De-risking Drug Discovery of Intracellular Targeting Peptides: Screening Strategies to Eliminate False-Positive Hits

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Cite This: *ACS Med. Chem. Lett.* 2020, 11, 1993–2001

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ABSTRACT: Nonspecific 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—in terms of both their binding affinities and cellular activities. Through multiple gold-standard biophysical techniques, we unambiguously show that both peptides lack specific binding to KRas and instead induce protein unfolding. Although these peptides inhibited cellular proliferation, the activities appeared to be off-target on the basis of a counterscreen 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 counterscreens are indispensable.

KEYWORDS: PAINS, Ras, macrocyclic peptides, screening

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 literature³ to cause a significant waste of resources, both by academic groups who may use these as chemical probes and by drug discovery teams who may attempt to advance these molecules toward the clinic.⁴

Nonspecific modulation of target activity has been recognized for some time and can occur through several mechanisms,⁵ including covalent reactivity, redox cycling, fluorescence interference, membrane disruption, and the formation of colloidal aggregates, with the last of these 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 enzymatic activities in unexpected ways. Aggregators are not exclusive to synthetic small molecules. They include natural products⁹ and marketed drugs.¹⁰ Besides enzymes, targets mediating protein–protein interaction are affected.^{11,12} Importantly, these artifacts 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 underexplored. Recent data have 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 peptides 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 macrocyclic peptides, SAH-SOS1_A and cyclorasin 9A5, that have been reported in widely cited publications^{18,19} as specific KRas inhibitors are in fact false positives in terms of both their binding affinities and cellular activities. Critically, our assays are validated with another macrocyclic peptide (KRpep-2d),²⁰ a positive control supported by structure–activity relationships²¹ and X-ray crystallography.²²

Special Issue: Medicinal Chemistry: From Targets to Therapies

Received: January 30, 2020

Accepted: May 1, 2020

Published: May 1, 2020

Figure 1. FP assays were performed in a polypropylene microplate using FAM-SAHSOS1_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 $\pm$ SD for triplicates. R^2 corresponds to the fitting of no-detergent data (solid circles).

Figure 2. The nature of the microplate significantly affects the FP assays. (A) Uncoated polypropylene and (B) polystyrene microplates are hydrophobic and adsorbed the peptide and protein in the absence of detergent. (C) In contrast, the apparent binding of FAM-SAHSOS1_A to KRas disappeared in microplate coated with a hydrophilic material (Corning NBS). (D–F) Binding of FAM-KRpep-2d to KRas is maintained under all circumstances. Data are mean $\pm$ SD for triplicates.

RESULTS AND DISCUSSION

FAM-SAHSOS1_A Is a Promiscuous Binder. To disrupt the KRas–SOS1 interaction, Walensky and co-workers¹⁸ designed an 18-residue stapled peptide, named SAHSOS1_A, by incorporating an *i, i + 4* hydrocarbon linker at the noninteracting 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 SAHSOS1_A (FAM-SAHSOS1_A) could bind to wild-type and five mutant KRas with submicromolar dissociation constants (100–175 nM). We were able to reproduce the binding isotherm of FAM-SAHSOS1_A using several forms of GDP-loaded KRas (G12D, Q61H, and wild-type; Figures 1A and 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 suboptimal fitting (R^2 = 0.812; Figure 1A). However, the apparent bindings were abolished upon addition of nonionic 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-SAHSOS1_A and demonstrated comparable binding affinities. These interactions were also detergent-sensitive. To ensure that 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, nonspecific activities occurred for unrelated proteins (Figure 1E,F) when the detergent was 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 nonbinder.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 (A⁸Q, Q⁹L), 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 of the proteins that we investigated, and the binding interactions were 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 nonspecifically adsorbs on the microplate's plastic surface and/or (ii) FAM-labeled peptide forms a colloidal aggregate that then sequesters protein. Under both circumstances, the apparent "hydrodynamic size" of the labeled peptides increases, leading to lower tumbling rates, increased FP signals, and the false impression of specific target binding.

To assess the effect of the microplate, we explored (i) an uncoated polypropylene microplate (Figures 1, S1, and S2), (ii) an uncoated polystyrene microplate, and (iii) a polystyrene microplate coated with a nonionic hydrophilic material. We observed apparent binding of FAM-SAH-SOS1_A regardless of whether a polystyrene or polypropylene microplate was used (Figure 2A,B). The apparent binding disappeared once we added detergent or used the polystyrene microplate with a nonbinding surface (Figure 2C). Although the amount of KRas protein added to the sample did affect the FP signal, we are confident that the effect is nonspecific for two reasons. First, addition of nonionic detergent abrogated the FP signal, presumably by preventing nonspecific 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), regardless of protein concentration. Thus, it appeared that the increased FP signal was related to nonspecific 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 in FP signal was observed when we delayed the signal measurement by including a preincubation time (Figure S3). To rule out the possibility that KRas contributed to the signal increase, we monitored the FP signals of FAM-SAH-SOS1_A alone over the course of 3 h (Figure S4). The signal should remain flat in the absence of protein. However, we observed a time-dependent signal increase, a phenomenon that likely occurs from adsorption of peptide to plastic surfaces. All of the 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 μ M concentrations of FAM-SAH-SOS1_A and FAM-SAH-SOS1_B (Figure 3). Both peptides gave strong scattering intensities (5700–

Figure 3. Normalized autocorrelation functions of dynamic light scattering. Each peptide (10 μ M) underwent three independent measurements at 25 $^{\circ}$ C. All of the data are plotted (the range of particle sizes are shown in parentheses).

7400 kcps for FAM-SAH-SOS1_A and 12600–14500 kcps for FAM-SAH-SOS1_B, which were much higher than that of the buffer alone (35–36 kcps). The 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 μ M. Interestingly, ATSP-7041 (A⁸Q, Q⁹L) showed aggregation at 10 μ M (Figure S5) yet is a specific inhibitor of MDM2 when used at lower concentrations (<100 nM, Figure 4).

Surface Plasmon Resonance Confirms That SAH-SOS1_A and Cyclorasin 9A5 Are Nonbinders 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 μ M (Figure 4). Similarly, FAM-SAH-SOS1_A also did not bind to KRas by SPR (Figure S6). To ensure that the immobilized KRas was 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 nonspecific SPR binding readouts that did not fit a 1:1 binding model (Figure 4; similar results for FAM-SAH-SOS1_A are shown in Figure S6). Indeed, at the highest concentration tested (1 μ M), the 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 nonsaturable and non-one-to-one binding SPR signals seen with SAH-SOS1_A and cyclorasin 9A5. In contrast, two control peptides ATSP-7041 (A⁸Q, Q⁹L) and PHAGESOL²⁴ bound with 1:1 saturated binding to their

Figure 4. Surface plasmon resonance. We injected the individual peptides (in columns) over the individual flow cells (in rows) immobilized with KRas (~400 RU), MDM2 (~1200 RU), and eIF4E (~1600 RU). SAH-SOS1_A (1 μ M from top, 2 $\times$ dilution) and cyclorasin 9A5 (1 μ M from top, 2 $\times$ dilution) did not bind to KRas but demonstrated promiscuous binding to MDM2 and eIF4E. In contrast, ATSP-7041 (A⁸Q, Q⁹L) (50 nM from top, 2 $\times$ dilution) and PHAGESOL (300 nM from top, 2 $\times$ dilution) demonstrated saturated 1:1 binding to their corresponding protein targets. The running buffer included 0.01% Tween 20. Black lines depict the double-referenced sensorgrams; red lines depict the global fit of the data to a 1:1 binding model.

Figure 5. Isothermal titration calorimetry. (A–D) Heat changes from titration of SAH-SOS1_A into (A) KRas G12D or (B) buffer alone and from titration of cyclorasin 9A5 into (C) KRas G12D or (D) KRas G12V. (E) KRpep-2d demonstrated tight binding to KRas G12D. Raf-RBD demonstrated (F) tight binding to activated wild-type KRas (GMPPNP) but (G) no binding to the inactive wild-type KRas (GDP). The assay buffer included 0.03 mM *n*-dodecyl β -D-maltoside as a detergent.

respective targets (MDM2 and eIF4E) but not to KRas (Figure 4).

Isothermal Titration Calorimetry and Thermal Shift Assay Confirm That SAH-SOS1_A and Cyclorasin 9A5 Are Nonbinders 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 homogeneous conditions. This technique further confirmed that neither SAH-SOS1_A nor cyclorasin 9A5 bound to KRas G12D (Figure 5A,C). Cyclorasin 9A5 also

did not bind to KRas G12V, the protein used for discovery of the macrocyclic peptide (Figure 5D). The heats released during the titrations of SAH-SOS1_A into the protein or 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 a stoichiometry close to unity (Figure 5E). Wild-type KRas adopts distinct conformations when bound to different nucleotides. As a confirmatory experiment, we showed that wild-type KRas binds to its downstream effector (Raf-RBD, K_D

Figure 6. HDX-MS. The Y axis represents differences in deuterium uptake (Da) in the unbound KRas subtracted from that of the peptide-bound KRas. The X axis represents pepsin-proteolyzed peptides (N- to C-terminus). Each dot represents the difference at 1 or 5 min of deuterium labeling. Gray bars represent the sums of the differences at the two time points. 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 mapped onto KRas (in blue). (F) Residues protected from deuterium exchange (red box in (D)) in the presence of KRpep-2d (Arg del) mapped onto KRas (PDB entry 5XCO).

= 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 positives came from thermal shift experiments since both peptides produced a negative shift in the melting temperature (ΔT_m) of KRas G12D (Figure S8), suggesting ligand-induced destabilization. At higher concentrations, SAH-SOS1_A displayed a high initial background, probably due to aggregated peptide–dye interactions (Figure S8A). In contrast, the positive control KRpep-2d stabilized KRas with a ΔT_m of +11.5 °C (Figure S8C), indicating that the assay was functional. To ensure that our biophysical observations were not unique to cyclorasin 9A5, we tested a closely related analogue, cyclorasin 9A16, a molecule with reported IC₅₀ values that were similar to those of cyclorasin 9A5 in both inhibition of the KRas–Raf interaction and an antiproliferative 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 that cyclorasin 9A16 is a nonbinder, *i.e.*, false positive, for KRas (Figure S9B,C).

HDX-MS Confirms That SAH-SOS1_A and Cyclorasins Destabilize KRas. The negative ΔT_m in the thermal shift

assay suggests that 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 on 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) because of its superior solubility versus KRpep-2d. The corresponding data gave an HDX protection pattern highly consistent with the binding site observed in the KRas/KRpep-2d cocrystal structure,²² thus validating the technique's ability to faithfully map the peptide-binding sites (Figure 6D,F). 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 (Figures S8 and S9A). The 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 colleagues¹⁸ were designed and docked to the structure of Ras complexed to SOS. These peptides were subjected 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 and S12), in agreement 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 (EC₅₀ = 10 μ M), SAH-SOS1_A with unstapled hydrocarbon (EC₅₀ = 8 μ M), and cyclorasin 9A5 (EC₅₀ = 30 μ M), potencies that are comparable to that of iDNA79 (EC₅₀ = 20 μ M), a cationic amphipathic stapled peptide we use as a positive control (Figure 7). In

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

contrast, LDH release was insignificant (<10%) for SAH-SOS1_B, Aib-SOS1_A, cyclorasin 12A, ATSP-7041 (A⁸Q, Q⁹L), and PHAGESOL, and minimal (<30%) for cyclorasin 9A54 up to 50 μ M.

We performed the LDH release assay under serum-free conditions, which are the same conditions as used in the published report.¹⁸ We incubated HCT116, a colorectal cell line that harbors mutant KRas G13D, with peptides for 4 h. We expect that rapid cell death within this time frame is unlikely to be a result of apoptosis due to KRas signaling blockade. The most likely cause is rupture of the cellular membrane induced by the peptides. Interestingly, these

undesired toxicities were only observed for SAH-SOS1_A and its unstapled hydrocarbon analogue but not for its linear version (Aib-SOS1_A) and the negative control (SAH-SOS1_B), where the stapled hydrocarbon is placed on a different face of the helix. Minimal LDH leakage was also observed for the analogues of cyclorasin 9A5, such as cyclorasin 9A54 and cyclorasin 12A. Coincidentally, the degree of membrane disruption caused by SAH-SOS1_A, cyclorasin 9A5, and their nontoxic analogues 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_A¹⁸ in that the staple length is different and two N-terminal arginine residues have been 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 genetic knockdown of KRas.^{26,27} At concentrations above 20 μ M, both peptides induced strong antiproliferative effects with very steep dose-response curves on both cell lines in the presence of 10% serum (Figure 8A), suggesting off-target cytotoxic activities.

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 μ M concentration upon treatment for just 15 min. 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 an EC₅₀ of 10 μ M. 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 antiproliferation of KRas-independent cell lines and reduced KRas signaling support the interpretation of cell death caused by the peptides as resulting from nonspecific and off-target effects.

Figure 8. Antiproliferation and inhibition of the downstream signaling in A549 and U-2 OS cell lines. (A) Treatment with SAH-SOS1_A and cyclorasin 9A5 at a threshold above 20 μ M concentration for 72-h resulted in strong antiproliferation in both cell lines as determined by measurement of the intracellular ATP level. Data are mean $\pm$ SD for triplicates. (B) Treatment with SAH-SOS1_A but not cyclorasin 9A5 for 15 min inhibited the downstream phosphorylation of Erk at 20 μ M concentration.

■ CONCLUDING REMARKS

FP is a popular technique²⁸ for measuring protein–protein interactions because of 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 a 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 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 a 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 antiproliferative 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 secondary assays for binding validation. SPR is very sensitive to nonspecific binding. Nonsaturable 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 the LDH release assay as a convenient counterscreen 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, CETSA³⁰ and NanoBRET³¹ 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, selected 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 and reveal promiscuous target-unrelated activity, thereby improving the quality of publications to promote a productive research community.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsmmedchemlett.0c00022>.

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 (PDF)

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Notes

The authors declare the following competing financial interest(s): S.K. and C.S.V. are founders of Sinopsee Therapeutics; the current work has no conflicts.

■ ACKNOWLEDGMENTS

We thank A*STAR (grant IDs H17/01/a0/010, IAF111213C, H18/01/a0/015) and MSD for financial support, NSCC for computational support and Brian Henry for his review of the manuscript.

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