De-risking drug discovery of intracellular targeting peptides: screening strategies to eliminate false-positive hits

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Abstract

Non-specific promiscuous compounds can mislead researchers and waste significant resources. This phenomenon, though well-documented for small molecules, has not been widely explored for the peptide modality. Here, we demonstrate that two purported peptide-based KRas inhibitors, SAH-SOS1$_A$ and cyclorasin 9A5, exemplify false-positive molecules – both in terms of their binding affinities and cellular activities. Through multiple gold-standard biophysical techniques, we unambiguously show that both peptides lack specific binding for KRas and instead induce protein unfolding. Although these peptides inhibited cellular proliferation, the activities appeared to be off-target based on counter-screen with KRas-independent cell lines. We further demonstrate that their cellular activities are derived from membrane disruption. Accordingly, we propose that, to de-risk false-positive molecules, orthogonal binding assays and cellular counter-screens are indispensable.

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Introduction

Drug discovery programs often initiate with screens designed to identify binding molecules that modulate their targets in a specific manner. Ensuring authentic binding is critical as false-positive binders occur with high frequency.[1-2] These molecules can propagate through the literature[3] to cause a significant waste of resources, both by academic groups who may use these as biological probes, and by drug discovery teams who may attempt to advance these molecules toward the clinic.[4]

Non-specific modulation of target activity has been recognized for sometime and can occur through several mechanisms[5] including covalent reactivity, redox cycling, fluorescence interference, membrane disruption, and the formation of colloidal aggregates, with the latter being perhaps the most common.[6-7] In seminal work,[8] Shoichet and colleagues proposed that the colloidal aggregates exert their effects by enzyme sequestration, thereby blocking and inhibiting their activities in unexpected ways. Aggregators are not exclusive to synthetic small molecules. They include natural products[9] and marketed drugs.[10] Besides enzymes, targets mediating protein-protein interaction are affected.[11-12] Importantly, these artefacts can be dose-dependent, reproducible, and challenging to identify. Therefore, biochemical assays often include detergents such that the negative effects of colloidal aggregates can be reduced or eliminated.[13]

While these issues are well characterized for small molecules, their impacts on peptides are relatively under-explored. Recent data has supported macrocyclic peptides as a promising modality for targeting protein-protein interactions, which are often intractable with the small-molecule modality.[14-15] Indeed, macrocyclic peptide can exhibit highly specific 1:1 stoichiometric binding and on-target cellular activities.[16] These efforts have translated ALRN-6924 into clinical trials.[17] Despite these encouraging developments, we describe herein that two widely cited publications[18-19] reporting macrocyclic peptides, SAH-SOS1$_A$ and cyclorasin 9A5, as specific KRas inhibitors, are in fact false positives, both in terms of their binding affinities and cellular activities. Critically, our assays are validated with another macrocyclic peptide (KRpep-2d),[20] a positive control supported by structural-activity-relationships[21] and x-ray crystallography.[22]

Results and Discussion

FAM-SAH-SOS1$_A$ is a Promiscuous Binder. To disrupt KRas–SOS1 interaction, Walensky and co-workers[18] designed an 18-residue stapled peptide, named SAH-SOS1$_A$, by incorporating an *i*, *i*+4 hydrocarbon linker at the non-interacting face of the SOS1 α-helix (929–944) and two extra arginines at the N-terminus.[18] Using fluorescence polarization (FP), the investigators showed that fluorescein-labeled SAH-SOS1$_A$ (FAM-SAH-SOS1$_A$) could bind to wild-type, and five others mutant KRas with sub-micromolar dissociation constants (100–175 nM). We were able to reproduce the binding isotherm of FAM-SAH-SOS1$_A$ using several forms of GDP-loaded KRas (G12D, Q61H, and wild-type, Figure 1A, S1), although the bindings appear to be weaker (K_D = 430–710 nM). The FP binding curve adopted a sigmoidal shape and seemed saturated, despite the sub-optimal fitting (R^2 = 0.812, Figure 1A). However, the apparent bindings were abolished upon addition of non-ionic detergent (0.01% v/v Tween 20) to the assay buffer. To check for promiscuous binding, we separately titrated two unrelated proteins, MDM2 (Figure 1B) and eIF4E (Figure 1C), into FAM-SAH-SOS1$_A$ and demonstrated comparable binding affinities. These interactions were also detergent-sensitive. To ensure this assay format is functional for KRas, we used KRpep-2d as the positive control.[20] Indeed, FAM-KRpep-2d bound tightly to KRas (K_D = 16 nM, Figure 1D) in the presence of detergent. Again, non-specific activities occurred for unrelated proteins (Figure 1E-F) when detergent is omitted. We also tested FAM-SAH-SOS1$_B$, whose hydrocarbon linker is installed at the interacting face of the helix and was reported as a KRas non-binder.[18] However, we found that FAM-SAH-SOS1$_B$ could bind to KRas as well as the unrelated proteins, albeit with lower FP signals (Figure S2) that could be abrogated by adding detergent. Conversely, fluorescein-labeled ATSP-7041 (A^8Q, Q^9L), a progenitor of the first stapled peptide entering clinical trials,[17] could bind to its target MDM2 in the presence of detergent, suggesting a *bona fide* interaction (Figure S2). In contrast, FAM-SAH-SOS1$_A$ and FAM-SAH-SOS1$_B$ bound to all proteins we have investigated, and the binding interactions are always partially or completely abolished by detergent.

Mechanisms for the False-positive Readout in Fluorescence Polarization. We hypothesize that, in the absence of detergent, two scenarios could contribute to the false-positive FP signals: (i) FAM-labeled peptide non-specifically adsorbs to the microplate's plastic surface, and/or (ii) FAM-labeled peptide forms a colloidal aggregate which then sequesters protein. Under both

circumstances, the apparent "hydrodynamic size" of the labeled peptides increase, leading to slower tumbling rates, an increased FP signals, and the false impression of specific target binding.

To assess the effect of microplates, we explored (i) uncoated polypropylene microplate (Figures 1, S1, and S2), (ii) uncoated polystyrene microplate, and (iii) polystyrene microplate coated with a non-ionic hydrophilic material. We observed apparent binding of FAM-SAH-SOS1$_A$ regardless of whether polystyrene or polypropylene microplates were used (Figure 2A and 2B). The apparent binding disappeared once we added detergent or used the polystyrene microplate with a non-binding surface (Figure 2C). Although the amount of KRas protein added to the sample did increase the FP signal, we are confident the effect is non-specific for two reasons. First, addition of non-ionic detergent abrogated the FP signal, presumably by preventing non-specific interactions between the peptide and the protein, plastic surface, or protein-coated plastic surface. Second, no binding was observed in the coated microplate (Corning NBS^{TM}), regardless of protein concentration. Thus, it appeared that the increased FP signal was related to non-specific adherence of FAM-SAH-SOS1$_A$ to the plastic surface or protein-coated plastic surface rather than a specific biomolecular interaction with KRas. In contrast, KRpep-2d always retained its binding to KRas under all circumstances (with or without detergent) regardless of the nature of the microplate (Figure 2D-F). Interestingly, we found that the adsorption of FAM-SAH-SOS1$_A$ to the uncoated microplate is time-dependent. Specifically, a global increase FP signal was observed when we delayed the signal measurement by including a pre-incubation time (Figure S3). To rule out the possibility that KRas was contributing to the signal increase, we monitored the FP signals of FAM-SAH-SOS1$_A$ alone over a course of three hours (Figure S4). The signal should remain flat in the absence of protein. However, we observed time-dependent signal increase, a phenomenon that likely occurs from adsorption of peptide to plastic surfaces. All data above, thus far, support our hypothesis that adsorption of FAM-labeled peptide to the plastic surface is responsible for the apparent binding observed in the FP assays.

Detection of Aggregators by Dynamic Light Scattering. Alternatively, colloidal aggregates of FAM-labeled peptides may sequester proteins and give false-positive FP readouts. Such interference is usually time- and concentration-dependent,[8] and the readouts are sensitive to detergent.[13] To characterize the peptides, we employed dynamic light scattering (DLS), a universal technique for monitoring aggregation. We observed strong and well-defined

autocorrelation functions suggesting aggregation at 10 μM concentrations of FAM-SAH-SOS1$_A$ and FAM-SAH-SOS1$_B$ (Figure 3). Both peptides gave strong scattering intensities (5700–7400 kcps for FAM-SAH-SOS1$_A$ and 12600–14500 kcps for FAM-SAH-SOS1$_B$), much higher than the buffer alone (35–36 kcps). Particle hydrodynamic diameters ranged from 350 to 370 nm for FAM-SAH-SOS1$_A$ and 1200 to 1600 nm for FAM-SAH-SOS1$_B$. However, we could not detect reproducible and well-defined DLS signals for SAH-SOS1$_A$, SAH-SOS1$_B$ and cyclorasin 9A5 (data not shown), suggesting an absence of stable aggregates at 10 μM. Interestingly, ATSP-7041 (A^8Q, Q^9L) showed aggregation at 10 μM (Figure S5), yet is a specific inhibitor of MDM2 when used at lower concentrations (<100 nM, Figure 4).

Surface Plasmon Resonance Confirms SAH-SOS1$_A$ and Cyclorasin 9A5 as Non-binders for KRas. To gain further insights into the binding behaviors of these peptides, we employed surface plasmon resonance (SPR) as an orthogonal assay. Consistent with FP, we found no KRas binding for SAH-SOS1$_A$ and cyclorasin 9A5, up to 1 μM (Figure 4). Similarly, FAM-SAH-SOS1$_A$ also did not bind to KRas by SPR (Figure S6). To ensure that the immobilized KRas is competently-folded, we tested KRpep-2d which bound to KRas with saturable binding responses (Figure S7). Paradoxically, when tested against the irrelevant proteins MDM2 and eIF4E, both SAH-SOS1$_A$ and cyclorasin 9A5, gave significant but non-specific SPR binding readouts that did not fit a 1:1 binding model (Figure 4; similar results for FAM-SAH-SOS1$_A$, Figure S6). Indeed, at the highest concentration tested (1 μM), binding signal of cyclorasin 9A5 is functionally irreversible, displaying extremely slow dissociation.

It is unclear how SAH-SOS1$_A$ and cyclorasin 9A5 interact with MDM2 and eIF4E. Previously, SPR was demonstrated as a sensitive technique to recognize aggregates early in the discovery process.[23] Adsorption of massive aggregates onto the biosensor surface can amplify the SPR signals.[23] We postulate that this is likely the cause of the non-saturable and non-one-to-one binding SPR signals seen with SAH-SOS1$_A$ and cyclorasin 9A5. In contrast, two control peptides ATSP-7041 (A^8Q, Q^9L) and PHAGESOL[24] bound, with 1:1 saturated binding, to their respective targets (MDM2/eIF4E) but not KRas (Figure 4).

Isothermal Titration Calorimetry and Thermal Shift Assay Confirms SAH-SOS1$_A$ and Cyclorasin 9A5 as Non-binders for KRas. To cross-validate our FP and SPR data, we used isothermal titration calorimetry (ITC), a rigorous technique for measuring biomolecular interactions directly under homogenous condition. This technique further confirmed that neither

SAH-SOS1$_A$ nor cyclorasin 9A5 bound to KRas G12D (Figures 5A and 5C). Cylcorasin 9A5 also did not bind to KRas G12V, the protein used for discovery of the macrocyclic peptide (Figure 5D). The heat released during the titration of SAH-SOS1$_A$ to the protein or to the buffer itself are almost identical (Figure 5A–B). In contrast, we showed that KRpep-2d binds to KRas G12D with a K_D of 21 nM and with a stoichiometry close to unity (Figure 5E). Wild-type KRas adopts distinct conformations when bound to different nucleotides. As a confirmatory experiment, we show that wild-type KRas binds to its downstream effector (Raf-RBD, K_D = 6 nM) only when KRas is activated with GMPPNP but not with GDP (Figure 5F–G).

Further evidence that SAH-SOS1$_A$ and cyclorasin 9A5 are false positive came from thermal shift experiments since both peptides produced a negative shift in the melting temperatures (ΔT_m) of KRas G12D (Figure S8), suggesting ligand-induced destabilization. At higher concentrations, SAH-SOS1$_A$ displayed high initial background probably due to aggregated peptide-dye interactions (Figure S8A). In contrast, positive control KRpep-2d stabilized KRas with a ΔT_m of +11.5 °C (Figure S8C), indicating the assay is functional. To ensure our biophysical observations were not unique to cyclorasin 9A5, we tested a closely related analog, cyclorasin 9A16, a molecule with reported IC_{50} values that were similar to cyclorasin 9A5's in both the inhibition of KRas-Raf interaction and anti-proliferative assay.[19] Once again, the thermal shift assay produced a negative ΔT_m of -5.9 °C for cyclorasin 9A16 (Figure S9A). SPR and ITC experiments further confirmed cyclorasin 9A16 as a non-binder, i.e., false positive, for KRas (Figure S9B–C).

HDX-MS Confirms SAH-SOS1$_A$ and Cyclorasins Destablize KRas. The negative ΔT_m in the thermal shift assay suggests the peptides may destabilize KRas. To confirm these observations, hydrogen-deuterium exchange mass spectrometry (HDX-MS) was used to monitor changes in deuterium uptake across the protein amides in response to peptide binding. HDX-MS experiments of KRas identified 88 pepsin-proteolyzed peptides with primary sequence coverage of 100 % (Figure S10), allowing us to assess binding or destabilization across the entire sequence.

As a validation experiment, we used an arginine-deleted variant of KRpep-2d, (KRpep-2d (Arg del), Figure S7), due to its superior solubility versus KRpep-2d. The corresponding data gave a HDX protection pattern highly consistent with the binding site observed in the KRas/KRpep-2d co-crystal structure,[22] thus validating the technique's ability to faithfully map

the peptide-binding sites (Figure 6D and 6F). In contrast, the experiments involving SAH-SOS1$_A$ and cyclorasin (9A5 and 9A16) demonstrated a complete absence of deuterium protection and agreed well with the destabilization (Figure 6E) observed in the thermal shift assay (Figure S8 and S9A). All three peptides showed similar deuterium uptake profiles suggestive of similar destabilizing effects on KRas.

Molecular simulations of the putative KRas-peptide complexes. A series of peptides inspired by the observations of Walensky and colleagues[18] were designed and docked to the structure of Ras complexed to SOS. These peptides were subject to MD simulations to monitor their stability. None of the peptides (SOS1$_A$ unstapled and stapled, cyclorasin 9A5) were found to remain in the initial (and speculated) locations and drifted away (Figures S11–12); agreeing with the lack of specific binding seen experimentally.

SAH-SOS1$_A$ and Cyclorasin 9A5 can Disrupt Cell Membranes and Trigger Cell Lysis. With multiple techniques showing that SAH-SOS1$_A$ and cyclorasin 9A5 are false-positive binders of KRas, we were perplexed by the reported biological activities.[18-19] Release of intracellular lactate dehydrogenase (LDH) is a common method used to directly quantify the degree of cell membrane rupture. We detected appreciable LDH leakage for SAH-SOS1$_A$ (EC_{50} = 10 μM), SAH-SOS1$_A$ with unstapled hydrocarbon (EC_{50} = 8 μM) and cyclorasin 9A5 (EC_{50} = 30 μM), potencies that are comparable to that of iDNA79 (EC_{50} = 20 μM), a cationic amphipathic stapled peptide we use as a positive control (Figure 7). In contrast, LDH release was insignificant (< 10%) for SAH-SOS1$_B$, Aib-SOS1$_A$, cyclorasin 12A, ATSP-7041 (A^8Q, Q^9L) and PHAGESOL, and minimal (< 30%) for cyclorasin 9A54, up to 50 μM of the concentration tested.

We performed the LDH release assay under serum-free condition which is the same condition used in the published report.[18] We incubated HCT116, a colorectal cell line which harbors mutant $KRas^{G13D}$, with peptides for 4 hours. We expect that rapid cell death within this time-frame is unlikely the result of apoptosis due to KRas signaling blockade. The most likely cause is the rupture of cellular membrane induced by the peptides. Interestingly, these undesired toxicities are only observed for SAH-SOS1$_A$ and its unstapled hydrocarbon analog, but not for its linear version (Aib-SOS1$_A$), and the negative control (SAH-SOS1$_B$) where the stapled hydrocarbon is placed at a different face of the helix. Minimal LDH leakage is also observed for the analogs of cyclorasin 9A5, such as cyclorasin 9A54 and cyclorasin 12A. Coincidentally, the degree of membrane disruption caused by the SAH-SOS1$_A$, cyclorasin 9A5 and their non-toxic

analogs is consistent with the cytotoxicity reported in the publications.[18-19] In a different report, Bird *et al.* disclosed that SAH-SOS1$_A$ did not induce LDH release.[25] However, the tested peptides are distinct from the original SAH-SOS1$_A$[18] in that the staple length is different and with two arginine residues removed.

SAH-SOS1$_A$ and Cyclorasin 9A5 Exhibit Off-target Cytotoxic Activities in KRas-independent Cell Lines. To further investigate the promiscuous cellular activities, we assessed the effects of SAH-SOS1$_A$ and cyclorasin 9A5 on cell lines (U-2 OS and A549) that do not require KRas for viability and downstream KRas signaling. These lines are not responsive to KRas genetic knockdown.[26-27] At concentrations above 20 μM, both peptides induced strong anti-proliferative effects with very steep dose-response curves on both cell lines in the presence of 10% serum (Figure 8A), suggesting off-target mechanisms.

We further investigated the effect of the peptides on the KRas signaling pathways using U-2 OS cells (Figure 8B). We showed that SAH-SOS1$_A$ but not cyclorasin 9A5 could inhibit the phosphorylation of Erk, a KRas downstream target, at 20 μM concentration with just 15 minutes of treatment. This result should be interpreted with caution. It is premature to regard the reduced downstream cellular responses as a confirmation of target engagement. Previously, we showed that SAH-SOS1$_A$ triggered rapid cell lysis at EC_{50} of 10 μM. Therefore, the attenuation of phosphorylated Erk observed here may arise from the lysed cells having disrupted membrane-bound KRas and/or compromised cellular machineries which become unresponsive to stimulation by recombinant human EGF. Consistent with the LDH data, our results for the anti-proliferation of KRas-independent cell lines and reduced KRas signaling support the interpretation of the cell death caused by the peptides as non-specific and off-target.

Concluding Remarks

FP is a popular technique[28] for measuring protein-protein interactions due to its advantages of high-throughput, mix-and-read format, and cost-effectiveness. With well-validated probes, competitive FP is indeed a powerful screen for large compound libraries. However, direct-binding FP assays are prone to false-positive results, which are most likely derived from the adherence of the peptide to the plastic surface or protein-coated plastic surface.To mitigate such interference, we urge researchers to include mild detergent and use coated microplates for FP assays. Furthermore, by employing SPR and ITC as the orthogonal assays, we unambiguously

showed that SAH-SOS1$_A$ and cyclorasin (9A5 and 9A16) do not bind to the mutant KRas whereas the positive control (KRpep-2d) does. These results were confirmed by both thermal shift assay and HDX-MS, where the false-positive peptides induced unfolding of KRas.

We also showed that SAH-SOS1$_A$ and cyclorasin 9A5 triggered LDH release by disrupting the cell membrane. Amphipathic peptides with positive charges at one face and hydrophobic patch at the other tend to disrupt cell membranes.[29] Indeed, SAH-SOS1$_A$ and cyclorasin 9A5 fit this pattern. Moreover, we observed strong anti-proliferative activities of SAH-SOS1$_A$ and cyclorasin 9A5 for KRas-independent cell lines, most likely through off-target effects by the disruption of the cell membrane, rather than specific blockade of KRas signaling.

Besides KRas, our screening strategies are also applicable to other target classes involved in protein-protein interactions. Our findings make a strong case for using reporter-free systems such as SPR and ITC as a secondary assay for binding validation. SPR is very sensitive to non-specific binding. Non-saturable SPR signals could imply aggregation or some arcane activity.[23] In such cases, ITC should be employed to ensure getting the ideal 1:1 stoichiometric binding. We also suggest LDH release assay as a convenient counter-screen to ensure that the observed biological activities do not arise from membrane disruption. In contrast to cell viability assays or indirect measurement of downstream cellular responses, direct intracellular target engagement assays are better tools to prove that the desired target is indeed bound in the cellular context. For instance, CETSA[30] and NanoBRET[31] are increasingly employed to distinguish on-target versus off-target cancer-killing effects.

This report documents an extreme case where the molecules investigated do not bind to their target *in vitro*, yet exhibit an apparent cellular activity. In other cases, *bona fide* binders may have confounding cellular activities because of membrane lysis.[32] However, molecules with cellular activity at concentrations well below their membrane lysis threshold remain of interest. Indeed, select examples have been demonstrated to have on-target activity *in vitro* and *in vivo*.[16, 33] We hope that adhering to the principles outlined herein will de-risk assay interference, reveal promiscuous target-unrelated activity, thereby improving the quality of publications to promote a productive research community.

Supporting Information

This material is available free of charge at http://pubs.acs.org.

Experimental details for peptide synthesis, FP assay, DLS, SPR, ITC, thermal shift assay, HDX-MS, LDH release assay, cellular growth assay, cell lysis, Western blotting, protein expression, biotinylation of protein, computational modeling, and HPLC/MS spectra of peptides.

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Figure 1. FP assays were performed in polypropylene microplate using FAM-SAH-SOS1$_A$ (15 nM) titrated with three distinct proteins separately. The peptide demonstrated binding to (A) KRas G12D, (B) MDM2, and (C) eIF4E. However, the bindings were attenuated in the presence of detergent. In contrast, FAM-KRpep-2d (15 nM) maintained its binding to (D) KRas G12D but not to (E) MDM2 and (F) eIF4E upon addition of detergent. Data are mean of triplicates ± SD. R^2 corresponds to the fitting of no-detergent data (solid circle).

Figure 2. The nature of microplate significantly affects the FP assays. (A) Uncoated polypropylene and (B) polystyrene microplate are hydrophobic and adsorbed the peptide and protein in the absence of detergent. (C) In contrast, the apparent binding of FAM-SAH-SOS1$_A$ to KRas disappeared in microplate coated with a hydrophilic material (Corning NBS^{TM}). (D-F) Binding of FAM-KRpep-2d to KRas is maintained under all circumstances. Data are mean of triplicates ± SD.

Figure 3. Normalized autocorrelation functions of dynamic light scattering. Each peptide (10 μM) underwent three independent measurements at 25 °C. All data are plotted (range of the particle size in bracket).

Figure 4. Surface plasmon resonance. We injected each peptide (in column) over the individual flow cell (in row) immobilized with KRas (~400 RU), MDM2 (~1200 RU) and eIF4E (~1600 RU). SAH-SOS1$_A$ (1 μM from top, 2× dilution) and cyclorasin 9A5 (1 μM from top, 2× dilution), did not bind to KRas but demonstrated promiscuous binding to MDM2 and eIF4E. Whereas, ATSP-7041 (A^8Q, Q^9L) (50 nM from top, 2× dilution) and PHAGESOL (300 nM from top, 2× dilution) demonstrated saturated 1:1 binding for their corresponding protein targets. Running buffer includes 0.01% Tween 20. Black lines depict the double-referenced sensograms; red lines depict the global fit of the data to a 1:1 binding model.

Figure 5. Isothermal titration calorimetry. The heat changes from titrating SAH-SOS1$_A$ into (A) KRas G12D or into (B) buffer alone. The heat changes from titrating cyclorasin 9A5 into (C) KRas G12D or (D) KRas G12V. (E) KRpep-2d demonstrated tight binding to KRas G12D. Raf-RBD demonstrated tight binding to (F) activated wild-type KRas (GMPPNP) but no binding to

the (G) inactive wild-type KRas (GDP). Assay buffer includes 0.03 mM n-dodecyl β-D-maltoside as detergent.

Figure 6. HDX-MS. Y-axis represents differences in deuterium uptake (Da) in the unbound KRas subtracted from that of the peptide-bound KRas. X-axis represents pepsin-proteolyzed peptides (N- to C-terminus). Each dot represents the difference at 1min or 5 min of deuterium labeling. Grey bars represent sum of difference at both timepoints. In the presence of (A) cyclorasin 9A5, (B) SAH-SOS1$_A$, and (C) cyclorasin 9A16, no protection of KRas is observed suggestive of global destabilization/unfolding. Whereas, (D) KRpep-2d (Arg del) confers protection in deuterium uptake at the expected binding regions of KRpep-2d (boxed in red). (E) Residues with absolute difference >0.5 Da in the presence of cyclorasin 9A5 are mapped onto KRas (in blue). (F) Residues protected from deuterium exchange (red box in (D)) in the presence of KRpep-2d (Arg del) are mapped onto KRas (PDB: 5XCO)

Figure 7. Lactate dehydrogenase (LDH) release assay. Maximum LDH release was defined as the amount of LDH released induced by the lytic peptide (iDNA79) and used to normalize the results.

Figure 8. Anti-proliferation and inhibition of the downstream signaling in A549 and U-2 OS cell lines. (A) 72-hour treatment with SAH-SOS1$_A$ and cyclorasin 9A5, at a threshold above 20 μM concentration, resulted in strong anti-proliferation in both cell lines by measuring the intracellular ATP level. Data are mean of triplicates ± SD. (B) 15-min treatment with SAH-SOS1$_A$ but not cyclorasin 9A5 inhibited the downstream phosphorylation of Erk at 20 μM concentration.