimer bound to ATP as reported ref. 7 (PDB code: 2Y4I). A crystallographic two-fold rotation axis produces the heterotetramer. r.m.s. deviation between the heterodimer and heterotetramers, respectively, of the ATP- and APS-2-79-bound KSR2–MEK1 complexes are listed below. c, A model for APS-2-79 function as a KSR-targeted antagonist of MAPK signalling. APS-2-79 shifts the equilibrium of KSR2–MEK1 complexes so to populate the OFF state (left), and thereby antagonizes RAF dimerization and subsequent phosphorylation of KSR-bound MEK (far right). The model for RAF dimerization and MEK phosphorylation are adapted from ref. 7. In this model, the role of RAFcat may be fulfilled by multiple active RAF-family kinases, such as C-RAF, bound within homo- or heterodimers of RAF–RAF or KSR–RAF, respectively.
Extended Data Figure 6 | See next page for caption.
Extended Data Figure 6 | The APS-2-79 binding site within KSR2 and possible basis for KSR over RAF selectivity. a, APS-2-79 and ATP are overlaid in the KSR2 and MEK1 active sites, respectively. ATP was shown here to emphasize the MEK active site, but ATP was not included in the final model. Positive (blue) and negative (red) $F_o - F_c$ electron density maps, calculated before modelling of APS-2-79, are contoured at 3.5σ. Strong-positive-difference density within KSR2 supported modelling of APS-2-79 bound to KSR2 within the KSR2–MEK1 complex. b, Electron density map (blue mesh) for APS-2-79 (sticks) contoured at 4.5σ. Map represents positive difference density within the KSR2 active site before modelling of APS-2-79. c, Superposition of KSR2 (ATP- and APS-2-79-bound) with BRAF monomer (PDB code: 4W05) and BRAF dimer (PDB code: 3C4C) co-crystal structures reveals the possible bases for selectivity of APS-2-79 for KSR over RAF proteins. Residues within the APS-2-79 binding pocket that diverge between KSR and RAF proteins, but which are highly conserved within both sub-families are indicated with arrows. Thr802 in KSR2, which is universally a Gly residue in all active RAF homologues, and also Phe516 and Phe793 in KSR2, which adopt distinct orientations from the equivalent Phe residues in RAF kinases, directly contact the biphenyl ether motif in APS-2-79. The T802G substitution, as well as the positional differences of the above-mentioned aromatic residues, would be predicted to reduce binding of active RAFs with APS-2-79. Another interaction that is probably favoured in KSR includes the contact mediated by the epsilon nitrogen of Arg692 with the –O- linker of the biphenyl motif; the placement of Arg692 is stabilized by Asp803 of the DFG motif. In RAF, the Arg-to-Lys substitution (Lys483 in subdomain II of BRAF), lacks the equivalent nitrogens to bond with both the –O- linker in APS-2-79 and the aspartate of the DFG motif. d, Positive (blue) and negative (red) $F_o - F_c$ electron density map contoured at ±2.5σ, before modelling of residues I809 to Q814 in KSR2, is shown. e, Sequence alignment of KSR and RAF proteins. Arrows highlight APS-2-79 contact residues.
Extended Data Figure 7 | In vitro ATP\textsuperscript{biotin} competition assays.
Representative western blot images of in vitro ATP\textsuperscript{biotin} competition assays using recombinant KSR2–MEK1 and analogues reported in this study. Chemical structures are shown adjacent to assay blots. IC\textsubscript{50} values (mean ± s.d.; n = 2 biological replicates) against ATP\textsuperscript{biotin} probe-labelling of KSR2 are listed below blots. Line graphs include data points from two biological replicates.
Extended Data Figure 8 | See next page for caption.
Extended Data Figure 8 | Bio-layer interferometry binding data between BRAF and free MEK1 or the KSR2–MEK1 complex. a, Mapping of residues with a r.m.s. deviation of greater than 2.0 Ångstrom between the ATP- and APS-2-79-bound states of KSR2–MEK1 (right, blue), highlights alterations at contact residues Trp685 and His686 within the KSR–KSR homodimer (left, yellow) and KSR–RAF heterodimer (middle, orange) interfaces. b, Movement of Trp685–His686 within KSR2 between the ATP- and APS-2-79-bound states. A single protomer of KSR2 in the ATP-bound state (yellow), and both protomers (green and cyan) of the KSR2 dimer within the APS-2-79-bound state, are shown. Negative density around W685 and His686 in early-stage maps supported the conformational change in this loop between the ATP- and APS-2-79-bound states. c, The negative control compound APS-3-77 (25 μM) does not impact assembly of BRAF(F667E) and KSR2–MEK1. These assays were performed identically to the experiments in Fig. 3b–g. Coloured curves indicate dose ranges of KSR2–MEK1 or MEK1 from 625 nM to 10 μM in the presence or absence of the indicated compounds. In all plots, association occurred from 0 to 660 s, and dissociation was monitored thereafter up to 1500 s. d–e, Biolayer interferometry of wild-type BRAF with MEK1 and KSR2–MEK1 in the presence of DMSO and 25 μM APS-2-79. These assays were performed identically to the experiments in Fig. 3b–g. Coloured curves indicate dose ranges of KSR2–MEK1 or MEK1 from 625 nM to 10 μM in the presence or absence of the indicated compounds. In all plots, association occurred from 0 to 660 s, and dissociation was monitored thereafter up to 1500 s. f, Table summary of BLI data in this figure and Fig. 3b–h. \( K_d \), \( K_{on} \), and \( K_{off} \) values represent the mean and s.e.m. measurements derived from global fitting of 5 binding curves. \( \chi^2 \) and \( R^2 \) describe experimental and model data correlations; < 3 and above 0.95, respectively, indicate good fits.
Extended Data Figure 9 | See next page for caption.
Extended Data Figure 9 | KSRI binder APS-2-79 synergizes with trametinib in Ras-mutant cells. 
a. Average Bliss score of the combination of trametinib, binimetinib, PD0325901, or AZD6244 with APS-2-79 in the Ras-mutant cell lines HCT116 and A549 versus the RAF-mutant cell lines A375 and SK-MEL-239. Full combination matrices of APS-2-79 (range: 100 nM to 3 μM in threefold dilutions) with trametinib (range: 0.01–100 nM in threefold dilutions), binimetinib (range: 0.1–10 μM in threefold dilutions), PD0325901 (range: 0.1–10 μM in threefold dilutions), and AZD6244 (range: 0.1–10 μM in threefold dilutions). Bars represent the mean Bliss scores calculated from two biological replicates of the depicted concentration matrices; points represent each calculated score. 
b. Average Bliss scores of APS-2-79 or APS-3-77 in combination with trametinib in RAF-mutant, RAS-mutant cell lines. SK-MEL-2 and HepG2 are N-Ras-mutant cell lines, and MEWO is a NF1-mutant cell line. Bars represent the mean Bliss scores calculated from two biological replicates of the depicted concentration matrices; points represent each calculated score. 
c. Complete cell viability analysis of APS-2-79 (range: 100–3,000 nM in threefold dilutions) plus trametinib (range: 0.01–100 nM in threefold dilution) over a full concentration matrix in the Ras-mutant LOVO, CALU-6, SW620, SK-MEL-2, and HEPG2 cell lines, the RAF-mutant COLO-205, H2087, and SW1417 cells, and the NF1-mutant MEWO cell line. Numbers listed within synergy matrices represent percentage of growth inhibition relative to DMSO control and are the mean of two biological replicates. Bliss scores represent the mean calculated from two biological replicates of the depicted concentration matrices.
Extended Data Figure 10 | APS-2-79 synergizes with trametinib specifically in Ras-mutant cells compared to the HER-family and SRC-family inhibitors lapatinib and sarcatinib. a, Chemical structures of APS-2-79 and quinazoline-containing kinase inhibitors sarcatinib and lapatinib. The primary targets for sarcatinib and lapatinib are c-Src and Her2, respectively. IC₅₀ values against ATPbiotin probe-labelling of KSR2 are listed below structures. b, Bliss score analysis of HCT-116, A549, A375, and SK-MEL-239 cells treated with APS-2-79, sarcatinib, or lapatinib (range: 100–3,000 in threefold dilutions) in combination with trametinib (range: 0.01–100 in threefold dilution). Bars represent the mean Bliss scores calculated from two biological replicates; points represent each calculated score. c, Absolute Bliss score of the indicated drugs in combination with trametinib in Ras-mutant relative to RAF-mutant cell lines demonstrates selective synergy in Ras-mutant cell lines for APS-2-79 compared to sarcatinib and lapatinib. d, log of the combination index graphs of APS-2-79 in combination with trametinib in HCT-116 versus SK-MEL-239 cells as compared to the fractional effect. Negative combination index over a broad fractional effect range within HCT-116, but not SK-MEL-239, indicates strong synergy.
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