Ternary Complex-Templated Dynamic Combinatorial Chemistry for the Selection and Identification of Homo-PROTACs

Claudia J. Diehl,[a] Alessandra Salerno[a] and Alessio Ciulli*[a]

[a] Centre for Targeted Protein Degradation, School of Life Sciences, University of Dundee
1 James Lindsay Place, DD1 5JJ Dundee, Scotland, U.K.
E-mail: a.ciulli@dundee.ac.uk

Abstract:

Dynamic combinatorial chemistry (DCC) leverages a reversible reaction to generate compound libraries from constituting building blocks under thermodynamic control. The position of this equilibrium can be biased by addition of a target macromolecule towards enrichment of bound ligands. While DCC has been applied to select ligands for a single target protein, its application to identifying chimeric molecules inducing proximity between two proteins is unprecedented. In this proof-of-concept study, we develop a DCC approach to select bifunctional proteolysis targeting chimeras (PROTACs) based on their ability to stabilize the ternary complex. We focus on VHL-targeting Homo-PROTACs as model system, and show that the formation of a VHL_2:Homo-PROTAC ternary complex reversibly assembled using thiol-disulfide exchange chemistry leads to amplification of potent VHL Homo-PROTACs with degradation activities which correlated well with their biophysical ability to dimerize VHL. Ternary complex templated dynamic combinatorial libraries allowed identification of novel Homo-PROTAC degraders. We anticipate future applications of ternary-complex directed DCC to early PROTAC screenings and expansion to other proximity-inducing modalities beyond PROTACs.

Introduction

Dynamic combinatorial chemistry (DCC) is a powerful approach to generate chemically complex compound libraries with applications ranging from drug discovery[1] to supramolecular chemistry.[2] Conceptually, DCC exploits a reversible reaction to form compound libraries (dynamic combinatorial libraries, DCLs) from constituting building blocks under thermodynamic control. The position of the equilibrium of this reversible reaction can be biased by addition of an interacting target macromolecule acting as template. Reversibly assembled library members that bind to that template will be thermodynamically stabilized, hence enriched in abundance in templated libraries. These stabilized library members can be identified and ranked by their enrichment in templated libraries when compared to the corresponding non-templated blank libraries (Figure 1A).

The utility of DCC as hit-identification strategy in drug discovery has been demonstrated for a range of biological macromolecules as targets, including enzymes, receptors, nucleotides, and transmembrane proteins.[3] While two-body DCC scenarios harnessing interaction of reversibly formed library members with one templating biomolecule are well-established, applications with multiple templates are rare.

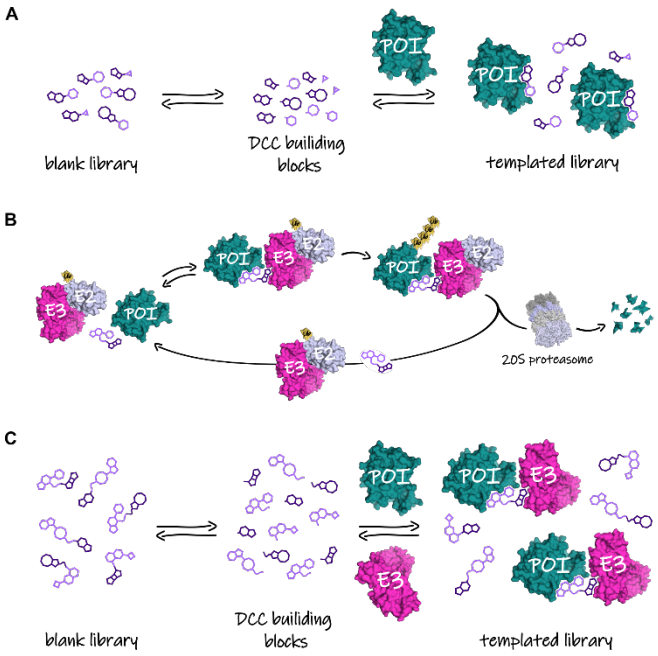


Figure 1. Schematic representation of DCC concept for PROTAC screening. A) Conventional DCC approach with one protein of interest (POI) acting as template; B) Mechanism of the mode of action of PROTAC degraders inducing an E3:PROTAC:POI ternary complex leading to ubiquitination and subsequent proteasomal degradation of target POI; C) This work: Proposed ternary complex-directed DCC approach for identification of ternary-complex inducing PROTACs.

Examples of multiple templates include an allosteric binding-guided DCC where macromolecular cyclic library members were assembled around one template, while encapsulated in γ-cyclodextrin as second template[4] and a multi-protein DCC approach where multiple proteins were introduced as templates of one DCL to simultaneously identify selective binders for each template.[5] To the best of our knowledge, application of DCC to the development of bivalent molecules directed and driven by ternary complex formation of library members with two templating proteins remains unprecedented.

Targeted protein degradation (TPD) describes chemically induced processes leading to the depletion of a target protein

(protein of interest, POI) in its cellular environment through co-opting of natural intracellular protein degradation mechanisms, such as the ubiquitin-proteasome system (UPS).[6] Over the past decade, TPD has evolved as promising alternative to conventional inhibitor-based therapies, mainly due to the successful development of proteolysis-targeting chimeras (PROTACs).[7] PROTACs are bifunctional molecules consisting of a ligand for a POI and a ligand for an E3 ligase joined by a linker. PROTACs are designed to induce proximity of POI and E3 ligase through formation of a E3:PROTAC:POI ternary complex, thus enabling POI ubiquitination and subsequent proteasomal degradation (Figure 1B).[8] Diverse approaches have been established to guide and streamline PROTAC development starting from an initial hit compound, including structure-based design[9] and rational design based on structure-activity relationships (SAR)[10] among others. Typically, molecular starting points are identified in direct screens for cellular target degradation of large libraries of previously synthesized PROTACs. Alternative screening approaches rely on cellular target engagement and/or biophysics to evaluate ternary complex formation either with recombinant proteins or in a cellular environment.[11] These initial screening libraries are usually designed by systematically changing E3 ligase and POI ligands, exit vectors for linker attachment on these ligands as well as linker length and nature. Hit identification through these approaches is usually highly time- and resource consuming for both synthesis and biological screening.

Substantive evidence supports the notion that optimizing for ternary complex affinity and stability provides a robustly validated and, in most cases, successful criterium for optimizing degradation activities of PROTACs.[12] For example, correlations have often been observed between a PROTAC's ability to form stable ternary complexes at low concentrations and its degradation efficiency and potency.[13] From this rationale, we therefore posited that DCC could be applied in a new fashion to screen for and identify most promising PROTACs in experiments where stabilization of reversibly assembled PROTAC library members is governed by E3:DCC-PROTAC:POI ternary complex formation. In this context, both E3 ligase and POI would act jointly as templating moiety to thermodynamically stabilize and consequently enrich DCC-PROTACs able to form a stable ternary complex (Figure 1C), while discriminating against species that are less able to induce ternary complex formation. We therefore embarked on a study to develop and apply a proof-of-concept for a DCC-PROTAC approach.

Results and Discussion

Design Rational for Proof-of-Concept DCC-PROTACs

To test the hypothesis of ternary complex-directed DCC PROTAC screening, our system had to fulfill the general requirements of DCL experiments: namely, reversible chemistry with reaction conditions that are tolerated by the proteins introduced as templates; the reaction rates should be sufficiently fast in the interest of protein stability and experimental efficiency; and the equilibrium state has to be frozen prior to library analysis without chemical modification of the reversibly assembled DCC-PROTACs. Beyond that, the reversible bond introduced into these PROTACs needs to be sufficiently stable to allow independent synthesis and isolation of the unmodified DCC-PROTACs for biophysical and biological testing. This last point is vital in this work to unambiguously correlate amplification observed in the DCC experiments with the biophysical and cellular activity of the PROTAC, thus allowing to validate the concept of ternary complex formation as a driving force for amplification in the DCC assay.

Based on these criteria, we selected the disulfide exchange reaction as reversible chemistry to build the DCL, because it features mild and protein-benign reversible reaction conditions, easy freezing of the equilibrium by acidification, and provides molecules expected to be sufficiently stable for isolation and testing.[1b, 2a, 14] As disulfides are formed through dimerization of thiols, the chemical handle on both constituting building blocks is identical in the disulfide exchange reaction. As such both homo-dimeric (A–A and B–B) and heterodimeric (A–B) products can be formed in this reaction. To allow exploiting not only heterodimeric, but also the homo-dimeric products of the reversible disulfide exchange and maximize the number of on-target PROTACs formed during building block scrambling, we decided to leverage our previously reported VHL-targeting Homo-PROTACs, where the protein VHL serves as both the E3 ligase and the target protein, mediating its own degradation.[15] We envisaged that, in a DCC setting, formation of homo-dimeric scrambling products will lead to symmetric Homo-PROTACs, while hetero-dimeric scrambling will give rise to asymmetric Homo-PROTACs.

Synthesis and Assessment of Symmetric Disulfide-Containing Homo-PROTACs

Since DCC experiments beyond 2-body systems had remained unprecedented, our first goal was to test the hypothesis that the relative stability of ternary complexes induced by DCC-PROTACs could influence their enrichment in templated DCC experiments. For this purpose, we designed and independently synthesized a small set of disulfide-containing Homo-PROTACs containing a DCC handle in their linker moiety, which were assessed regarding their ability to induce ternary complex formation *in vitro* and degradation of the target protein in cells.

We began by synthesizing a small set of disulfide-containing Homo-PROTACs as starting compounds for the later-developed DCC assay and for biological and biophysical profiling. The initial Homo-PROTAC set was designed to contain both degrading and non-degrading PROTACs to represent the wide range of outcomes of early-stage DCC-derived PROTACs. We leveraged our previously revealed SAR of VHL Homo-PROTACs, with compound CM11 (Figure 2A), featuring an 18-atom PEG-5 linker, forming the most stable VHL_2:CM11 ternary complex and being the most potent degrader in cell, while analogous compounds with shorter linkers, e.g. PEG-3 and PEG-4, exhibited poorer performance in the same assays.[15] We therefore designed ten disulfide-containing Homo-PROTACs, featuring either the same VHL ligand as in CM11 (namely, VH032) or its benzyl-methylated analog, and alkoxyether or alkyl linkers of various length (Figure 2B). In line with previous SAR of VHL-targeting Homo-

PROTACs,[15] the 18-atom linkers were expected to perform better than those with shorter linkers. To be able to decipher the role of the underexplored disulfide motif, we also designed and synthesized analogs featuring the benzyl-methylated derivative of VH032 as VHL ligand on each side with the same linker as in CM11 (MeCM11) and an 18-atom alkyl chain equivalent as linker with VH032 as VHL ligand on either side (alCM11) (Figure 2C).

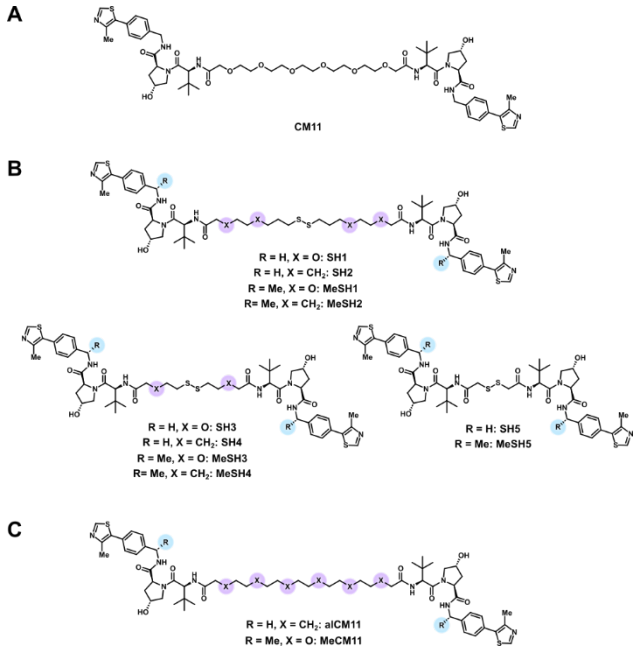
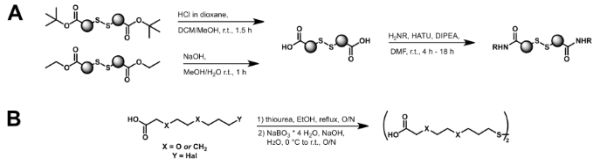


Figure 2. Design of a disulfide-containing VHL-targeting Homo-PROTAC library. A) Structure of the reported Homo-PROTAC CM11; B) Overview of the initially designed series of disulfide-containing Homo-PROTACs; C) Disulfide-free derivatives of CM11 as control compounds.

All symmetric Homo-PROTACs were assembled from established VHL ligands[16] and disulfide-containing linkers in a 2:1-ratio using HATU-mediated amide couplings (Scheme 1A).



Scheme 1. Synthetic strategy. A) General assembly of symmetric Homo-PROTACs from VHL ligands and linkers *via* HATU-mediated amide coupling; B) Method to synthesize disulfide-linkers from alkyl halide precursors.

Two general strategies were employed to synthesize the disulfide-containing linkers. If the corresponding thiols were commercially available, such as 2-mercaptoacetic acid, the disulfides were formed *via* oxidative dimerization using hydrogen peroxide as oxidant. Alternatively, disulfide linkers were synthesized from corresponding alkyl halide precursors by reaction with thiourea to form *S*-alkylisothiouronium salts, which were then oxidatively hydrolyzed to the desired disulfides (Scheme 1B).

All synthesized Homo-PROTACs were subsequently tested in both cellular degradation and biophysical ternary complex formation assays. First, VHL levels were determined *via* Western Blot after compound treatment for 8 h in two different cell lines (HEK 293 and HeLa) and compared to the degradation induced by reference compound CM11 (Figures 3A and S1). Efficient degradation of the long isoform of VHL (pVHL30) was observed at 1 μM concentration for four compounds (SH1, SH2, MeSH1, MeSH2), featuring – like CM11 – 18-atom linkers. In line with previous reports,[15] degradation efficacy decreased with shorter linkers. Interestingly unprecedented degradation of the short isoform of VHL (pVHL19) was induced by compound SH2. Comparison with the degradation behavior of the disulfide-free analog alCM11 inducing only < 35% degradation of pVHL19 (Figure S2), and SH1 with a disulfide-alkoxy linker inducing 11% degradation of pVHL19, suggested that combination of the disulfide motif and the alkyl linker leads to the cooperative improvement in pVHL19 degradation observed with SH2.

Next, to assess and rank the ability of Homo-PROTACs to induce VHL dimerization, we developed and validated a novel rapid and convenient native gel electrophoresis assay (see SI, Figure S3). The recombinant VCB protein complex (VHL:ElonginC:ElonginB) was incubated with the respective Homo-PROTAC for 30 min at room temperature and then separated *via* gel electrophoresis under nondenaturing conditions. Ternary complex formation was associated with an up-shift of the protein band in the gel, as observed for CM11. The 18-atom linker featuring Homo-PROTACs SH1, SH2, MeSH1 and MeSH2 led to the largest formation of the ternary complex, while other PROTACs only induced incomplete dimerization of VHL (MeCM11, alCM11, SH3, SH5) or formation of aggregates (SH5, MeSH3) (Figure 3B).
Plotting the percent of ternary complex detected in the native gel electrophoresis assay against the pVHL30 degradation level observed in the initial PROTAC screen in HEK 293 cells at 1 μM for 8 h (see Figure 3A) reveals a clear correlation between the PROTACs' ability to induce ternary complex formation *in vitro* and inducing target degradation in cells (Figure 3C).

These results demonstrate that the introduced disulfide motif is well-tolerated, and that the previously observed trend of diminished degrader potency with shortened linker length is maintained with these disulfide-containing Homo-PROTACs. Crucially, the degradation potency of the new compounds correlated well with their ability to induce VHL dimerization, thus supporting our working hypothesis for the ternary complex-directed DCC assay.

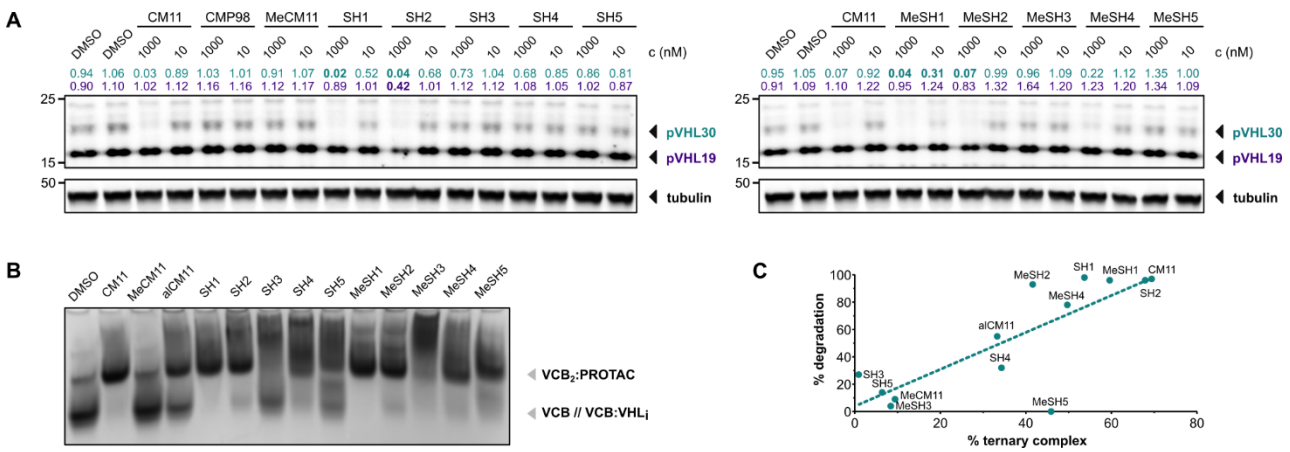


Figure 3. Cellular and *in vitro* assessment of the initial series of Homo-PROTACs. A) Representative immunoblots of VHL protein levels after treatment of HEK 293 cells at indicated concentrations and 0.01% DMSO for 8 h. VHL protein levels were obtained by quantifying the ratios of pVHL30/tubulin or pVHL19/tubulin, respectively, normalized to the average of the DMSO-treated samples. Data obtained from two independent biological experiments; B) Representative native gel of VCB preincubated with Homo-PROTAC or 5% DMSO for 30 min; C) Correlation of degradation levels of pVHL30 at 1 μM after 8 h in HEK 293 cells (presented in Figure 3A) with *in vitro* formed fraction of ternary complex from native gel electrophoresis assay (presented in Figure 3B). Values of two biological replicates of each experimental set were averaged for plotting.

Having established the suitability of disulfides as DCC motif in stable, isolatable compounds and the vital correlation between induction of ternary complex formation and degrader potency, we set out to characterize the identified novel degraders in depth and selected SH2 as representative for this extended characterization studies. To gain mechanistic insight into the mode of action of disulfide-containing Homo-PROTACs, we performed co-treatments of degrader SH2 along with the proteasome inhibitor MG132,[17] the neddylation inhibitor MLN4924[18] as well as with the potent VHL inhibitor VH298[19] (Figure 4A). VHL protein levels were rescued in all three cases, indicating that VHL degradation is dependent on the proteasomal degradation pathway, on the activity of the CRL^{VHL} ligase and specifically on binding to VHL, respectively, as the expected PROTAC mode of action.

We next examined both time- and dose-dependent degradation of VHL in HEK 293 cells induced by SH2 (Figure 4B and S4). SH2 exhibited double-digit nanomolar half-degradation concentrations (DC_{50}) for both isoforms of VHL after 8 h treatment (DC_{50} = 11 nM, D_{max} = 94% for pVHL30; DC_{50} = 52 nM, D_{max} = 66% for pVHL19), as such comparing favourably with CM11. Remarkably, SH2 induced degradation of the long isoform of VHL at considerable faster rate compared to CM11 ($t_{½}$ (pVHL30, SH2) = 35 min, $t_{½}$ (pVHL30, CM11) = 116 min[15]) while still maintaining low VHL levels for up to 48 h, qualifying SH2 overall as a superior VHL degrader relative to CM11.

To validate the ability of SH2 to induce ternary complex formation, we turned to size exclusion chromatography (SEC) as orthogonal method to the previously performed native gel electrophoresis assay. Using CM11 as benchmark, we confirmed the formation of a VCB_2:SH2 ternary complex, which migrated quicker than the VCB vehicle control (Figure 4C).

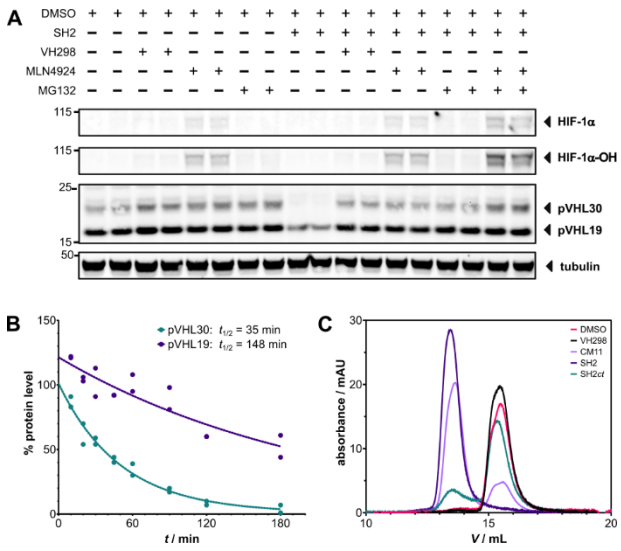


Figure 4. Detailed characterization of Homo-PROTAC SH2. A) Cellular mechanistic studies. Immunoblot after treatment of HEK 293 cells with 1 μM of SH2 in the absence and presence of 50 μM of VHL inhibitor VH298, 3 μM of neddylation inhibitor MLN4924 or 12.5 μM of proteasome inhibitor MG132. B) Time-dependent VHL degradation. VHL protein levels were obtained by quantifying the ratios of pVHL30/tubulin or pVHL19/tubulin, respectively, normalized to the average of the DMSO-treated samples. Data obtained from two independent biological experiments. C) Size exclusion chromatography (SEC) UV chromatograms of complex formation after incubation of VCB with VH298 (black), CM11 (lavender), SH2 (lilac), and negative control SH2*ct* (green).

Furthermore, we synthesized a negative control compound (SH2*ct*) featuring the VH032 binder as one ligand and its non-

binding derivative *cis*-VH032, which abrogates binding through inverted stereochemistry at the hydroxyl group of hydroxyproline, as second ligand (see synthesis overview in Scheme S1). As anticipated, SH2*ct* did not affect the retention volume of VCB during SEC, confirming the requirement of two binding ligands in SH2 for ternary complex formation.

Development of a Ternary-Complex Directed DCC Assay

Having established a suitable set of compounds with well understood SAR, we next moved on to developing and optimizing a DCC experimental set-up. Initial criterium for the reaction optimization was detectable formation of novel unsymmetric DCC-Homo-PROTACs through scrambling of the symmetric disulfide-containing Homo-PROTACs used as starting materials. Further optimization aimed at reducing the equilibration time to a reasonable time frame while maintaining conditions that will be tolerated by VCB. In order to achieve rapid scrambling of the DCL members, we used 1,4-dithiothreitol (DTT) as initiating reagent. Under optimized exchange conditions (100 mM DTT in tris buffer at pH = 8.5 with 10% DMSO under argon atmosphere) the libraries equilibrated at 37 °C within 24 h. With these conditions in hand, we attempted the first templated DCC experiment at the smallest possible library size using only two symmetric Homo-PROTACs, the potent VHL-degrader SH2 and the much less potent Homo-PROTAC SH4, as starting point (Figure 5A). In this scenario, our DCL would be composed of three Homo-PROTACs, i.e., the two symmetric starting materials along with the unsymmetric scrambling product, along with the two corresponding monomeric thiols. While only small amounts of the unsymmetric DCL-Homo-PROTAC H2-H4 formed in the blank library, considerable larger amounts of H2-H4 were detected in the VCB-templated library after equilibration. Overall, 67% relative enrichment of the novel, unsymmetric Homo-PROTAC H2-H4 was observed.

To validate that the unsymmetric Homo-PROTAC H2-H4 is indeed capable of inducing both ternary complex formation and VHL degradation, we synthesized this hit independently for biological testing (see synthesis overview in Scheme S2). As with the initial set of synthesized Homo-PROTACs, native gel electrophoresis was used to evaluate H2-H4's ability to induce VCB_2:H2-H4 ternary complex formation *in vitro* (Figure 6B & C). Incubation of VCB with H2-H4 for 30 min led to quantitative formation of the ternary complex, while SH2 only induced incomplete ternary complex formation under these conditions.

Encouraged by the successful ternary complex formation observed *in vitro*, H2-H4 was further studied in cells. Pleasingly, H2-H4 induced 94% degradation of pVHL30 in HEK 293 cells already after 4 hours at 1 μM and maintained pVHL30 levels at < 6% for at least 24 hours (Figure S5A). Subsequent dose- and time-dependent treatments (Figure 6D and S5B) characterized H2-H4 as potent degrader of pVHL30 (DC_{50} = 35 nM, D_{max} = 98%) inducing remarkably rapid depletion of pVHL30 ($t_{½}$ = 28 min). Though performed on conceivable smallest scale, this initial DCL experiment led already to identification of a novel, potent VHL degrader through substantial amplification of this library member in the presence of a protein template.

We next decided to increase the size of the DCL. By adding three symmetric Homo-PROTACs as starting materials, the number of PROTAC library members increases from three to 15 for this second library, alongside five monomeric thiols (Figure 7A). Aiming to portray the whole chemical space covered by the previously synthesized symmetric Homo-PROTACs, we included symmetric Homo-PROTACs with good (MeSH2), average (MeSH3), and very poor (MeSH5) potency as starting materials in this extended DCC experiment (Figure 7B).

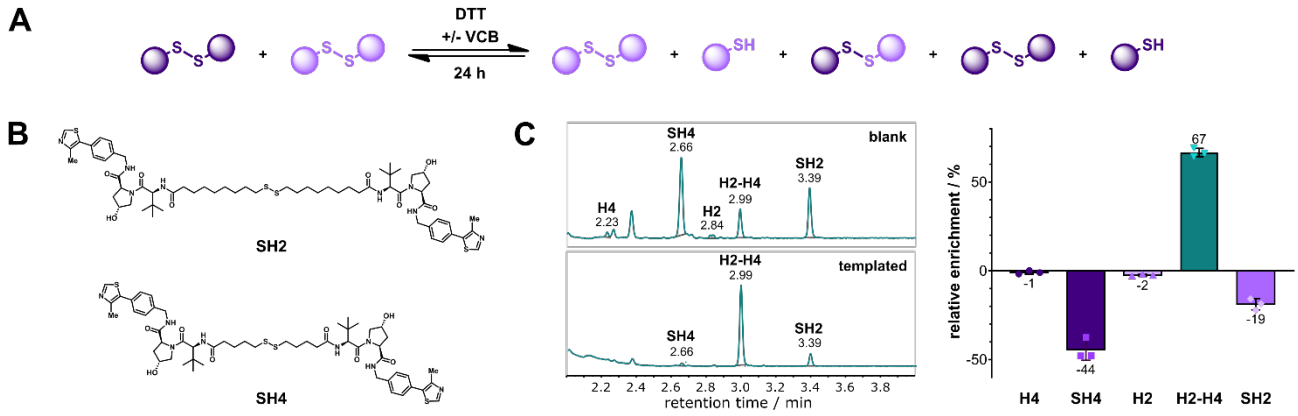


Figure 5. Initial DCC experiment. A) General scheme; B) Structures of the starting molecules SH2 and SH4 for scrambling; C) UV Chromatograms of the blank (top) and VCB templated (bottom) library after 24 h equilibration time and plot of the relative enrichment of all library components. Values are averaged over three independent experiments.

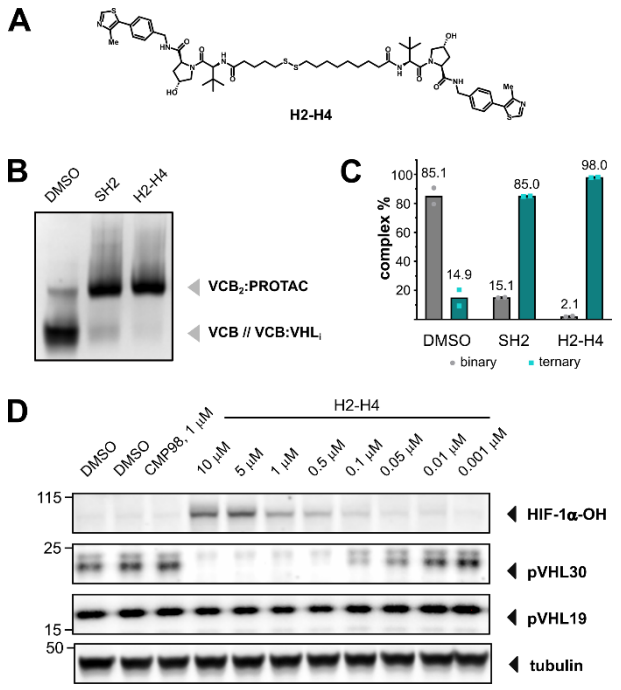


Figure 6. Validation of H2-H4 as a hit from initial DCC experiment. A) Structure of H2-H4; B) Representative native gel of VCB preincubated with 0.5 equiv. of SH2, H2-H4 or 5% DMSO for 30 min; C) Quantification of complex bands in the native gel assay. Averaged results of two independent biological replicates were plotted; D) Representative immunoblot of treatment of HEK 293 cells with H2-H4 or CMP98 at indicated concentrations and 0.1% DMSO for 8 h.

As in the first DCC experiment, addition of VCB as templating biomolecule led to significant changes in the distribution of species in the equilibrated library (Figure S6). While all monomeric species (grey) as well as DCL-PROTACs with very short linkers (four to seven atoms, lilac and lavender) were depleted in the templated library, 14- and 18-atom species (green and pink) were enriched in presence of VCB (Figure 7C).

A plot of the enrichment/depletion in the templated library of the initially synthesized symmetric Homo-PROTACs as well as H2-H4 against the degradation level induced at 1 µM, shows a clear correlation between degrader potency and amplification observed in the templated DCL (Figure 7D). Similarly, the amplification in the templated DCL informs about the ability of the Homo-PROTAC to induce ternary complex formation (Figure S7). We observed significant depletion of monomeric species in the templated DCL, which is consistent with enrichment in the DCC experiment being derived from thermodynamic stabilization of Homo-PROTAC species *via* induced VHL dimerization rather than binary binding of monomeric species or DCC-Homo-PROTACs to only one templating VHL. Besides confirming previously identified Homo-PROTACs (SH2, MeSH2) as hits and demonstrating the envisioned correlation between signal amplification in a templated DCL with degrader potency, two additional and novel, asymmetric DCC-Homo-PROTACs were identified in this second DCC experiment, namely MeH2-MeH3 and H2-MeH3, showing strong enhancement in the templated DCL.

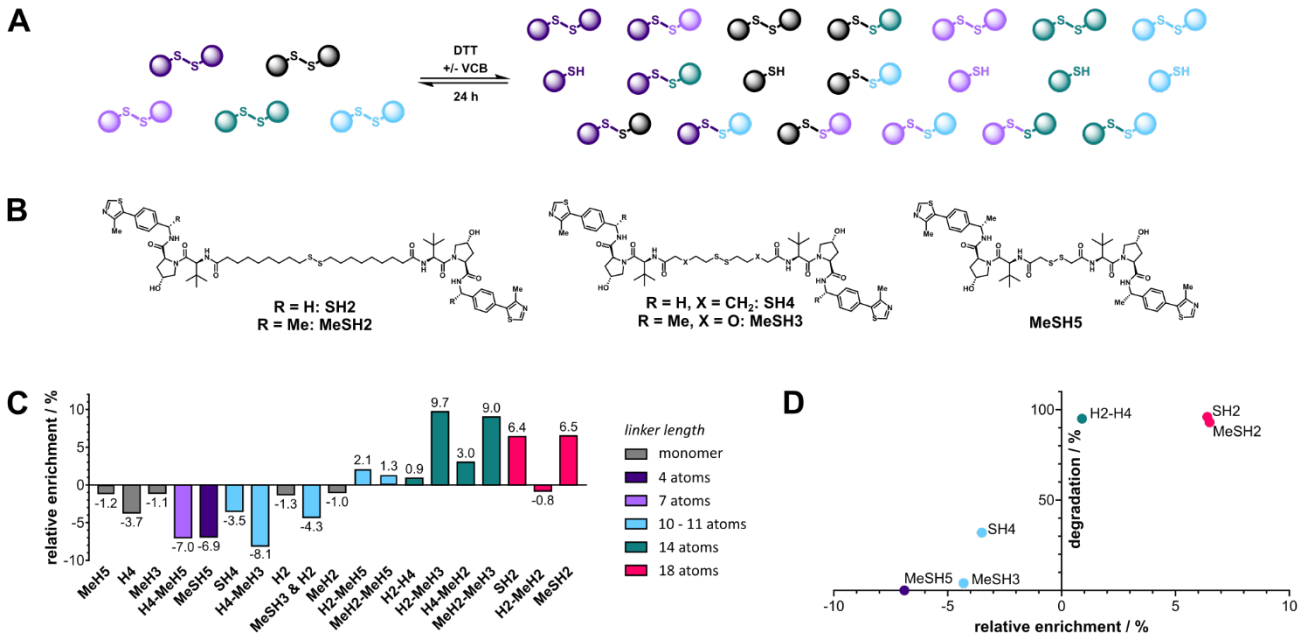


Figure 7. Expanded DCC experiment. A) General scheme; B) Structures of the starting molecules SH2, MeSH2, MeSH3, SH4 and MeSH5 for scrambling; C) Plot of the relative enrichment of all library components after 24 h equilibration time. Values are averaged over two independent experiments; D) Plot of relative enrichment of independently characterized Homo-PROTACs against degradation induced at 1 µM after 8 h in HEK 293 cells (see Figure 3A).

As validation of our ternary-complex directed DCC approach, we independently synthesized one of the hits, MeH2-MeH3 (Figure 8A, see synthesis overview in Scheme S3), of this expanded DCC experiment for further evaluation. As before, native gel electrophoresis was utilized to assess the capability of MeH2-MeH3 in prompting VCB_2:MeH2-MeH3 ternary complex formation *in vitro.* Following incubation of VCB with MeH2-MeH3 for 30 min, partial formation of the ternary complex was observed at the initial ratios of 1:1 to 1:4, and full conversion was observed with increasing excess of MeH2-MeH3 (Figure 8B & C). Based on the *in vitro* findings suggesting the compound's ability to induce ternary complex formation – albeit to a less extent than SH2 – MeH2-MeH3 was tested in cells and shown to induce degradation of pVHL30 after 8 hours in a dose-dependent treatment with DC_{50} = 1.32 µM and full degradation at 10 µM (Figure 8D). The evidenced activities of MeH2-MeH3, though not as potent as SH2 or H2-H4, further validates the DCC approach in expediting the identification of dimerizers as active degraders.

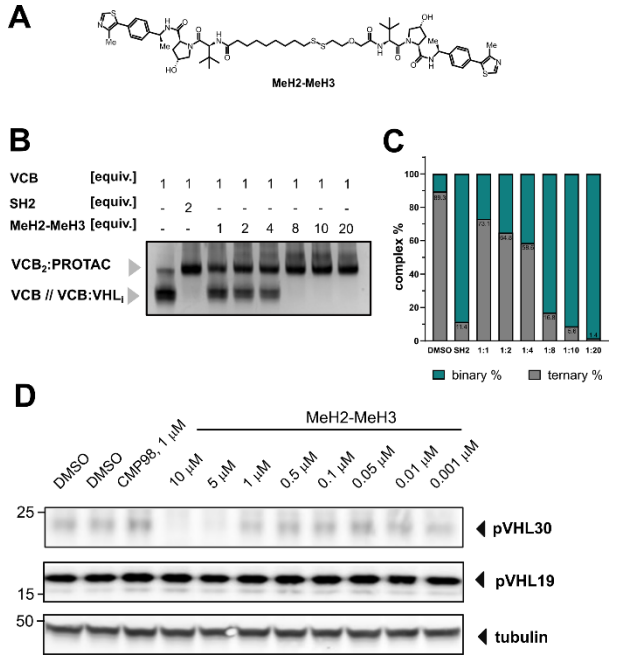


Figure 8. Validation of MeH2-MeH3 as a hit from the second DCC experiment. A) Structure of MeH2-MeH3; B) Representative native gel of VCB preincubated with SH2, MeH2-MeH3 or 5% DMSO for 30 min; C) Quantification of complex bands in the native gel assay. Averaged results of two independent biological replicates were plotted; D) Representative immunoblot of treatment of HEK 293 cells with MeH2-MeH3 or CMP98 at indicated concentrations and 0.1% DMSO for 8 h.

Conclusions

The success of proximity-inducing modalities, such as PROTACs, crucially depends on the formation of a stable ternary complex. Herein, we establish that this ternary complex can be exploited as selection criterium in multi-protein templated DCLs by driving enrichment of proximity-inducing library members. Using a disulfide-containing VHL-targeting Homo-PROTAC system as proof-of-concept, we demonstrate that the developed ternary complex-directed DCC assay enables quick identification of novel VHL degraders from library mixtures. Comparison of the performance of DCL members in a templated library with their biophysical and cellular potency shows overall good correlation between their ability to induce VHL dimerization and cellular degradation activity and their enrichment in the DCC assay. Conversely, non-degrading PROTACs and monomeric species were depleted in the templated DCL, indicating that amplification is indeed driven by cooperative ternary complex formation, and not a result of binary protein-library member binding events. Besides confirming previously tested degrading disulfide-containing Homo-PROTACs as hits, *i.e.,* SH2 and MeSH2, further novel hits were identified using this strategy. For instance, H2-H4 identified in the very first ternary complex-directed DCC experiment, was validated as excellent mediator of ternary complex formation outperforming SH2, and as potent VHL degrader, leading to rapid depletion of pVHL30 levels in cells.

Although the herein implemented ternary complex-templated DCC approach is currently applied to only one E3 ligase as target, we envisage it to be readily transferable to the development of Homo-PROTACs targeting other E3 ligases, especially those with available high-quality non-covalent small molecule ligands, such as for CRBN,[20] MDM2,[21] cIAP,[22] DCAF1,[23] TRIM58[24] or SOCS2.[25] Beyond Homo-PROTACs, this disulfide-based ternary complex directed DCC approach could be further adopted to hetero-PROTACs, though requiring careful building block design (*i.e.*, VHL binding building blocks featuring short linkers would be suitable for the development of hetero-PROTAC DCC approaches, as depletion of Homo-PROTACs with short linkers was observed in templated DCLs). Taking the concept of ternary complex-directed DCC assays one step further, other reversible chemistries used in conventional two-body DCC setting could be envisioned for this approach, *e.g.* oxime formation or boronate ester formation, which were recently established for reversibly assembled PROTACs by the Kodadek group.[26] While generalizability beyond VHL as target remains to be shown, we believe that the ternary complex-directed DCC approach developed here offers the opportunity to fast-track hit identification in early-stage discovery programs for PROTACs and beyond for the identification of other bifunctional molecules as proximity-inducing modalities.

Supporting Information

Supporting information including additional figures and schemes, general experimental details,synthetic protocols and characterization data are provided in a separate file.

Acknowledgements

The work of the Ciulli laboratory on VHL ligands and PROTACs has received funding from the European Research Council (ERC) under the European Union's Seventh Framework Programme (FP7/2007-2013) as a starting grant to A.C. (grant agreement

ERC-2012-StG-311460 DrugE3CRLs) and the Innovative Medicines Initiative 2 (IMI2) Joint Undertaking under grant agreement no. 875510 (EUbOPEN project to A.C.) from the European Union's Horizon 2020 Research and Innovation Programme. C.J.D. received funding from the European Union's Horizon 2020 Research and Innovation Programme under the Marie Skłodowska–Curie Actions Individual Fellowship, grant agreement no. 101026833. Biophysics and drug discovery activities at Dundee were supported by Wellcome Trust strategic awards 100476/Z/12/Z and 094090/Z/10/Z, respectively. The authors thank Mark Nakasone and Zoe Rutter for helpful discussions and support with development of the native gel electrophoresis assay, Oliver Hsia for guidance with cellular work, and Charlotte Crowe, Xingui Liu and Kevin Haubrich for gifting VCB protein.

Conflict of Interest

The Ciulli laboratory receives or has received sponsored research support from Almirall, Amgen, Amphista Therapeutics, Boehringer Ingelheim, Eisai, Merck KgaA, Nurix Therapeutics, Ono Pharmaceutical and Tocris-Biotechne. A.C. is a scientific founder, shareholder, and advisor of Amphista Therapeutics, a company that is developing targeted protein degradation therapeutic platforms.

Keywords: dynamic combinatorial chemistry • targeted protein degradation • PROTACs • VHL • assay development

[1] a) M. Mondal, A. K. Hirsch, *Chem. Soc. Rev* **2015**, *44*, 2455; b) A. Canal-Martin, R. Perez-Fernandez, *ACS Omega* **2020**, *5*, 26307; c) G. Bagnolini, B. Balboni, F. Schipani, D. Gioia, M. Veronesi, F. De Franco, C. Kaya, R. P. Jumde, J. A. Ortega, S. Girotto, A. K. H. Hirsch, M. Roberti, A. Cavalli, *ACS Med. Chem. Lett.* **2022**, *13*, 1262; d) S. D. Volpe, R. Listro, F. A. Ambrosio, M. Garbagnoli, P. Linciano, D. Rossi, G. Costa, S. Alcaro, F. Vasile, A. K. H. Hirsch, S. Collina, *ACS Med. Chem. Lett.* **2023**, *14*, 1509.

[2] a) P. T. Corbett, J. Leclaire, L. Vial, K. R. West, J. L. Wietor, J. K. Sanders, S. Otto, *Chem. Rev.* **2006**, *106*, 3652; b) S. P. Black, J. K. M. Sanders, A. R. Stefankiewicz, *Chem. Soc. Rev.* **2014**, *43*, 1861.

[3] a) D. E. Scott, G. J. Dawes, M. Ando, C. Abell, A. Ciulli, *ChemBioChem* **2009**, *10*, 2772; b) J. D. McAnany, J. P. Reichert, B. L. Miller, *Bioorg. Med. Chem.* **2016**, *24*, 3940; c) L. Monjas, L. J. Y. M. Swier, I. Setyawati, D. J. Slotboom, A. K. H. Hirsch, *ChemMedChem* **2017**, *12*, 1693; d) S. Jana, D. Panda, P. Saha, G. D. Pantoş, J. Dash, *J. Med. Chem.* **2019**, *62*, 762; e) A. M. Hartman, W. A. M. Elgaher, N. Hertrich, S. A. Andrei, C. Ottmann, A. K. H. Hirsch, *ACS Med. Chem. Lett.* **2020**, *11*, 1041.

[4] J. Li, P. Nowak, S. Otto, *Angew. Chem., Int. Ed.* **2015**, *54*, 833.

[5] M. Das, T. Yang, J. Dong, F. Prasetya, Y. Xie, K. H. Q. Wong, A. Cheong, E. C. Y. Woon, *Chem. Asian J.* **2018**, *13*, 2854.

[6] R. Verma, D. Mohl, R. J. Deshaies, *Mol. Cell* **2020**, *77*, 446.

[7] a) D. A. Nalawansha, C. M. Crews, *Cell Chem. Biol.* **2020**, *27*, 998; b) M. Békés, D. R. Langley, C. M. Crews, *Nature Rev. Drug Discov.* **2022**, *21*, 181.

[8] A. Ciulli, N. Trainor, *Biochem (Lond)* **2021**, *43*, 74.

[9] a) W. Farnaby, M. Koegl, M. J. Roy, C. Whitworth, E. Diers, N. Trainor, D. Zollman, S. Steurer, J. Karolyi-Oezguer, C. Riedmueller, T. Gmaschitz, J. Wachter, C. Dank, M. Galant, B. Sharps, K. Rumpel, E. Traxler, T. Gerstberger, R. Schnitzer, O. Petermann, P. Greb, H. Weinstabl, G. Bader, A. Zoephel, A. Weiss-Puxbaum, K. Ehrenhofer-Wolfer, S. Wohrle, G. Boehmelt, J. Rinnenthal, H. Arnhof, N. Wiechens, M. Y. Wu, T. Owen-Hughes, P. Ettmayer, M. Pearson, D. B. McConnell, A. Ciulli, *Nat. Chem. Biol.* **2019**, *15*, 672; b) A. Testa, S. J. Hughes, X. Lucas, J. E. Wright, A. Ciulli, *Angew Chem. Int. Ed. Engl.* **2020**, *59*, 1727; c) C.-w. Chung, H. Dai, E. Fernandez, C. P. Tinworth, I. Churcher, J. Cryan, J. Denyer, J. D. Harling, A. Konopacka, M. A. Queisser, C. J. Tame, G. Watt, F. Jiang, D. Qian, A. B. Benowitz, *ACS Chem. Biol.* **2020**, *15*, 2316; d) X. Yu, D. Li, J. Kottur, Y. Shen, H. S. Kim, K.-S. Park, Y.-H. Tsai, W. Gong, J. Wang, K. Suzuki, J. Parker, L. Herring, H. Ü. Kaniskan, L. Cai, R. Jain, J. Liu, A. K. Aggarwal, G. G. Wang, J. Jin, *Sci. Transl. Med.* **2021**, *13*, eabj1578; e) J. Schiemer, R. Horst, Y. Meng, J. I. Montgomery, Y. Xu, X. Feng, K. Borzilleri, D. P. Uccello, C. Leverett, S. Brown, Y. Che, M. F. Brown, M. M. Hayward, A. M. Gilbert, M. C. Noe, M. F. Calabrese, *Nat. Chem. Biol.* **2021**, *17*, 152.

[10] a) V. Zoppi, S. J. Hughes, C. Maniaci, A. Testa, T. Gmaschitz, C. Wieshofer, M. Koegl, K. M. Riching, D. L. Daniels, A. Spallarossa, A. Ciulli, *J. Med. Chem.* **2019**, *62*, 699; b) X. Han, C. Wang, C. Qin, W. Xiang, E. Fernandez-Salas, C.-Y. Yang, M. Wang, L. Zhao, T. Xu, K. Chinnaswamy, J. Delproposto, J. Stuckey, S. Wang, *J. Med. Chem.* **2019**, *62*, 941; c) X. Han, L. Zhao, W. Xiang, C. Qin, B. Miao, T. Xu, M. Wang, C.-Y. Yang, K. Chinnaswamy, J. Stuckey, S. Wang, *J. Med. Chem.* **2019**, *62*, 11218; d) X. Liu, A. F. Kalogeropulou, S. Domingos, N. Makukhin, R. S. Nirujogi, F. Singh, N. Shpiro, A. Saalfrank, E. Sammler, I. G. Ganley, R. Moreira, D. R. Alessi, A. Ciulli, *J. Am. Chem. Soc.* **2022**, *144*, 16930.

[11] a) D. L. Daniels, K. M. Riching, M. Urh, *Drug Discov. Today Technol.* **2019**, *31*, 61; b) X. Liu, X. Zhang, D. Lv, Y. Yuan, G. Zheng, D. Zhou, *Future Med. Chem.* **2020**, *12*, 1155; c) D. Zollman, A. Ciulli, in *Protein Degradation with New Chemical Modalities*, 10.1039/9781839160691-00014 (Eds.: H. Weinmann, C. Crews), The Royal Society of Chemistry, **2020**, p. 0; d) R. Casement, A. Bond, C. Craigon, A. Ciulli, in *Targeted Protein Degradation: Methods and Protocols*, 10.1007/978-1-0716-1665-9_5 (Eds.: A. M. Cacace, C. M. Hickey, M. Békés), Springer US, New York, NY, **2021**, pp. 79-113.

[12] a) M. S. Gadd, A. Testa, X. Lucas, K.-H. Chan, W. Chen, D. J. Lamont, M. Zengerle, A. Ciulli, *Nat. Chem. Biol.* **2017**, *13*, 514; b) D. P. Bondeson, B. E. Smith, G. M. Burslem, A. D. Buhimschi, J. Hines, S. Jaime-Figueroa, J. Wang, B. D. Hamman, A. Ishchenko, C. M. Crews, *Cell Chem. Biol.* **2018**, *25*, 78; c) M. J. Roy, S. Winkler, S. J. Hughes, C. Whitworth, M. Galant, W. Farnaby, K. Rumpel, A. Ciulli, *ACS Chem. Biol.* **2019**, *14*, 361.

[13] a) T. H. Pillow, P. Adhikari, R. A. Blake, J. Chen, G. Del Rosario, G. Deshmukh, I. Figueroa, K. E. Gascoigne, A. V. Kamath, S. Kaufman, T. Kleinheinz, K. R. Kozak, B. Latifi, D. D. Leipold, C. Sing Li, R. Li, M. M. Mulvihill, A. O'Donohue, R. K. Rowntree, J. D. Sadowsky, J. Wai, X. Wang, C. Wu, Z. Xu, H. Yao, S.-F. Yu, D. Zhang, R. Zang, H. Zhang, H. Zhou, X. Zhu, P. S. Dragovich, *ChemMedChem* **2020**, *15*, 17; b) S. Imaide, K. M. Riching, N. Makukhin, V. Vetma, C. Whitworth, S. J. Hughes, N. Trainor, S. D. Mahan, N. Murphy, A. D. Cowan, K. H. Chan, C. Craigon, A. Testa, C. Maniaci, M. Urh, D. L. Daniels, A. Ciulli, *Nat. Chem. Biol.* **2021**, *17*, 1157; c) P. S. Dragovich, T. H. Pillow, R. A. Blake, J. D. Sadowsky, E. Adaligil, P. Adhikari, J. Chen, N. Corr, J. dela Cruz-Chuh, G. Del Rosario, A. Fullerton, S. J. Hartman, F. Jiang, S. Kaufman, T. Kleinheinz, K. R. Kozak, L. Liu, Y. Lu, M. M. Mulvihill, J. M. Murray, A. O'Donohue, R. K. Rowntree, W. S. Sawyer, L. R. Staben, J. Wai, J. Wang, B. Wei, W. Wei, Z. Xu, H. Yao, S.-F. Yu, D. Zhang, H. Zhang, S. Zhang, Y. Zhao, H. Zhou, X. Zhu, *J. Med. Chem.* **2021**, *64*, 2576; d) P. S. Dragovich, T. H. Pillow, R. A. Blake, J. D. Sadowsky, E. Adaligil, P. Adhikari, S. Bhakta, N. Blaquiere, J. Chen, J. dela Cruz-Chuh, K. E. Gascoigne, S. J. Hartman, M. He, S. Kaufman, T. Kleinheinz, K. R. Kozak, L. Liu, L. Liu, Q. Liu, Y. Lu, F. Meng, M. M. Mulvihill, A. O'Donohue, R. K. Rowntree, L. R. Staben, S. T. Staben, J. Wai, J. Wang, B. Wei, C. Wilson, J. Xin, Z. Xu, H. Yao, D. Zhang, H. Zhang, H. Zhou, X. Zhu, *J. Med. Chem.* **2021**, *64*, 2534; e) R. P. Law, J. Nunes, C.-w. Chung, M. Bantscheff, K. Buda, H. Dai, J. P. Evans, A. Flinders, D. Klimaszewska, A. J. Lewis, M. Muelbaier, P. Scott-Stevens, P. Stacey, C. J. Tame, G. F. Watt, N. Zinn, M. A. Queisser, J. D. Harling, A. B. Benowitz, *Angew. Chem., Int. Ed.* **2021**, *60*, 23327; f) C. Kofink, N. Trainor, B. Mair, S. Wöhrle, M. Wurm, N. Mischerikow, G. Bader, K. Rumpel, T. Gerstberger, Y. Cui, P. Greb, G. Garavel, M. Scharnweber, J. Fuchs, G. Gremel, P. Chette, S. Hopf, N. Budano, J. Rinnenthal, G.

Gmaschitz, E. Diers, R. McLennan, M. Roy, C. Whitworth, V. Vetma, M. Mayer, M. Koegl, A. Ciulli, H. Weinstabl, W. Farnaby, *Nat. Commun.* **2022**, *13*, 5969; g) S. Ichikawa, N. C. Payne, W. Xu, C.-F. Chang, N. Vallavoju, S. Frome, H. A. Flaxman, R. Mazitschek, C. M. Woo, *Cell Chem. Biol.* **2024** *S2451-9456(24)00039-4*.

[14] S. Otto, R. L. E. Furlan, J. K. M. Sanders, *J. Am. Chem. Soc.* **2000**, *122*, 12063.

[15] C. Maniaci, S. J. Hughes, A. Testa, W. Chen, D. J. Lamont, S. Rocha, D. R. Alessi, R. Romeo, A. Ciulli, *Nat. Commun.* **2017**, *8*, 830.

[16] a) C. Galdeano, M. S. Gadd, P. Soares, S. Scaffidi, I. Van Molle, I. Birced, S. Hewitt, D. M. Dias, A. Ciulli, *J. Med. Chem.* **2014**, *57*, 8657; b) K. Raina, J. Lu, Y. Qian, M. Altieri, D. Gordon, A. M. K. Rossi, J. Wang, X. Chen, H. Dong, K. Siu, J. D. Winkler, A. P. Crew, C. M. Crews, K. G. Coleman, *Proc. Natl. Acad. Sci. U. S. A.* **2016**, *113*, 7124.

[17] S. Tsubuki, Y. Saito, M. Tomioka, H. Ito, S. Kawashima, *J. Biochem.* **1996**, *119*, 572.

[18] T. A. Soucy, P. G. Smith, M. A. Milhollen, A. J. Berger, J. M. Gavin, S. Adhikari, J. E. Brownell, K. E. Burke, D. P. Cardin, S. Critchley, C. A. Cullis, A. Doucette, J. J. Garnsey, J. L. Gaulin, R. E. Gershman, A. R. Lublinsky, A. McDonald, H. Mizutani, U. Narayanan, E. J. Olhava, S. Peluso, M. Rezaei, M. D. Sintchak, T. Talreja, M. P. Thomas, T. Traore, S. Vyskocil, G. S. Weatherhead, J. Yu, J. Zhang, L. R. Dick, C. F. Claiborne, M. Rolfe, J. B. Bolen, S. P. Langston, *Nature* **2009**, *458*, 732.

[19] a) J. Frost, C. Galdeano, P. Soares, M. S. Gadd, K. M. Grzes, L. Ellis, O. Epemolu, S. Shimamura, M. Bantscheff, P. Grandi, K. D. Read, D. A. Cantrell, S. Rocha, A. Ciulli, *Nat. Commun.* **2016**, *7*, 13312; b) P. Soares, M. S. Gadd, J. Frost, C. Galdeano, L. Ellis, O. Epemolu, S. Rocha, K. D. Read, A. Ciulli, *J. Med. Chem.* **2018**, *61*, 599.

[20] C. Steinebach, S. Lindner, N. D. Udeshi, D. C. Mani, H. Kehm, S. Köpff, S. A. Carr, M. Gütschow, J. Krönke, *ACS Chem. Biol.* **2018**, *13*, 2771.

[21] S. He, J. Ma, Y. Fang, Y. Liu, S. Wu, G. Dong, W. Wang, C. Sheng, *Acta Pharm. Sin. B* **2021**, *11*, 1617.

[22] H. Sun, Z. Nikolovska-Coleska, J. Lu, J. L. Meagher, C.-Y. Yang, S. Qiu, Y. Tomita, Y. Ueda, S. Jiang, K. Krajewski, P. P. Roller, J. A. Stuckey, S. Wang, *J. Am. Chem. Soc.* **2007**, *129*, 15279.

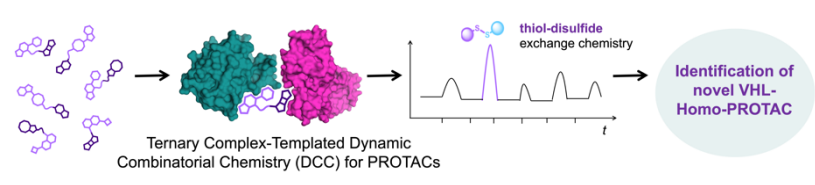
[23] a) A. S. M. Li, S. Kimani, B. Wilson, M. Noureldin, H. González-Álvarez, A. Mamai, L. Hoffer, J. P. Guilinger, Y. Zhang, M. von Rechenberg, J. S. Disch, C. J. Mulhern, B. L. Slakman, J. W. Cuozzo, A. Dong, G. Poda, M. Mohammed, P. Saraon, M. Mittal, P. Modh, V. Rathod, B. Patel, S. Ackloo, V. Santhakumar, M. M. Szewczyk, D. Barsyte-Lovejoy, C. H. Arrowsmith, R. Marcellus, M.-A. Guié, A. D. Keefe, P. J. Brown, L. Halabelian, R. Al-awar, M. Vedadi, *J. Med. Chem.* **2023**, *66*, 5041; b) A. Vulpetti, P. Holzer, N. Schmiedeberg, P. Imbach-Weese, C. Pissot-Soldermann, G. J. Hollingworth, T. Radimerski, C. R. Thoma, T.-M. Stachyra, M. Wojtynek, M. Maschlej, S. Chau, A. Schuffenhauer, C. Fernández, M. Schröder, M. Renatus, *ACS Med. Chem. Lett.* **2023**, *14*, 949.

[24] K. Hoegenauer, S. An, J. Axford, C. Benander, C. Bergsdorf, J. Botsch, S. Chau, C. Fernández, S. Gleim, U. Hassiepen, J. Hunziker, E. Joly, A. Keller, S. Lopez Romero, R. Maher, A.-S. Mangold, C. Mickanin, M. Mihalic, P. Neuner, A. W. Patterson, F. Perruccio, S. Roggo, J. Scesa, M. Schröder, D. Shkoza, B. Thai, A. Vulpetti, M. Renatus, J. S. Reece-Hoyes, *ACS Med. Chem. Lett.* **2023**, 10.1021/acsmedchemlett.3c00259.

[25] S. Ramachandran, N. Makukhin, K. Haubrich, M. Nagala, B. Forrester, D. M. Lynch, R. Casement, A. Testa, E. Bruno, R. Gitto, A. Ciulli, *Nat. Commun.* **2023**, *14*, 6345.

[26] a) W. Gui, T. Kodadek, *ChemBioChem* **2022**, *23*, e202200275; b) W. Gui, S. F. Giardina, M. Balzarini, F. Barany, T. Kodadek, *ACS Chem. Biol.* **2023**, *18*, 1582.

Entry for the Table of Contents



Dynamic Combinatorial Chemistry meets PROTAC screening: Stability of ternary complexes formed by PROTACs is shown to act as selection criterion in multi-protein templated dynamic combinatorial libraries. Reversible thiol-disulfide exchange chemistry allows rapid identification of novel VHL-targeting Homo-PROTACs from complex library mixtures.

Institute and/or researcher Twitter usernames: @UoDCeTPD, @CJ_Diehl, @ales_salerno, @alessiociulli