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Design and Combinatorial Development of Shield-1 Peptide Mimetics Binding to Destabilized FKBP12

Daniel Madsen, Frederik P. Jørgensen, Daniel Palmer, Milena E. Roux, Jakob V. Olsen, Mikael Bols, Sanne Schoffenel, Frederik Diness,* and Morten Meldal*

ABSTRACT: On the basis of computational design, a focused one-bead one-compound library has been prepared on microparticle-encoded PEGA1900 beads consisting of small tripeptides with a triazole-capped N-terminal. The library was screened towards a double point-mutated version of the human FKBP12 protein, known as the destabilizing domain (DD). Inspired by the decoded library hits, unnatural peptide structures were screened in a novel on-bead assay, which was useful for a rapid structure evaluation prior to off-bead resynthesis. Subsequently, a series of 19 compounds were prepared and tested using a competitive fluorescence polarization assay, which led to the discovery of peptide ligands with low micromolar binding affinity towards the DD. The methodology represents a rapid approach for identification of a novel structure scaffold, where the screening and initial structure refinement was accomplished using small quantities of library building blocks.

KEYWORDS: destabilizing domain, one-bead one-compound library, encoded beads, solid-phase synthesis

INTRODUCTION

The engineered and double point-mutated version of the human FK506-binding protein, F36V-L106P-FKBP12, is known as the destabilizing domain (DD). This domain can confer its instability to proteins-of-interests (POIs) when fused to it. When expressed in cells, the DD and the fusion protein will rapidly be degraded by the proteasome, resulting in low levels of the POI. Upon stabilization of the DD by addition of a small ligand, the degradation is reduced, which induces a dose-dependent accumulation of the POI within the cell. Hereby, the level of gene product can be chemically controlled, creating a powerful tool for interrogating biological systems. The compound Shield-1 (Shld1, Figure 1) is a small molecule derived from the natural ligand FK506 and has been shown to interact selectively with the DD. The Shld1–DD system has proven its vast utility in several biological systems, including living mice, plants, parasites, mammalian cells, and yeast. Previous studies have reported Ki values of 7.5 nM from in vitro experiments between Shld1 and the DD. However, in plant systems genetically modified to express DD-POI fusion proteins, high concentrations of Shld1 were required to induce accumulation of the destabilized complex. In studies with rice and wheat, treatment with 3–10 μM Shld1 solutions was required to achieve sufficient diffusion of the small Shld1 molecule through the plant cuticle and subsequent stabilization of DD-POI. A recent study highlighted the challenges of improving the plant cell penetrating properties of Shld1 derivatives, while preserving sufficient potency of the modified compounds. In connection to this project, alternative and novel stabilizing compounds with a less demanding synthetic route were envisioned. We describe, in this study, a method for using computational design, combined with a series of encoded combinatorial synthesis and screening...
steps, as a rapid approach for developing new peptide-based ligands that stabilize the DD protein.

The FKBP12s have been described as being “undruggable” for low molecular mass ligands with sufficient affinity,15 and most synthetic ligands of the FKBP12s are derived from the natural product FK506. Structure 1 of the peptide mimetics involves standard solid-phase synthesis in the C- to N-direction, whereas in strategy 2, the ligand is anchored via a carboxylic linker attached to a tyrosine sidechain.

![Illustration of the development process for small molecule peptide mimetics and their respective attachment strategies.](image)

Figure 1. Illustration of the development process for small molecule peptide mimetics and their respective attachment strategies. Prior to this study, DD-ligands, such as SLF* and Shield-1 (Shld1), were derived by structure-based design originating from the natural product FK506. Strategy 1 of the peptide mimetics involves standard solid-phase synthesis in the C- to N-direction, whereas in strategy 2, the ligand is anchored via a carboxylic linker attached to a tyrosine sidechain.

The structural insight gained from the on-bead assay assisted the design of 19 unnatural peptides which were screened in a competitive fluorescence polarization assay, and our findings has led to the discovery of novel and small unnatural peptides with low micromolar binding affinity towards the DD.

**RESULTS AND DISCUSSION**

With the aim of developing a core structure with modifiable moieties for combinatorial library development, a tripeptide with a triazole capped N-terminal was designed by computer modeling to adopt a similar binding pose to that of Shld1 in the DD binding site.5 The reference compound SLF* and an example of the computational designed ligand 1 is depicted in Figure 2. Modeling was performed with Molecular Operating Environment (MOE) and Amber 10 at 230 K, 1 ns, 230 K, 3 ns. In the final round, the coordinates of F36V–FKBP12 were fixed, and in the subsequent rounds, the contact residues in F36V–FKBP12 were relaxed to allow an induced fit of F36V–FKBP12 and the ligand.

![Comparison of the binding modes of SLF* (A) and an example of peptide mimetic 1 (B) to the crystal structure of the SLF*-stabilized F36V–FKBP12 complex.](image)

Figure 2. Comparison of the binding modes of SLF* (A) and an example of peptide mimetic 1 (B) to the crystal structure of the SLF*-stabilized F36V–FKBP12 complex. In the modeling of the complex between F36V–FKBP12 and the peptide mimetic, key features of the ligand were maintained while building the structure. The water-soaked complexes of F36V–FKBP12 with the peptide mimetic was subjected to several rounds simulated annealing (MOE, Amber 10, ETH; T = 430 K, 1 ns, 430–230 K, 1 ns, 230 K, 3 ns). In the first round, the coordinates of F36V–FKBP12 were fixed, and in the subsequent rounds, the contact residues in F36V–FKBP12 were relaxed to allow an induced fit of F36V–FKBP12 and the ligand.
Scheme 1

**Strategy 1:**

Reagents and conditions: (a) Fmoc-4-Amb-OH, TBTU, NEM, DMF, 2 h; (b) piperidine/DMF (1:4), 20 min; (c) Fmoc-L-Phe-OH, TBTU, NEM, DMF, 2 h; (d) Fmoc-L-Pip-OH, TBTU, NEM, DMF, 2 h; (e) Fmoc-D-Abu-OH, TBTU, NEM, DMF, 2 h, then piperidine/DMF (1:4), 20 min; (f) 2-(3,4,5-trimethoxyphenyl)butanoic acid, TBTU, NEM, DMF, 2 h; (g) phenylacetylene, CuSO₄₅₂₅, ascorbic acid, t-BuOH/H₂O (3:1), 16 h; (h) Ac₂O/pyridine/DMF (1:1:8), 30 min; (i) 2-(3,4,5-trimethoxyphenyl)butanoic acid, TBTU, NEM, DMF, 2 h; (j) benzylamine, PyBOP, NEM, DMF, 2 h; (k) ethyl-2-bromoacetate, Cs₂CO₃, DMF, 3 h, 86%; (l) TFA/CH₂Cl₂ (1:1), 0 °C to rt, 1.5 h, 93% (2 steps); (m) MgI₂, THF, mw 120 °C, 15 min, quant; (n) TBTU, NEM, DMF, 2 h.

**Strategy 2:**

Environ (MOE, CCG) using the crystal structure of SLF bound to F36V–FKBP12 (PDB 1BL4) as a starting point. Upon fixing the coordinates of the protein, the SLF compound was replaced with peptide mimetics, soaked in a water droplet, and relaxed by energy minimization as described in detail in the Supporting Information. Modifications were explored on key features of the peptides during structural development. The pepticolic moiety is located in a groove on the active site of the protein and has in several studies been shown to be important for binding to the protein.

A range of alternative ring sizes and substitution patterns were sampled during the modeling, but the pipelicolic residue presented a superior fit to the pocket. Originating from an α-aminobutyric acid, the ethyl part adjacent to the triazole is designed to dock into the hydrophobic cavity formed after the F36V mutation, and various lengths of alkyl groups were investigated and selected to explore its capacity. Those alkyl groups providing favorable interactions in the pocket during modeling were included in subsequent library synthesis. For the remaining residues, interaction over the entire binding site was evaluated and structural components displaying appropriate contact with the protein were selected for combinatorial library synthesis.

The design of the peptide mimetic scaffold allowed usage of the large pool of natural and unnatural amino acid building blocks in combination with the orthogonal CuAAC click chemistry to survey the pharmacophore interactions. To screen for optimal interaction with the protein for the immobilized substrates in the library, two different linker strategies were employed. As presented in Scheme 1, strategy 1 involve standard solid-phase peptide synthesis (SPPS) in the C- to N-direction. Whereas, strategy 2 is anchored via a linker piece from the tyrosine side chain, protruding from the other end of the compound and resembling the part of the SLF* used for

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*Reagents and conditions: (a) Fmoc-4-Amb-OH, TBTU, NEM, DMF, 2 h; (b) piperidine/DMF (1:4), 20 min; (c) Fmoc-L-Phe-OH, TBTU, NEM, DMF, 2 h; (d) Fmoc-L-Pip-OH, TBTU, NEM, DMF, 2 h; (e) Fmoc-D-Abu-OH, TBTU, NEM, DMF, 2 h, then piperidine/DMF (1:4), 20 min; (f) 2-(3,4,5-trimethoxyphenyl)butanoic acid, CuSO₄₅₂₅, ascorbic acid, t-BuOH/H₂O (3:1), 16 h; (g) phenylacetylene, CuSO₄₅₂₅, ascorbic acid, t-BuOH/H₂O (3:1), 16 h; (h) Ac₂O/pyridine/DMF (1:1:8), 30 min; (i) 2-(3,4,5-trimethoxyphenyl)butanoic acid, TBTU, NEM, DMF, 2 h; (j) benzylamine, PyBOP, NEM, DMF, 2 h, 99%; (k) ethyl-2-bromoacetate, Cs₂CO₃, DMF, 3 h, 86%; (l) TFA/CH₂Cl₂ (1:1), 0 °C to rt, 1.5 h, 93% (2 steps); (m) MgI₂, THF, mw 120 °C, 15 min, quant; (n) TBTU, NEM, DMF, 2 h.
immobilization of 18. Presumably, attachment from this part of the peptide would exhibit a smaller degree of steric interaction with the DD. To initially verify the scaffold design and test the two linking strategies, 2–3 examples of the scaffold linked by each of the methods were synthesized on solid support (Scheme 1). The building block for the strategy 2 linker was synthesized by coupling of 9 with benzylamine, followed by a substitution of ethyl 2-bromoacetate to give 10. A Boc to Fmoc protection group exchange of 10 was performed, and after ester hydrolysis orthogonal to the Fmoc protection group,18 11 was formed in five steps with an overall yield of 79%, starting from Boc-protected L-tyrosine. A set of solid-supported compounds were then synthesized on a PEGA1900 resin (500−550 μm)29 and subjected to a maltose binding protein (MBP) fused DD, which had been fluorescently labeled with the rhodamine-X fluorophore (MBP-DD-(ROX)). The MBP was fused to the DD to prevent aggregation of the destabilized protein and to increase the overall polarity of the protein, hence reducing the nonspecific binding. The unique swelling properties of the porous PEGA1900 resin in aqueous media enabled the large fusion protein to gain access to the bead interior.30 As a reference compound for the initial test screen, a solid-supported version of the SLF* compound was synthesized by adapting known procedures (see Supporting Information).18

The carboxylic acid part of the SLF* has been shown to have minor influence on the binding to the DD protein and has previously been used as an anchor for solid-support while retaining its interaction with the protein.31 To estimate any level of nonspecific binding, three different control tripeptides presenting negative, positive, and neutral charge (19−21, see Figure 3) were tested in the on-bead binding assay. As binding saturation is presumably observed for strong binding ligands. Indeed, compound 6 provided the same if not more fluorescence intensity than resin bound SLF*. However, these results indicated that the triazole-based mimetics of the 2-trimethoxyphenylbutyric acid would be a good starting point for development of a combinatorial library.

The main advantage of this approach was that the azido acid could be generated on bead from amino acid building blocks, hereby removing the need for introducing a stereocenter through enantioselective alkylation of a range of phenylacetic acid derivatives.39,40 Because of the anticipation of slightly better interaction and the ease of synthesis, strategy 1 was selected for the development of the combinatorial library.

**Library Screening.** Using the split-and-mix approach, a library of in total 896 different compounds were synthesized on PEGA1900 beads (500−550 μm) that were optically encoded by MicroParticleMatrix (MPM)-encoding (Figure 4).32 Encoding of the immobilized peptides was performed by recording three orthogonal images of every bead in each portion of the split process, to generate a microparticle coordinate matrix used as a barcode for the individual beads. The images were recorded using a custom-made MPM decoding instrument comprising a solid state laser, three CCD-cameras with telecentric optics and a carousel for individual bead handling.21 Subsequently, the isolated hit-

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**Figure 4.** Development and screening of encoded one-bead one-compound library. (A) Using the split-and-mix approach, an 896-member library was synthesized on encoded PEGA1900 beads (500−550 μm) using the same conditions as presented in Scheme 1. (B and C) Bright field and fluorescence images, respectively, of library beads after being subjected to a 1 μM solution of Shld1-stabilized MBP-DD-(ROX) for 48 h. The images presents a clear hit bead among inactive beads. (D) Typical encoded bead used in the present study. Three orthogonal images of the same bead are recorded and used in construction of the 3-dimensional matrix represented by the microparticle coordinates. These are used as a unique digital code to identify the structure of the compound immobilized on the bead. The code of the bead is recorded at each synthetic step and used for hit identification.

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**Figure 3.** Single concentration binding tests of solid-supported ligands subjected to a fluorescently labeled DD protein (550 nM) for 16 h. The three tripeptides 19 (Ac-Glu-Phe-Gly-OH), 20 (Ac-Lys-Phe-Gly-OH), and 21 (Ac-Ala-Phe-Gly-OH) were attached to the PEGA resin via the HMBA linker and used as controls for nonspecific binding.
beads were recorded and the 3-dimensional matrix represented by their microparticle coordinates were determined, allowing tracking through the synthetic history of the immobilized compound.\textsuperscript{21} Pidolic acid and 4-(aminomethyl) benzoic acid were maintained as structural features during the library development. The remaining amino acid building blocks were varied and coupled using either coupling reagents TBTU or HATU. Subsequently, a solid-phase diazotransfer to the N-terminal amine was carried out to form the corresponding azide,\textsuperscript{33} which in turn was reacted by the CuAAC click reaction using various alkynes (see Table S1 for the full list of library building blocks).

The selection of structure variations of R, R1, and R2 were guided by computational design as described above. The moieties on the R-position were primarily aliphatic and aromatic cyclic structures, whereas the R2-position consisted of small linear and branched alkyl groups. Presumably, the enlargement of the hydrophobic cavity formed upon F36V mutation of FKBP12 could allow binding of longer alkyl groups, particularly in combination with the less bulky triazole moiety, as compared to the reference trimethoxy phenylbutyric acid residue of SLF\textsuperscript{8}. To induce the correct folding of the protein, the MBP-DD-(ROX) was preincubated for 30 min with a stoichiometric amount of Shld1 prior to the incubation of the library beads. A final concentration of 1 μM MBP-DD-(ROX) was used, and the beads were incubated for 48 h to establish equilibrium between the individual compounds in a binding-competition assay. As presented in Figure 4, a clear difference in interaction with the protein among the solid-supported ligands was observed, which was visualized by recording the fluorescence intensity of the bead collections. The binding was quite selective and provided a number of bright beads on a background of many inactive beads. The identified hit-beads were manually sorted based on their apparent fluorescence intensity. The structures attached to the isolated encoded hit-beads were decoded giving a total of 16 different hit-structures out of 17 isolated beads (see Supporting Information for all the hit structures).\textsuperscript{21} Nine of the hit compounds contained a 4-trifluoromethyl phenylalanine derivative as the second amino acid. In addition, 12 compounds had an ethyl group on the R1-position and primarily either a 4-methoxy phenyl or a cyclopropyl on the R2-position.

**On-Bead Assay and Structure Evaluation**. To distinguish and verify the potency among the structures of the solidsupported hit compounds, a well-based on-bead binding assay was employed.\textsuperscript{34} Guided by the results from the library screen, a set of immobilized substrates were resynthesized on PEGA\textsubscript{1900} beads (125−200 μm). The beads were singly sorted into a 96-well plate using a COMPAS bead sorter (Union Biometrica) and subjected to a dilution series of MBP-DD-(ROX). After 16 h of incubation, the fluorescence intensity was measured on wide-field fluorescence microscope. The individual binding profile of the resynthesized peptide mimetics was compared to the solid-supported SLF\textsuperscript{5} and their potency was graded in a scale from 1 to 4 (Table 1). In general, it was found that the most abundant substituents from the hits in the library screen, also proved to be the most potent in the bead assays. Compounds containing a 4-trifluoromethyl phenylalanine as the second amino acid, and an ethyl group on the R1-position displayed the most promising binding profile. On the R2-position, neither the phenyl group of 23 of nor the 3-benzoic acid of 24 were identified as hits during the screening but were selected for comparison with the binding of 25. These two compounds displayed a comparable binding profile to the anisole of 25, which indicated that aromatic moieties on this position was preferred. Resynthesis of the peptide mimetics in the solid-supported screening format was realized on a low micromolar scale, requiring a minimal amount of the unnatural amino acid building-blocks, which in combination with the library screening gave an efficient and rapid structure evaluation.

### Table 1. On-Bead Screening of Compounds Derived from the Hits Identified through Optically Encoded Split-and-Mix Library Technology

<table>
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<th>R1</th>
<th>R2</th>
<th>Potency\textsuperscript{a}</th>
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<tbody>
<tr>
<td>18\textsuperscript{b}</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++++</td>
</tr>
<tr>
<td>23</td>
<td>F</td>
<td>C</td>
<td>(R)</td>
<td>-</td>
</tr>
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<td>24</td>
<td>F</td>
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<td>35</td>
<td>(R)</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

\textsuperscript{a}Binding profile of the peptide mimetics was compared to the solid-supported SLF\textsuperscript{5} and their potency was graded in a scale from 1 to 4.

\textsuperscript{b}Immobilized SLF\textsuperscript{5} (see Scheme 1).
Off-Bead Peptide Resynthesis and Screening. The next step was to survey the nonlinked scaffold for binding to the MBP-DD in solution. On the basis of the results obtained from the well-based on-bead assay, a set of 19 unnatural peptides were synthesized in solution or from solid-support (see Supporting Information). Using the same synthetic approach as presented in Scheme 1, the compounds containing a primary amide or a benzoic acid on the C-terminal of the peptides were synthesized from the Rink-amide linker and the 2-chloro trityl linker, respectively. The solid-supported compounds were cleaved upon acid treatment to yield the 2-chloro trityl linker, respectively. The solid-supported peptides were synthesized from the Rink-amide linker and the (see Supporting Information). Using the same synthetic peptides were synthesized in solution or from solid-support from the well-based on-bead assay, a set of 19 unnatural the MBP-DD in solution. On the basis of the results obtained this position may be advantageous to the binding of the 3-step compound 37 found the active derivative 37, determining factor, as introducing the aliphatic amide substituent found the active derivative 37, suggesting that a hydrogen bond donor in this position may be advantageous to the binding of the protein. Testing rather small moieties on the R1-position in combination with or without changing the R- and R2- substituents (50–54) interestingly revealed the two most potent compounds in the series (51 and 53) with Kd-values of 5.13 and 11.2 μM, respectively. Both compounds are among the most hydrophilic, but again, this effect is not the only determining factor, as introducing the aliphatic amide substituent found the active derivative 40 in the R1-position of 51 led to loss of activity (41). This underlines that further optimization of the scaffold has to be multidimensional, which exactly is the strength of combinatorial screening. However, the presented data demonstrates that the combination of computational design and combinatorial synthesis and screening is a powerful concept for developing new active scaffolds towards a given biological target.

Binding Evaluation of Peptide Ligands. The mode of interaction of the two ligands, 37 and 51 was analyzed in detail. The interactions between ligand 37 and F36V–FKBP12 involve two hydrophobic pockets (see Figure 5A). One for the

Table 2. Kd Values of Resynthesized Peptide Mimetics 36–54

<table>
<thead>
<tr>
<th>Cmp</th>
<th>R1</th>
<th>R2</th>
<th>Kd (μM)</th>
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<tr>
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<tr>
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<td></td>
<td></td>
<td>21.5 ± 4.0</td>
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<tr>
<td>38</td>
<td>H</td>
<td>CF3</td>
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<td>45</td>
<td></td>
<td></td>
<td>&gt;200</td>
</tr>
</tbody>
</table>

“A competitive fluorescence polarization assay was used to obtain the reported Kd values ± SD from three independent experiments.”
The alternate binding mode presented by compound and ligand 37 FKBP12. The overlay of SLF network of interacting residues between the complex interaction between presented by computational modeling. (A) Contact residues of crystal structure of F36V Tyr-26, and Leu-97 of F36V −ethyl group, which interact with Ile-91, Gly-28, Val-36, Phe-99, Tyr-26, and Leu-97 of F36V−FKBP12, and one for the pipercolic acid, which is lined by Phe-46, Phe-99, Tyr-82 and the triazole binds perpendicularly to the carbonyl of the pipecolic amide. This in turn binds amide NH of Ile-56. Tyr-82 forms a hydrogen bond to the deeply buried carbonyl of the triazole modified butyric acid, while the triazole forms a hydrogen bond to the NH of the Phe(CF₃) residue. Overall the fit of 37 is not as tight as that of SLF* 5,13 The optimized interaction of the three peripheral aromatic rings seem to lift the core pipecolic amide and α-aminobutyric acid residue slightly from their binding pockets, since it cannot reach over the rim of the binding pocket. Hereby, interaction with the protein is compromised, and as visualized in Figure SC, the SLF* is in significant closer contact with the protein in the deep hydrophobic binding pocket. The complex of F36V−FKBP12 with compound S1 presents an alternative binding mode in which the cyclopropane ring interacts with Ile-90, His-97, and Tyr-82 and the triazole binds perpendicularly to the NH of the Phe(CF₃). This redirects Tyr-82 to form a hydrogen bond to the carbonyl of Phe(CF₃), allowing optimal interaction of the morpholine residue with Ile-56. Surprisingly, the smaller compound S1 showed the highest binding affinity. However, since all peripheral residues of S1 are relatively small, they may allow optimal interaction of the core pipecolic amide and α-aminobutyric acid residues with the F36V−FKBP12. These observations suggest that the scaffold could be even further improved by extending the core framework to position the peripheral aromatic residues at more favorable regions of the interaction interface, thus providing a better match of binding to the active site of the DD.

In summary, an 896-member one-bead one-compound library was synthesized on microparticle encoded PEGA₁⁹₀₀ beads using the split-and-mix approach. The library was screened towards a fluorescently labeled version of the DD-protein and the screen resulted in 16 different structures, which were identified as hits and deconvoluted using in-house developed decoder equipment. General trends among the hits included the presence of a 4-trifluoromethyl-1-phenylalanine as the second amino acid and an ethyl-group on the R₁-position (Figure 4) originating from an α-α-aminobutyric acid. Guided by the library hits, solid supported peptide mimetics were resynthesized on small scale and screened in a novel on-bead binding format to confirm and distinguish the potency among the library hits. The structure information was then used for the synthesis of 19 peptide mimetics. By exploiting the fixed stereochemistry of the unnatural amino acids, the small peptide-like compounds were synthesized in few steps using either solution- or solid-phase synthesis. The peptide mimetics were tested in a competition fluorescence polarization assay and one compound with a low micromolar binding affinity towards the DD was identified. Analysis of the identified low potency ligand (37) indicates that binding may be partly compromised by the small displacement of the pipercolic and butyric acids caused by rim interaction of the peripheral residues. This may also add to the explanation, why a small compound like S1 bind better than 37, while displaying less interaction. Our high-throughput screening approach to identify a novel peptide-like structure scaffold for the DD has involved the combination of computational modeling,
combinatorial library screening, and on-bead structure evaluation.

With a focus on improving the solubility of the peptide mimetics, the identified ligand S1 could serve as a starting point for development of unnatural peptides that mimic the Shld1–DD interaction. We envision that the present methodology can be applied to the synthesis and screening of larger peptide and nonpeptide libraries, where the screening and initial structure refinement can be accomplished routinely and rapidly using small quantities of library building blocks in a high throughput manner using the MPM-encoding technology.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscombsci.9b00197.

Full description of the materials and methods, including HPLC, NMR, and MS data (PDF)

**AUTHOR INFORMATION**

Corresponding Authors

Frederik Diness — Department of Chemistry and Center for Evolutionary Chemical Biology, University of Copenhagen, 2100 Copenhagen, Denmark; orcid.org/0000-0001-5098-7198; Email: fdi@chem.ku.dk

Morten Meldal — Department of Chemistry and Center for Evolutionary Chemical Biology, University of Copenhagen, 2100 Copenhagen, Denmark; orcid.org/0000-0001-6114-9018; Email: meldal@chem.ku.dk

Authors

Daniel Madsen — Department of Chemistry and Center for Evolutionary Chemical Biology, University of Copenhagen, 2100 Copenhagen, Denmark

Frederik P. Jørgensen — Department of Chemistry, University of Copenhagen, 2100 Copenhagen, Denmark

Daniel Palmer — Department of Chemistry and Center for Evolutionary Chemical Biology, University of Copenhagen, 2100 Copenhagen, Denmark

Milena E. Roux — Department of Biology, University of Copenhagen, 2100 Copenhagen, Denmark

Jakob V. Olsen — Department of Biology, University of Copenhagen, 2100 Copenhagen, Denmark

Mikael Bols — Department of Chemistry, University of Copenhagen, 2100 Copenhagen, Denmark; orcid.org/0000-0002-6561-6952

Sanne Schoffelen — Department of Chemistry and Center for Evolutionary Chemical Biology, University of Copenhagen, 2100 Copenhagen, Denmark; orcid.org/0000-0003-2366-8561

Complete contact information is available at: https://pubs.acs.org/10.1021/acscombsci.9b00197

**Author Contributions**

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**Notes**

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**ABBREVIATIONS**

Ac, acetyl; Abu, α-aminobutyric acid; Amb, aminomethylbenzoic acid; Boc, tert-butoxycarbonyl; Comp, compound; DD, destabilizing domain; DMF, N,N-dimethylformamide; FKBP, FK506-binding protein; Fmoc, 9-fluorenylethoxycarbonyl; HATU, 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate; HMBA, hexamethylenbenzoic acid; MBP, maltose-binding protein; NEM, N-ethylmorpholine; NSB, nonspecific binding; PEGA, polyethylene glycol dimethyl acrylamide; Pip, pipicolic acid; POI, protein of interest; PyBOP, benzotriazole-1-yl-oxytrityrrolidinophosphonohexafluorophosphate; ROX, 5(6)-rhodamine-X; rt, room temperature; Shld1, Shld-1; SPPS, solid-phase peptide synthesis; EPS, solid-phase synthesis; TBTU, N-(1H-benzotriazol-1-yl)(dimethylamino)-methylene]-N-methyl methanaminium tetrafluoroborate; TFA, trifluoroacetic acid; THF, tetrahydrofuran

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