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Discovery of Small-Molecule Stabilizers of 14-3-3 Protein–Protein Interactions via Dynamic Combinatorial Chemistry

Alwin M. Hartman, Walid A. M. Elgaher, Nathalie Hertrich, Sebastian A. Andrei, Christian Ottmann, and Anna K. Hirsch*

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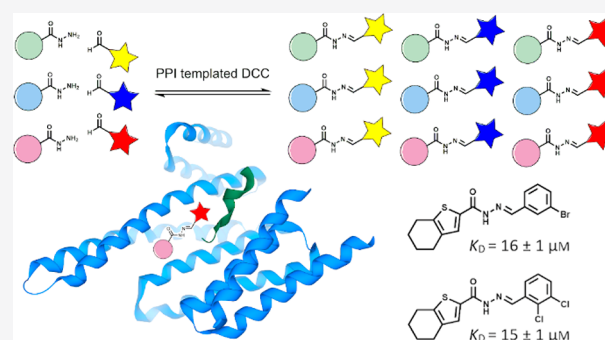
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ABSTRACT: Protein–protein interactions (PPIs) play an important role in numerous biological processes such as cell-cycle regulation and multiple diseases. The family of 14-3-3 proteins is an attractive target as they serve as binding partner to various proteins and are therefore capable of regulating their biological activities. Discovering small-molecule modulators, in particular stabilizers, of such complexes via traditional screening approaches is a challenging task. Herein, we pioneered the first application of dynamic combinatorial chemistry (DCC) to a PPI target, to find modulators of 14-3-3 proteins. Evaluation of the amplified hits from the DCC experiments for their binding affinity via surface plasmon resonance (SPR), revealed that the low-micromolar (K_D 15–16 μM) acylhydrazones are 14-3-3/synaptopodin PPI stabilizers. Thus, DCC appears to be ideally suited for the discovery of not only modulators but even the more elusive stabilizers of notoriously challenging PPIs.

KEYWORDS: Protein–protein interactions, DCC, hit-identification strategy, small-molecule stabilizers



The family of 14-3-3 proteins is present in all eukaryotic cell types, and its members are involved in a myriad of processes in the human body.¹ They play significant roles, ranging from signal transduction, to regulation of metabolism, to cell-death, and they are correlated to diseases such as Alzheimer's and Noonan syndrome.^{2,3} This is attributed to the ability of 14-3-3 to establish protein–protein interactions (PPIs) with more than 500 protein partners.⁴ There are seven known human isoforms of 14-3-3: beta (β), epsilon (ϵ), eta (η), gamma (γ), tau (τ), sigma (σ), and zeta (ζ). The binding partners feature three conserved binding motifs for the binding groove in 14-3-3: RSXpSXP (mode 1), RXXXpSXP (mode 2), and pS/TX-COOH (mode 3), where pS/T denotes a phosphoserine or threonine residue.^{5–7} In this study, we used the actin binding protein synaptopodin as a binding partner of 14-3-3 using mode 2 for interaction. Synaptopodin maintains the actin cytoskeleton and urine-filtering capabilities of podocytes in the kidney glomerulus. It is regulated through phosphorylation and subsequent 14-3-3 binding.⁸ 14-3-3 proteins are potential drug targets and an increasing number of chemical classes that modulate 14-3-3 PPIs have been reported, as recently reviewed.^{9,10} Modulators of 14-3-3 PPIs can be inhibitors, mostly small synthetic molecules, and stabilizers, which include bigger scaffolds, e.g., pyrrolidone1 and (semi-) natural products, e.g., fusicoccin-A.^{9,11–14} Dynamic combinatorial chemistry (DCC) has become an established technique for hit identification. Briefly, it allows a

target-based amplification of the best binder(s) from a pool of reacting building blocks and the corresponding products existing under thermodynamic equilibrium. The types of reversible linkages that can be applied in DCC, reaction conditions, and analysis of the dynamic combinatorial library (DCL) have been comprehensively reviewed before.^{15–17} In the present work, we set out to demonstrate the full potential of DCC by pioneering its application to the hitherto unexplored class of PPI target, thus we exploited the power of DCC to identify new PPI modulators targeting the 14-3-3(ζ) isoform. For the DCC experiments, we used acylhydrazone formation from the corresponding hydrazide and aldehyde as a reversible reaction. The acylhydrazone linkage can take part in binding with the desired target, as it offers H-bond donor and -acceptor sites. The acylhydrazone formation is sufficiently reversible in acidic media, but also stable against hydrolysis.¹⁸ In basic media, the reaction is very slow, therefore high pH values are used to freeze the equilibrium prior to DCL analysis. Bhat et al. showed that the use of aniline can accelerate the formation of the equilibrium to only 6 h while

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applying a closer to physiological pH of 6.2.^{19,20} As a prerequisite for acylhydrazone-based DCC, we systematically monitored the stability of our target protein 14-3-3(ζ) under acidic conditions using different buffers and pH values.²¹ We found that in MES buffer at pH 6.5, 14-3-3(ζ) is stable at room temperature up to 7 days. Therefore, we selected these conditions in the presence of 10 mM aniline as a nucleophilic catalyst for the DCC experiments. In order to confirm that the protein is folded correctly, we recorded the circular dichroism (CD) of 14-3-3. The obtained CD-spectrum matches that reported in the literature (Supporting Information, Figure S4).²²

RESULTS AND DISCUSSION

The initial design of the DCL and choice of building blocks were inspired by compound **1**, a small-molecule inhibitor of 14-3-3 discovered by virtual screening.²³ We envisioned the acylhydrazone linkage between the two aromatic rings, resulting in compound **2** (Figure 1). This modification should

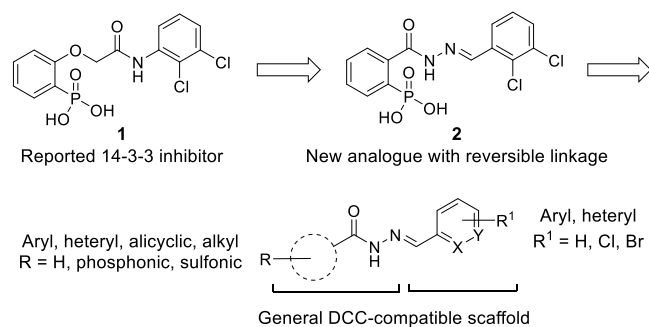
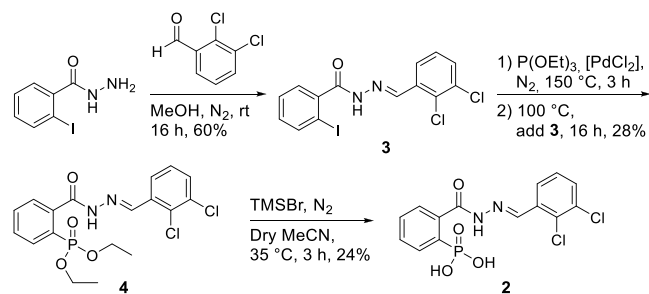


Figure 1. Design of DCL for 14-3-3 PPIs modulation based on the known small-molecule inhibitor **1**.

maintain the length of the linker between the two aromatic moieties of **1** owing to the restricted flexibility of the acylhydrazone group. Accordingly, compound **2** was synthesized in three consecutive steps starting with a condensation reaction between the commercially available 2-iodobenzhydrazide and 2,3-dichlorobenzaldehyde to form the acylhydrazone **3** (Scheme 1). A palladium-catalyzed coupling

Scheme 1. Synthetic Route toward Compound **2**



between the aryl iodide **3** and triethyl phosphite followed, to afford the phosphate ester **4**. This was achieved by first formation of a palladium phosphonate complex at 150 °C, followed by the addition of the acylhydrazone **3** at 100 °C. It was important to lower the temperature before adding the acylhydrazone **3** in order to prevent its decomposition. Deprotection of **4** was achieved by TMSBr, resulting in the target compound **2**. It is noteworthy that trials to prepare the

hydrazide building block with *o*-phosphonic acid moiety, required for DCC, using the above-mentioned coupling conditions were unsuccessful, and only an intramolecular *N*-arylation product was obtained. We therefore had to use compound **2** in our DCC experiments for *in situ* generation of the corresponding hydrazide and aldehyde.

We evaluated compound **2** for its biochemical properties via surface plasmon resonance (SPR) and fluorescence polarization (FP) assay. For the SPR study, we first determined the binding affinity (K_D) of a synaptopodin fragment containing the binding motif to 14-3-3(ζ). The 29-amino acid peptide showed a clear binding response with the same K_D value of 1.38 μ M obtained from either the Langmuir isotherm or the kinetic curves (Figure S7). This showed us that the immobilized protein is still in the native folded state and can engage in PPIs. Next, we assessed compound **2**, which showed low millimolar affinity (K_D value 1.01 mM) toward 14-3-3(ζ) (Figure 2). In the FP assay, compound **2** was titrated to fixed concentrations of 14-3-3(ζ) and a fluorescently labeled 29-mer peptide fragment of synaptopodin. A known 14-3-3 PPI stabilizing molecule, fusicoccin-A, was used as a positive control resulting in a dose-dependent increase in FP signal (Figure S5). In agreement with our design approach, titrating compound **2** to the protein–peptide complex decreases the signal indicating an inhibitory effect with an EC_{50} value of 120 μ M (Figure S5).

Encouraged by these results we designed a DCL based on compound **2** (Figure 1 and Scheme S1). Besides the *o*-phenylphosphonic acid moiety, we used diverse aryl-, heteroaryl-, alicyclic-, and alkylhydrazides to explore other scaffolds for potential binding to 14-3-3(ζ). As an aldehyde component, four building blocks with similar reactivity were selected including haloaryl- and heteroarylaldehydes. However, LC-MS analysis of the DCC experiment did not reveal all of the possible acylhydrazones bearing *o*-phosphonic or *o*-sulfonic acid moieties (Figure S1). We therefore modified the DCL to include three aldehydes **A1**–**A3** and six hydrazides **H1**–**H6** and exclude the acidic motifs as shown in Scheme 2. Consequently, we ran three DCC experiments: (a) a library in which building blocks were present in combination with the 14-3-3(ζ)/unlabeled synaptopodin complex as a PPI model; (b) a library containing the building blocks and 14-3-3 protein; and (c) a “blank” in which only building blocks were present (Scheme 2).

LC-MS analysis of the DCLs that were allowed to equilibrate for 6 h resulted in the chromatograms shown in Figure 3. The most obvious differences are the two peaks at retention times of 13.5 and 13.9 min, corresponding to the compounds **A1H3** and **A2H3**, respectively. In the presence of 14-3-3(ζ) only and in the PPI-complex (14-3-3(ζ)/synaptopodin), these two acylhydrazones show a significant amplification (about 148%) compared to the DCL in the absence of protein. Table 1 shows the ratios of the relative areas of each peak compared to the blank DCL. These two hits were then synthesized to confirm the identity of peaks (Figure S3) and for biochemical characterization.

Synthesis was accomplished through the reaction of the hydrazide **H3** with a stoichiometric amount of the appropriate aldehyde at reflux overnight to afford the corresponding acylhydrazones in good to quantitative yield (Scheme 3).

We used SPR binding assays to analyze binding events (affinity and binding kinetics) of the synthesized DCC hits to the 14-3-3(ζ) protein. The acylhydrazones **A1H3** and **A2H3**

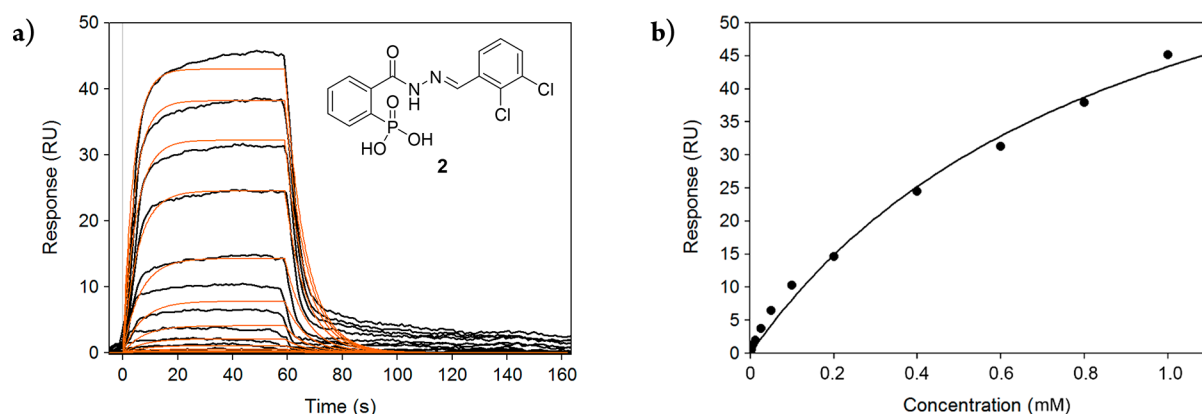
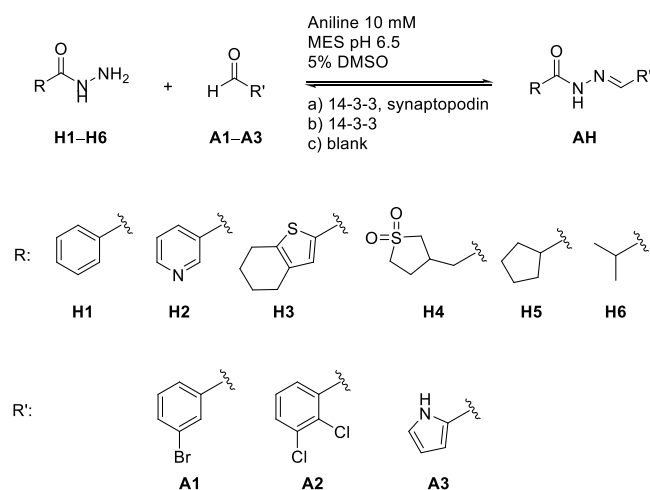


Figure 2. SPR binding assay of **2**: (a) Overlay of sensorgrams (black) of **2** at concentrations of 1.6–1000.0 μM running over an immobilized 14-3-3(ζ). Global fitting of the association and dissociation curves (red), (b) Langmuir binding isotherm ($K_D = 1.01 \pm 0.03 \text{ mM}$). Data obtained from single experiment.

Scheme 2. Dynamic Combinatorial Library (DCL) of Acylhydrazones with Aldehydes (100 μM Each), Hydrazides (300 μM Each), DMSO (5%), Aniline (10 mM), and (a) 14-3-3(ζ) (10 μM) and Synaptopodin (10 μM); (b) Control with 14-3-3(ζ) (10 μM); and (c) Control without Protein or Synaptopodin



showed low micromolar affinity to 14-3-3(ζ) (K_D values 16 and 15 μM , respectively). Interestingly, compound **A2H3** exhibited different on- and off-rates with a longer residence time compared to **A1H3** although with equal binding affinities (Table 2, Figure S8 and S9).

Intrigued by these findings, we next investigated the mechanism of action and binding site of the new 14-3-3 PPI modulators (compounds **2**, **A1H3**, and **A2H3**) by SPR competition assays using synaptopodin as a reference, which occupies the 14-3-3 main binding pocket. Modulators that inhibit 14-3-3 PPIs bind in the phosphorylation binding pocket, whereas stabilizers bind allosterically to the binding pocket or at the interface between 14-3-3 and its protein partners.²⁴ To check whether the compounds bind to the active site or elsewhere, we injected compound **2** (1000 μM), synaptopodin (1 μM), and a mixture of both at the same concentration in sequence over immobilized 14-3-3(ζ). The obtained response unit (RU) value of the mixture was compared to the theoretical sum of the RU values for the individual compounds. If **2** bound to the main binding pocket of 14-3-3, it would compete with synaptopodin and the response of the mixture should be less than the sum of RU values determined for the single compounds. On the other hand, if **2** bound allosterically to the active site, no competition

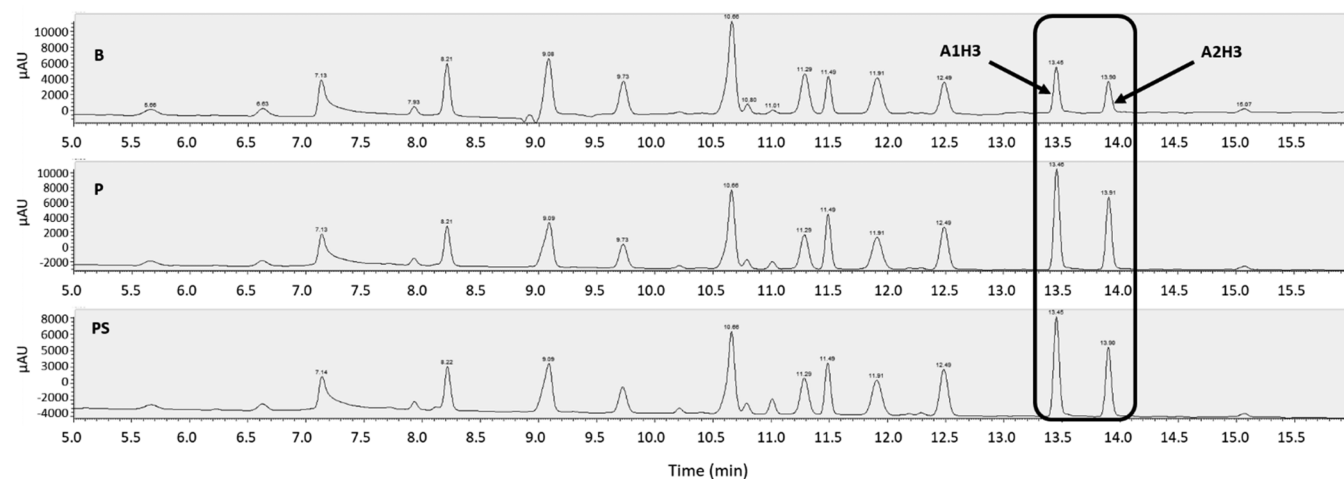
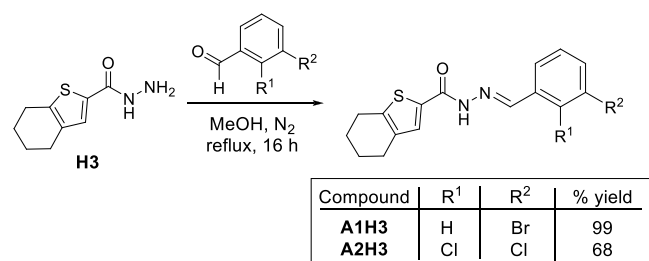


Figure 3. UV-chromatograms at 290 nm of DCLs in duplicate, blank (B), in the presence of protein (P), and in the presence of protein plus synaptopodin (PS) after 6 h. Compounds **A1H3** and **A2H3** are amplified in P as well as PS compared to B.

Table 1. Amplification Factors of the Products Formed in the DCC Experiments Analyzed via the Relative Areas of Peaks in the UV-Chromatograms^a

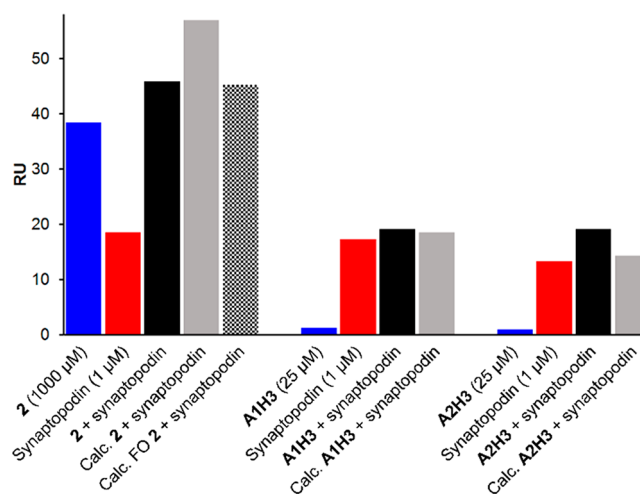
| Compd | t_R ^b (min) | % Amplification in the presence of 14-3-3 ^c | % Amplification in the presence of 14-3-3-synaptopodin complex ^d |
|------------|--------------------------|--|---|
| A3H2 | 4.76 | -59.0 | -44.0 |
| A3H4 | 5.66 | -33.9 | -35.9 |
| A3H6 | 6.62 | -25.7 | -16.4 |
| A3H1 | 7.93 | -33.1 | -18.1 |
| A1H2, A3H5 | 8.21 | -32.3 | -35.1 |
| A2H2, A1H4 | 9.09 | -28.0 | -26.0 |
| A2H4 | 9.73 | -36.7 | -32.9 |
| A1H1, A1H6 | 10.66 | -20.4 | -23.1 |
| A3H3 | 10.79 | -1.3 | -15.0 |
| A2H6 | 11.29 | -22.5 | -23.6 |
| A2H1 | 11.49 | 32.2 | 29.5 |
| A1H5 | 11.91 | -12.2 | -16.0 |
| A2H5 | 12.49 | 12.8 | 14.6 |
| A1H3 | 13.46 | 148.0 | 148.4 |
| A2H3 | 13.91 | 148.5 | 153.6 |

^aValues represent the average of two independent experiments.^bRetention time. ^cCalculated as $\left(\frac{RPA_{\text{protein}} - RPA_{\text{blank}}}{RPA_{\text{blank}}}\right) \times 100\%$. ^dCalculated as $\left(\frac{RPA_{\text{complex}} - RPA_{\text{blank}}}{RPA_{\text{blank}}}\right) \times 100\%$.**Scheme 3. Synthetic Route toward Compounds A1H3 and A2H3**

would occur and the response of the mixture should be equal to the sum of RU values of the individual compounds. We found that the RU value of **2** in combination with synaptopodin was less than the sum of the individual responses (Figure 4 and S10). This suggests that compound **2** competes with synaptopodin for the same binding pocket. No compound, however, could completely displace the other at the tested concentrations. To verify this, we recalculated the sum of responses considering a fractional occupancy (FO) of **2** and synaptopodin according to Perspicace et al.²⁵ Indeed, the experimental RU value of the mixture was equal to the new estimated one (Figure 4), indicating that the compounds compete for the same binding site. Using different concentrations of **2** and synaptopodin (1000 μM vs 25 μM ; and 200 μM vs 1 μM) afforded the same results (Figure S11 and S12).

Table 2. Kinetic Parameters of Hit Compounds A1H3 and A2H3 to 14-3-3(ζ)^a

| Compd | R_{max} (RU) | k_{on} ($\text{M}^{-1}\text{s}^{-1}$) | k_{off} (s^{-1}) | $t_{1/2}$ (s) | K_D (μM) |
|-------|-----------------------|--|--------------------------------------|---------------|-------------------------|
| A1H3 | 4.6 ± 0.2 | $2.6 \pm 0.2 \times 10^3$ | $4.1 \pm 0.1 \times 10^{-2}$ | 17 | 16 ± 1 |
| A2H3 | 4.5 ± 0.2 | $5.8 \pm 0.5 \times 10^2$ | $8.7 \pm 0.4 \times 10^{-3}$ | 80 | 15 ± 1 |

^a R_{max} : maximum analyte binding capacity; k_{on} : association rate constant; k_{off} : dissociation rate constant; $t_{1/2}$: dissociative half-life; K_D : equilibrium dissociation constant.**Figure 4.** SPR responses of compounds **2**, **A1H3**, and **A2H3** in the competition assays, using synaptopodin as a reference compound binding to the active site of 14-3-3.

In line with the FP-assay data, these results clearly indicate that compound **2** binds to the active site of 14-3-3, leading to disruption of PPIs.

Using the same approach, we injected the DCC hit compounds **A1H3** (25 μM) and **A2H3** (25 μM) alone and as a mixture with synaptopodin (1 μM) over immobilized 14-3-3, and analyzed their binding responses (Figure 5). Interestingly, the RU values of the mixtures containing **A1H3** or **A2H3** with synaptopodin were equal to or more than the sum of the individual responses (Figure 4 and 5). This indicates that these two compounds bind to 14-3-3 in a different pocket than that of synaptopodin. Moreover, the increased binding response of the mixture compared to the calculated sum indicates a stabilizing effect of the acylhydrazones to the complex of synaptopodin with 14-3-3(ζ). This confirms our findings from the DCC experiments, where the same amplification factors for these hits were obtained in the presence of 14-3-3(ζ) alone as well as the 14-3-3(ζ)/synaptopodin complex (Table 1). Therefore, their binding site could be allosteric to the main binding pocket, e.g. at the interface of the 14-3-3(ζ)/synaptopodin complex.

CONCLUSIONS

We set out to develop novel PPI modulators targeting the versatile 14-3-3 protein family. First, we pursued a ligand-based design of the acylhydrazone **2**, which turned out to be an inhibitor of 14-3-3 PPIs. Next, we applied a DCC approach, using 14-3-3(ζ) in complex with synaptopodin as a PPI model, resulting in the discovery of two modulators **A1H3** and **A2H3**. No significant change was observed in the DCL composition in the presence of only 14-3-3 compared to the 14-3-3(ζ)/synaptopodin complex, indicating that the hit compounds bind independently to a different site than the main binding groove

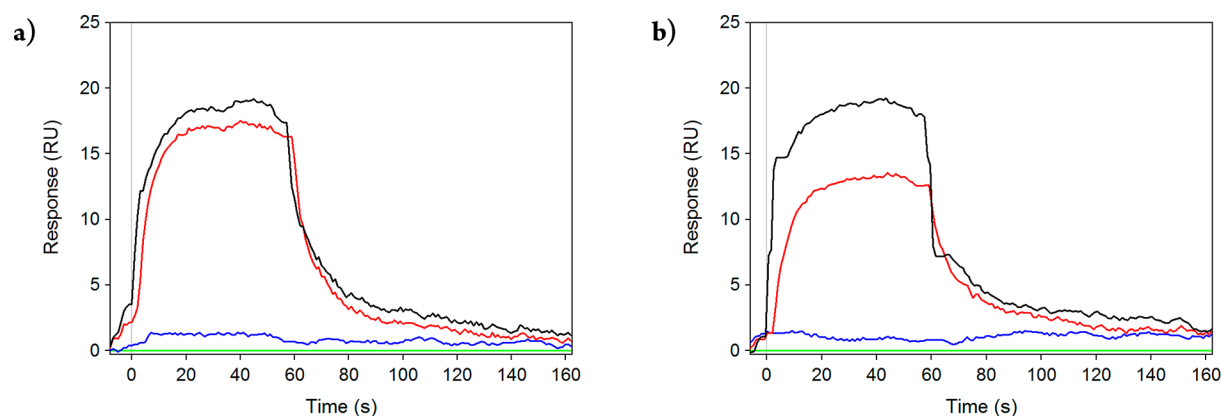


Figure 5. SPR competition assays: (a) Sensorgram overlay of **A1H3** (25 μM , blue), synaptopodin (1 μM , red), and a **A1H3**–synaptopodin mixture (black) showing an additive effect, indicating a noncompetitive binding; (b) sensorgrams of **A2H3** (25 μM , blue), synaptopodin (1 μM , red) and a **A2H3**–synaptopodin mixture (black) showing a synergistic effect, indicating a noncompetitive binding and a stabilizing effect.

of 14-3-3 involved in PPIs. Finally, we determined the binding affinities and kinetics of the hits via SPR and investigated their binding site on 14-3-3. Results of the SPR competition assays support our initial findings from the DCC experiments, suggesting that these small molecules stabilize the 14-3-3(ζ)/synaptopodin complex either directly or allosterically.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsmmedchemlett.9b00541>.

Synthesis and characterization of the described compounds, biochemical assays, protein expression, purification details, and NMR spectra (PDF)

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Author Contributions

A. M. Hartman was involved in the design of the project, performing the DCC experiments, synthesis of compounds, binding study via SPR, and writing of the manuscript. W. A. M. Elgaher was involved in the design and analysis of the SPR study and editing the manuscript. N. Hertrich was involved in the DCC experiments and the synthesis of the compounds. S. A. Andrei was involved in the expression and purification of 14-3-3, the synthesis of the synaptopodin fragment, and the FP assay. C. Ottmann and A. K. H. Hirsch were involved in the design of the project, supervision of the project, and editing the manuscript.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

PPIs, protein–protein interactions; DCC, dynamic combinatorial chemistry; DCL, dynamic combinatorial library; SPR, surface plasmon resonance; RU, response unit; FO, fractional occupancy

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