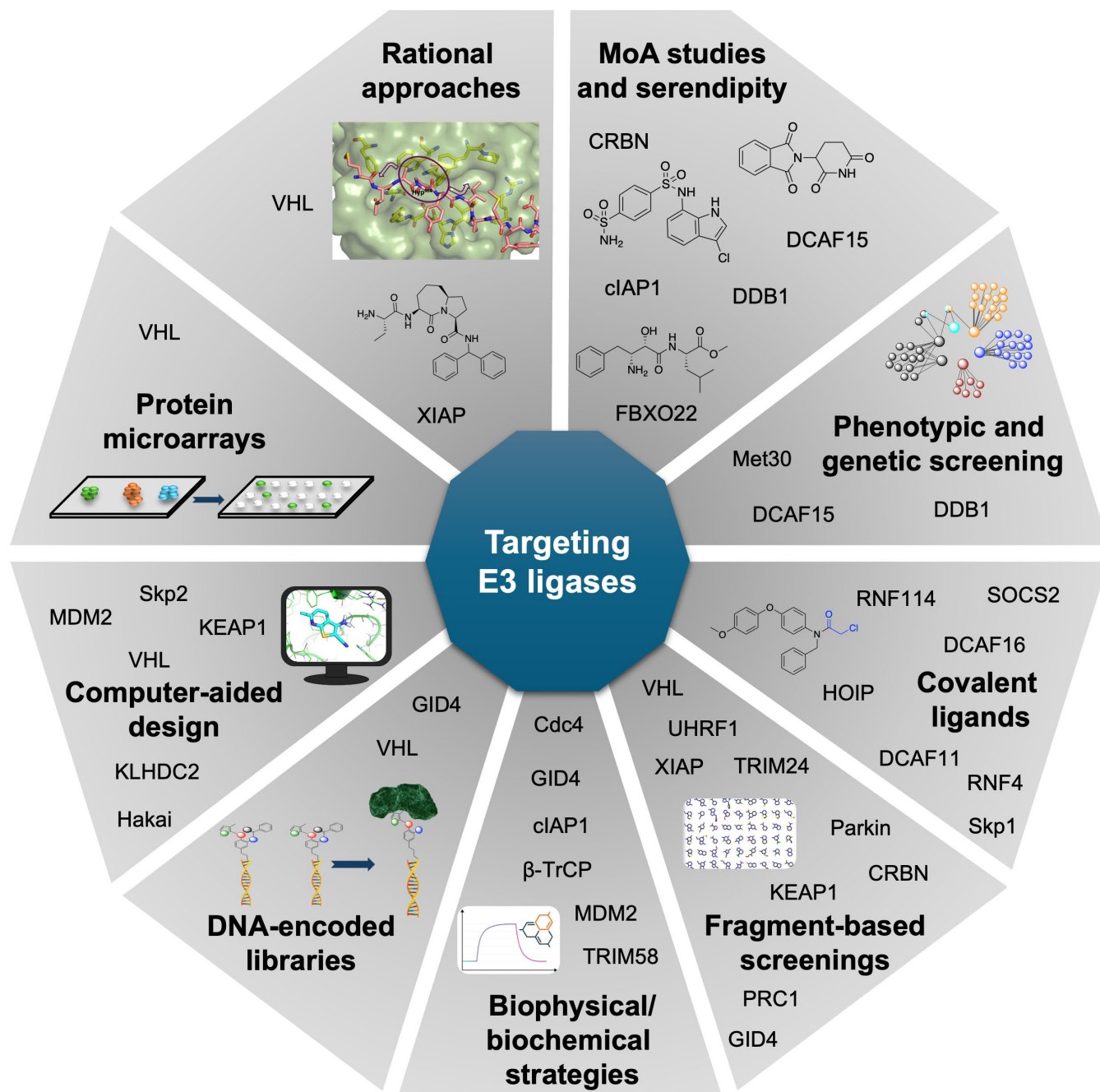


Drug Discovery Approaches to Target E3 Ligases

Alejandra Rodríguez-Gimeno^[a, b] and Carles Galdeano^{*[a, b]}



Targeting E3 ligases is a challenging area in drug discovery. Despite the human genome encoding for more than 600 E3 ubiquitin ligases, only a handful of E3 ligases have been pharmacologically modulated or exploited for targeted protein degradation (TPD) strategies. The main obstacle for hijacking these E3 ligases is the lack of small-molecule ligands. As research into this field advances, the identification of new small molecules capable of binding to E3 ligases has become an essential pursuit. These ligases not only expand the repertoire

of druggable targets but also offer the potential for increased specificity and selectivity in protein degradation. The synergy between academia and industry is key, as it combines academic expertise in fundamental research with the industrial capabilities of translating these findings into novel therapeutics. In this review, we provide an overview of the different strategies employed in academia and industry to the discovery of new E3 ligases ligands, showing them with illustrative cases.

1. Introduction

E3 ubiquitin ligases have been correlated with numerous pathogenic mechanisms. Interestingly, E3 ligases confer substrate specificity to the ubiquitination pathway, making these proteins highly appealing targets *per se* for specific therapeutic approaches.^[1] Even though E3 ligases are involved in the final step of covalent ubiquitylation of target proteins, they vary in both structure and mechanism. Generally, they can be categorized in three families: RING, HECT and RBRs.^[2] In contrast to other target families (e.g. kinases), E3 ligases are structurally heterogeneous, even within the same family.^[3] Thus, a widely used and general approach to find small molecule ligands that target E3 ligases does not exist.

Small molecules targeting E3 ligases could be developed into two different main therapeutic strategies. A first strategy is modulating the E3 ligase activity. In this case both inhibition and activation of the activity of the E3 ligase could be interesting. However, the development of drugs able to modulate E3 ligases has been recompensed with limited success. A second strategy is hijacking the E3 ligase for TPD strategies. In fact, the irruption of TPD strategies has situated E3 ligases at the forefront of research efforts.^[4] However, today only 14 E3 ligases that have been hijacked for proteolysis targeting chimeras (PROTACs[®], PROTACs[®] is a registered trademark of Arvinas, for simplicity in this review it will be named PROTACs) and molecular glues. PROTACs are bivalent molecules where one head of the molecule binds to an E3 ligase while the other binds to any target of interest forming a ternary complex that allows the ubiquitination and subsequent degradation of the target. In contrast, molecular glues are monovalent molecules that insert into a naturally occurring protein-protein interaction (PPI), making interactions with both the target and in this case the E3 ligase with an unique molecular scaffold. The Von Hippel–Lindau (VHL) and cereblon (CRBN) E3 ligases are

currently the most hijacked ones in TPD. Expanding the repertoire beyond these two E3 ligases holds promise for improving PROTAC selectivity, for example with unique expression patterns to enable tissue-, disease-, organelle- or cell-type specific target degradation, and potentially overcoming PROTAC resistance mediated by mutations on the target E3 ligase,^[5] a prevalent challenge in cancer therapy.

In this scenario, the identification of novel drug-like small-molecule hits capable to directly bind to E3 ligases has become an essential pursuit in both academia and industry settings. The collaboration between academia and industry is vital in this endeavor, as it can merge the academic expertise in fundamental research with the industrial experience of translating these discoveries into the drugs of the future. These collaborative efforts are exemplified by numerous partnerships and initiatives focused on identifying and validating novel E3 ligases ligands, characterizing PROTACs and molecular glues, and advancing these molecules through preclinical and clinical development. In this review, while not providing an exhaustive revision of all the E3 ligases targeted, we aim to give an overview of the different drug discovery approaches employed to discover new small molecule directly targeting E3 ligases, showing them with illustrative cases.

2. Rational Approaches: Structure-Guided Ligand Design

Experimental structural information is currently available for many E3 ligase domains or very structurally similar E3 ligases, which makes the rational structure-based approach attractive for guiding the rational design or identification of novel small molecule ligands of E3 ligases. Moreover, with the recent developments in cryo-EM and in artificial intelligence software's able to predict 3D structures (i.e. Alphafold), opportunities to rationally develop E3 ligase binders have increased exponentially. In this line, structural information about the recognition of substrate degrons by E3 ligases is crucial. Revealing the structural basis of the post-translational modifications that confer substrate specificity and governate protein recognition will also help to develop novel E3 ligands. In this sense, ligands targeting VHL E3 ligase continue being the best example of how to develop potent E3 ligase molecules using this approach.

Small molecule ligands targeting VHL. The VHL protein is a substrate receptor protein of the cullin 2 E3 ligase. Among its

[a] A. Rodríguez-Gimeno, C. Galdeano
Departament de Farmàcia i Tecnologia Farmacèutica, I Físicquímica,
Universitat de Barcelona, Av. Joan XXIII, 27–31, E-08028 Barcelona, Spain
E-mail: cgaldeano@ub.edu

[b] A. Rodríguez-Gimeno, C. Galdeano
Institute of Biomedicine (IBUB), Universitat de Barcelona, Av. Joan XXIII, 27–
31, 08028 Barcelona, Spain

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substrates, hypoxia-inducible factor 1 α (HIF-1 α) holds particular significance. Given HIF-1 α 's role in controlling proangiogenic factors, an inhibitor of VHL recognition would end up increasing erythropoietin and could be potentially used in the treatment of chronic anaemia and cancer chemotherapy.

In 2012, novel peptidomimetic inhibitors targeting VHL were described by Ciulli and Crews laboratories for the first time.^[6–8] They rationally designed inhibitors of the VHL/HIF-1 α PPI guided by the crystal structure of a hydroxylated HIF-1 α peptide bound to VCB (VHL, elongin C and elongin B, Figure 1).^[6,12] The authors hypothesized that small-molecule inhibitors of this PPI could be rationally designed starting from the crucial hydroxyproline (Hyp), since residue Hyp564 on HIF-1 α makes key interactions with VHL and is also central to HIF-1 α binding. They were able to identify a VHL ligand (15, Figure 1) with single-digit micromolar activity through a Fluorescence Polarization (FP) assay by starting from the structure of bound HIF-1 α peptide and through structure-guided medicinal chemistry by growing around the minimal Hyp recognition unit and *in silico* design. The cocrystal structure of compound 15 bounds to VHL evidenced that the ligand bind at the same binding site of HIF-1 α to VHL, keeping the vital H-bonds between the hydroxyl group of the pyrrolidine with the His115 and Ser111. Even though these molecules bound to VHL with moderate potency (one-digit micromolar affinity) and showed very low lipophilicity due to the hydrophilic contribution of the Hyp scaffold, this work was seminal and provided the basis for the development of a novel series of potent VHL ligands able to disrupt the VHL/HIF PPI.

Later, Galdeano *et al.* developed second-generation VHL ligands via structure-based drug design helped by the information of cocrystal structures and isothermal titration calorimetry (ITC). They improved binding affinity and lipophilicity by optimizing the interactions at the left-hand side of the VHL ligand, obtaining compounds with higher cell permeability and higher binding affinity.^[12] The structure-guided rational optimization study resulted in the discovery of VH032, a strong VHL binder with nanomolar binding affinity (Figure 1). Subsequent structure-guided medicinal chemistry work optimizing VH032, led to the discovery of novel compounds with

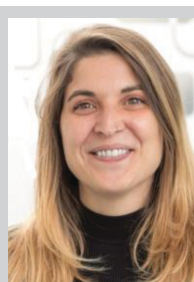
sub-100 nM activity, VH298 and VH101. Biophysical and cellular studies demonstrated VH298 as a potent, selective, and cell-active chemical probe VHL inhibitor showing a functional dose-dependent response downstream of HIF- α hydroxylation in the hypoxia-signalling cascade inside cells.^[13,14] In this work, the lab also developed the corresponding negative control compound that does not bind to VHL, the *cis*-hydroxyproline analogue *cis*VH298. This change in the hydroxyl stereochemistry is known to result in pronounced loss of binding of the hydroxylated HIF-1 α substrate to VHL.^[15] As expected, no binding of *cis*VH298 was detected by ITC, confirming the stereospecific effect is retained in VHL inhibitors. The discovery of VHL ligands, together with the identification of the mechanism of action of immunomodulatory drugs (IMiDs, i.e. thalidomide, lenalidomide, pomalidomide), represent a crucial milestone in the expansion of the PROTAC field. In fact, VH032 and VH298 are still heavily used in the development of VHL-based PROTACs.

Small molecule ligands targeting XIAP. Inhibitors of apoptosis proteins (IAPs) are regulators of cell death that act by directly inhibiting members of the caspase family. Structurally, the IAP family presents three baculovirus IAP repeat (BIR) domains at the N-termini. Some IAPs, including cellular inhibitor of apoptosis-1 (cIAP1) and X-linked inhibitor of apoptosis protein (XIAP), possess E3 ligase activity since they contain RING domains able to bind to the corresponding E2 proteins.

In 2000, two independent research groups described a mammalian protein called SMAC (also called DIABLO) that induces apoptosis by cancelling the inhibitory effects of IAPs on caspases.^[16,17] SMAC competes with the caspase for the same pocket on the surface of the BIR3 domain and promotes caspase-9 activation. Structural insights of the binding of SMAC protein to the BIR3 domain of XIAP were obtained by Nuclear Magnetic Resonance Spectroscopy (NMR) and X-ray showing that the SMAC N-terminus binds to the BIR3 domain with only the first four amino acid residues (AVPI) (Figure 2).^[18–20] The structural information of the tetrapeptide was envisaged as a starting point for the development of peptidomimetic ligands that would mimic the binding of the protein SMAC to XIAP and induce apoptosis. Using this information, scientists from Abbot synthesized peptide libraries by high-throughput solid-phase



Dr Carles Galdeano is currently an Associate Professor at University of Barcelona. He obtained his PhD in medicinal chemistry at University of Barcelona. After that, he spent three years post-doc in the Alessio Ciulli's lab (first at University of Cambridge and later at University of Dundee) where together they develop the first potent VHL ligands described. His postdoctoral discoveries represented a breakthrough in the PROTACs field. In 2015, he returned to the University of Barcelona to work in collaboration with Prof Barril until 2019, when he started his independent research lab. Dr Galdeano is also co-founder of Oniria Therapeutics.



Dr Alejandra Rodríguez-Gimeno obtained her PhD in Organic Chemistry at University of Valencia. She has since combined academic and pharmaceutical Medicinal Chemistry research experience. She worked in IIT (Genova, Italy) as a post-doc on the discovery of new modulators for Cystic Fibrosis disease, from hit-to-lead to preclinical development phase. Her roles in industry have included research scientist and project leader roles in Pharma companies in Spain. In 2021, she was awarded a "NextGenerationEU" Postdoctoral fellowship and joined Galdeano's Lab to focus on the development of new degraders for "undrugable" targets by combining computational chemistry, biophysics, medicinal chemistry, and cell biology.

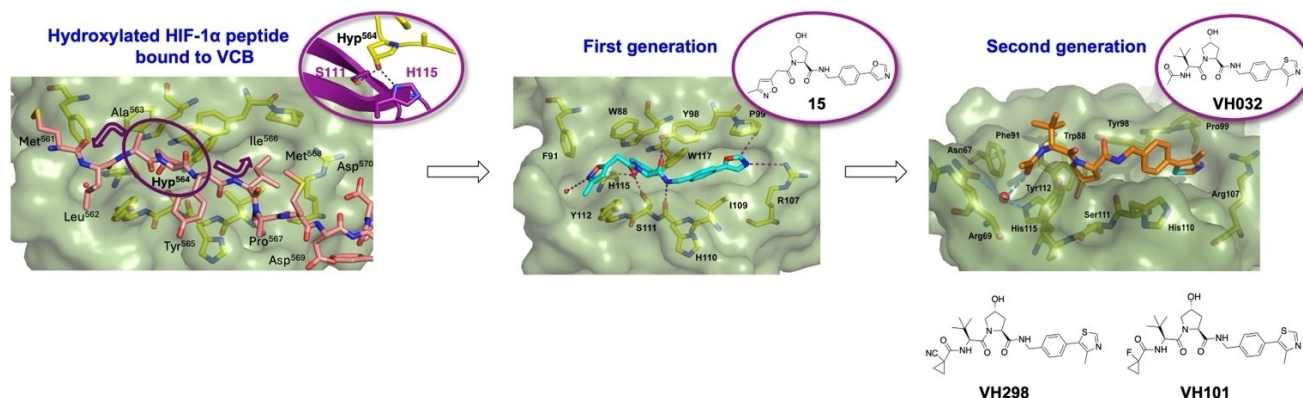


Figure 1. VHL ligands development.

peptide synthesis to determine the structure-activity relationships (SAR) and contribution for the binding of the individual residues of the SMAC tetrapeptide and evaluated their binding to the BIR3 domain of XIAP by fluorescent polarization assay.^[21]

Following a similar approach, Sun and collaborators designed and synthesized highly potent conformationally constrained nonpeptidic SMAC mimetics based on the 3D structures of SMAC in complex with XIAP BIR3.^[22] Structure-based design led to a potent compound (7, Figure 2) that showed a K_i value of 25 nM to XIAP BIR3, 23 times more potent than natural SMAC peptide in a FP-based binding assay. Furthermore, they confirmed by NMR HSQC spectra that 7 binds to the same binding pocket in the XIAP BIR3 where SMAC peptide binds.

3. Mechanism of Action Studies and Serendipitous Discoveries

We currently know that serendipity has driven the discovery of several E3 ligase binders, with examples of compounds shown to be efficacious and approved without prior knowledge of the specific mechanism of action. This fact is well-illustrated in the field by the molecular glue degraders. The protein degradation strategy based on molecular glues arose from the observation that immunomodulatory imide drugs (IMiDs) binding to the E3

ligase cereblon (CRBN) promoted the degradation of important non-native substrates (neosubstrates), such as Ikaros, Aiolos, GTP1 and CK1 α , which structurally only share a common β -hairpin containing a glycine residue. Biophysical and chromatographic techniques, genetic approaches and transcriptional expression analysis have been used to disentangle the mechanism of action of known drugs and therefore the identification of novel E3 ligase ligands by serendipity.

Small molecule ligands targeting CRBN. In the 1950s, thalidomide (Figure 3) was first developed by the company Grünenthal as a sedative for morning sickness in pregnant women. However, thalidomide showed severely teratogenic effects and was withdrawn from the market in the early 1960s.^[23] Fifty years later, thalidomide was further studied extensively and rediscovered as a promising IMiD, and approved for the treatment of erythema nodosum leprosum (ENL) and multiple myeloma in 1998 and 2006 respectively.^[24] The thalidomide derivatives, such as pomalidomide and lenalidomide (Figure 3) developed by Celgene, have also shown an excellent efficacy in the treatment of multiple myeloma with their immunomodulatory activities. However, the mechanism of action of thalidomide had not been disentangled until 2010 when Ito *et al.* discovered by affinity chromatography using nano-magnetic beads that thalidomide binds to CRBN, a subunit of the E3 ubiquitin ligase CUL4-RBX1-DDB1-CRBN(CRL4^{CRBN})^[25] that is important for limb outgrowth and expression of the fibroblast growth factor Fgf8 in *in vivo*

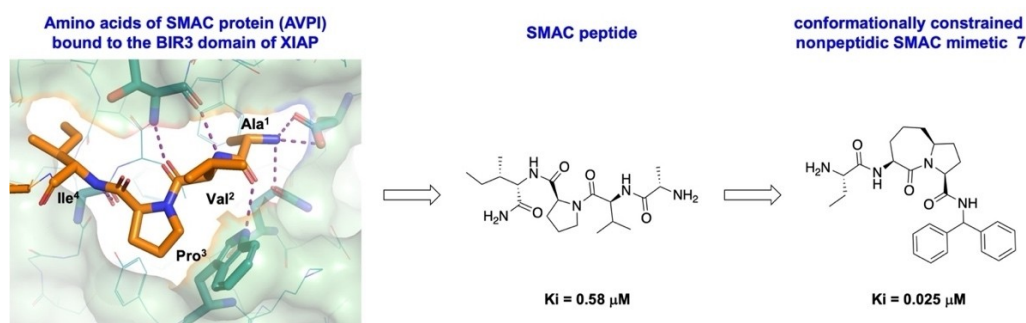


Figure 2. SMAC peptide and conformationally constrained mimetic compound 7.^[17]

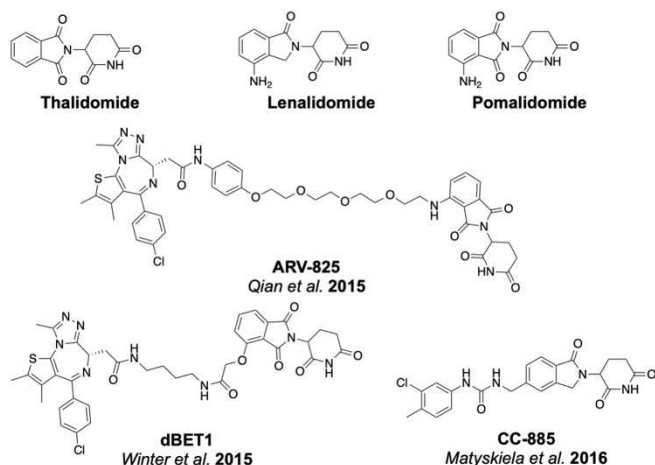


Figure 3. CRBN ligands and first CRBN-based PROTACs.

models. They demonstrated that thalidomide initiates its teratogenic effects by binding to CRBN and inhibiting its auto-ubiquitination activity. After that, two independent laboratories separately reported that lenalidomide (but also its analogues), a derivative of thalidomide and a marketed drug used for the treatment of haematological malignancies, could induced degradation of neo-substrates, in this case transcription factor Ikaros family zinc finger protein-1 (IKZF1) and Ikaros family zinc finger protein-3 (IKZF3).^[26,27] In 2014, the laboratory of Thomä (FMI) and Celgene disclosed two X-ray cocrystal structures of DDB1–CRBN–IMiDs complexes.^[28,29] The crystal structures showed that the glutarimide chemical group of thalidomide derivatives, through interactions with key residues, plays an important role in CRBN binding. The structures also revealed that the phthalimide ring on thalidomide is solvent exposed and opened the possibility for extending a substituent or linker and thus for the design of PROTACs without a loss of the binding activity. Thalidomide binds to CRBN with an affinity of 249 nM, while lenalidomide and pomalidomine bind with affinities of 178 nM and 157 nM, respectively.^[28] Crucially, IMiD binding to CRBN does not abolish the E3 ligase ability to target substrates for subsequent degradation. In parallel, in the last years, several studies have been dedicated to study the different IMiD-dependent degradation of neosubstrates.^[30,31] These studies have led to important discoveries in the field such as that the transcription factor SALL4 is the responsible of the teratogenesis caused by thalidomide, since SALL4 is a neosubstrate of CRBN upon IMiDs treatment.^[32,33] In fact, mutations in SALL4 produce a phenotype similar to that caused by thalidomide in humans, such as in Duane-Radial Ray Syndrome or Okihiro Syndrome.^[34]

In 2015, laboratories of Crews and Bradner reported PROTACs build from IMiDs that induced degradation of relevant proteins (Figure 3)^[35,36] Since then, the development of IMiDs derivatives has been accelerated and have been widely used for PROTAC design, being the most common E3 ligase hijacked for PROTACs. In parallel, structural information and medicinal chemistry efforts have spurred the development of neosubstrate-selective IMiDs. A good example is CC-885 (Figure 3), a

selective helios (IKZF2) degrader towards ikaros (IKZF1) and aiolos (IKZF3). In fact, currently several selective CRBN-based molecular glues can be found in clinical trials, including eragidomide, a GSTP1 degrader from Bristol Myers, CFT455, a IKZF1/3 degrader from C4Therapeutics or NVP-DKY709, also a IKZF2 degrader from Novartis.

Until recently, there was an important unknown when targeting CRBN with small molecules: what is the natural function and substrate of CRBN? A recent paper by Ichikawa *et al.* has shed much needed light, demonstrating that dipeptides with glutarimide or aspartimide ends can serve as CRBN recruiters, considering them as a post-translation modification.^[37] Consequences from a drug discovery perspective of this finding are still somehow unpredictable but it can open new doors in how to modulate CRBN activity with small molecules and TPD strategies.

Small molecule ligands targeting DCAF15. Sulfonamide derivatives such as indisulam (E7070),^[38] E7820,^[39] and chloroquinoxaline (CQS)^[40] have drawn attention in drug discovery principally due to their anticancer activities and have been tested in clinical trials (Figure 4). However, it was not until 2017 that the primary target and mode of action of these sulphonamides was elucidated in two independent studies. Uehara *et al.*^[41] identified by a single amino acid substitution and CRISPR-Cas9-based knockout that the sulfonamide derivatives induce ubiquitination and proteasomal degradation of CAPER α (coactivator of activating protein-1 and estrogen receptors) through the formation of a CAPER α -sulfonamide-DCAF15-DDB1-CUL4 complex. Han *et al.*^[42] also reported that these compounds trigger an interaction between the splicing factor RBM39 (RNA-binding motif protein 39) and DCAF15 in a molecular glue fashion. Crucially, although indisulam binds with weak affinity to DCAF15 (17 μ M), this affinity is sufficient to lead to the subsequent ubiquitination and proteolytic degradation of RBM39. To unravel the molecular mechanism by which these sulfonamides trigger the DCAF15-RBM39 interaction, three independent laboratories released cocrystal structures of DDB1-DCAF15-DDA1 core ligase complex bound to RBM39 and E7820 and indisulam.^[43–45] The structural information and kinetic studies derived from these studies confirmed that aryl sulfonamides act as a molecular glues. In parallel, other ligands able to bind to DCAF15 have been identified following other approaches (see below).

Small molecule ligands targeting cIAP1. cIAP1 (cellular inhibitor of apoptosis protein 1) is overexpressed in several human cancers and because of its genetic amplification it is considered as an oncogene.^[46–49]

In 2007, Sekine *et al.* reported that methyl bestatin (ME-BS, Figure 5) binds to cIAP1 and induces self-ubiquitination and

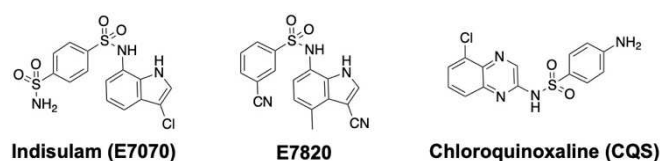


Figure 4. Sulfonamide derivatives targeting DCAF15.

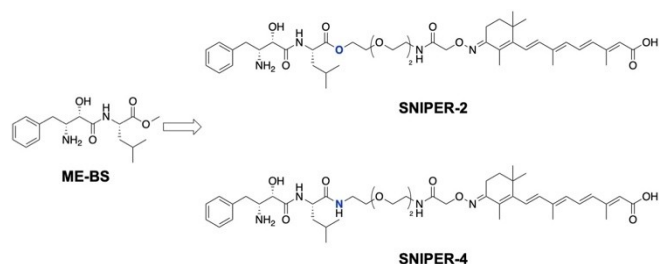


Figure 5. Methyl bestatin (ME-BS) and ME-BS-based PROTACs (SNIPER-2 and SNIPER-4).

proteasomal degradation of the latter.^[50] Bestatin was previously described as an inhibitor of aminopeptidase N with immunomodulatory activity, which induces apoptosis in human leukaemia cells^[51] and increases death ligand-induced apoptosis in human solid tumour cell lines.^[52] Mechanistic analysis also showed that ME-BS directly interacts with the BIR3 domain of cIAP1 while other IAPs, such as XIAP and cIAP2, are not affected.^[53] SAR studies of ME-BS suggested that the bestatin part of the molecule binds to cIAP, while the methyl ester is solvent exposed. Based on that, Itoh and collaborators developed bifunctional small molecules by linking ME-BS and ATRA (*all-trans* retinoic acid) with a polyethylene glycol (PEG) chain.^[54] These molecules induced the degradation of both cIAP1 and cellular retinoic acid binding protein-II (CRABP-II) and the authors named them SNIPERs (Specific and Non-genetic IAP-dependent Protein Eraser, SNIPER-2 and SNIPER-4, Figure 6).^[53,55] This degradation strategy, very similar to PROTACs, was then expanded to other targets such as ER,^[56] BRD4,^[57] and BCR-ABL.^[58]

Small molecule ligands targeting FBXO22. In a recent work, Hanley *et al.* reported the discovery of the molecule UNC8153 as an effective degrader of NSD2, a histone methyltransferase overexpressed in multiple myeloma and acute lymphoblastic leukaemia among other cancers.^[59–61] UNC8153 degrades NSD2 in cells in a proteasome- and neddylation-dependent manner, containing a simple primary alkylamine as an E3 recruiting moiety. However, the mechanism of action of how this compound promoted NSD2 degradation was unknown. The researchers also developed a second-generation of NSD2 degraders with a SAR exploration of the linker of UNC8153 and discovered UNC8732 (Figure 6).^[62] This second-generation of degraders also had a primary alkylamine that can be metabo-

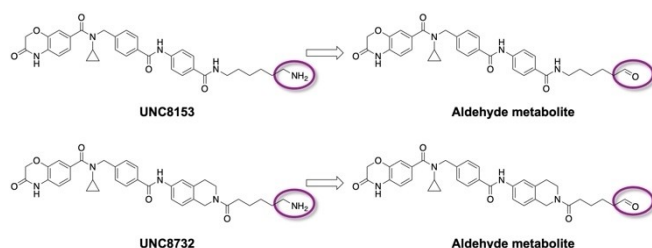


Figure 6. Structures of NSD2 degraders UNC8153 and UNC8732 and their corresponding aldehyde metabolites.

lized into different active species in a cellular context. To confirm the active metabolites, the authors conducted a LCMS-based metabolite identification and observed the transformation of the primary amine to an aldehyde, discovering the metabolite promoting NSD2 degradation. The researchers continued with the characterization of the mode of degradation of UNC8732 and discovered by proximity-dependent biotin identification (BioID) that the compound recruited FBXO22, a E3 ligase recognition subunit of a SKP1-CUL1-F-box complex (SCF^{FBXO22}) to promote the subsequent degradation. Ternary complex formation was confirmed by NanoBRET (bioluminescence resonance energy transfer), bio-layer interferometry (BLI), size exclusion chromatography and hydrogen deuterium exchange-mass spectrometry (HDX-MS). Furthermore, the authors showed that the aldehyde group of the compounds likely interacted via a reversible covalent interaction with Cys326 of FBXO22 FIST_C domain.

Small molecule ligands targeting DDB1. In 2020, three different studies identified CDK12 inhibitors with different chemical scaffolds that induce CDK12-DDB1 interactions to trigger cyclin K degradation following different strategies.^[63–65] Aiming to identify novel molecular glues, Ślabicki *et al.* carried out bioinformatic correlations between drug-sensitivity data for 4,518 clinical and preclinical drugs against the mRNA levels of 499 E3 ligase components across 578 different cancer cell lines.^[63] To validate the correlations discovered between drug toxicity and E3 ligase components expression, they analysed the rescue of the drug-induced toxicity observed after CRISPR-mediated inactivation of the corresponding E3 ligase component. With this approach, they observed, for example, a connection between the cytotoxic effect of (*R*)-CR8, a CDK inhibitor,^[66] and the mRNA levels of the DDB1, the CUL4 E3 ubiquitin ligase adaptor (Figure 7). Through quantitative proteome-wide mass spectrometry, the authors found that (*R*)-CR8 triggers cyclin K for subsequent proteasomal degradation through the activity of a DDB1. *In vitro* co-immunoprecipitation experiments and analysis of the crystal structure of CDK12–cyclin K bound to CR8 revealed that this compound binds to the ATP binding site of CDK12 and glues the CDK12–DDB1 interface. Their structural data showed that when (*R*)-CR8 is bound to CDK12, the pyridyl moiety is solvent exposed and promotes the formation of a complex between CDK12–cyclin K DDB1, leading to cyclin K ubiquitination and

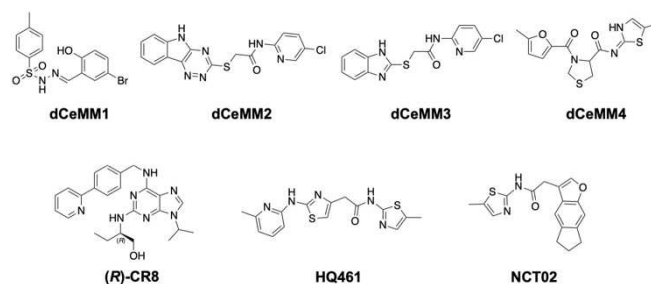


Figure 7. DCAF15-recruiting molecular glue degrader of RBM39 dCeMM1 and representative CDK12-DDB1 molecular glue degraders dCeMM2-4, (*R*)-CR8, HQ461 and NCT02.

degradation. In parallel, other ligands able to bind to DDB1 have been identified following other approaches (see below).

4. Phenotypic and Genetic Screening

The molecular glue strategy shows the potential of how E3 ligase agnostic and degradation-based phenotypic screenings can be applied to identify novel ligands of E3 ligases. Knowledgeable cellular screenings and the development of target deconvolution methodologies can fuel the identification and development of novel ligands following this approach.

Small molecule ligands targeting DCAF15. To identify novel E3 ligands, the lab of Georg Winter employed hyponeddylated cell lines that are characterized by a promiscuous defect in Cullin-RING ligases (CRL) activity.^[64] Under physiological conditions, the function and activation of most CRLs is dependent on the reversible ubiquitin-like protein NEDD8 (neural precursor cell expressed developmentally downregulated protein 8) conjugation. With this strategy, they performed a comparative screening of a library of 2000 compounds based on the chemical profiling in hyponeddylated cells versus neddylation-proficient cells. With this approach they identified four different compounds (dCeMM1/2/3/4) that were functionally dependent on the neddylation levels (Figure 7). Specifically, dCeMM1, was identified to act as a RBM39 degrader by inducing the interaction between this protein and the E3 ligase DCAF15. Mutations induced by CRISPR in DCAF15 made cells unresponsive to dCeMM1 treatment and reversed RBM39 destabilization, further confirming the mechanism of dCeMM1 as DCAF15 molecular glue degrader.

Small molecule ligands targeting DDB1. The Winter's lab also further characterized dCeMM2/3/4 (Figure 7) by coupling functional genomic screening strategies, quantitative proteomics, drug-affinity chromatography, and cellular proximity assays. These orthogonal experimental strategies revealed that the binding of compounds dCeMM2/3/4 to the CDK12-cyclin K complex induce a dimerization with the DDB1-CUL4B E3 ligase.^[64] In parallel, Lv *et al.* identified HQ461 as a molecular glue through a phenotype-based high-throughput screening (HTS) and chemical genetics (hypermutation in HCT-116 and CRISPR-Cas9 screenings) (Figure 7).^[65] The authors showed that HQ461 binds to the kinase domain of CDK12 modifying CDK12 surface to recruit DDB1 triggering the ubiquitination and posterior degradation of cyclin K. In 2021, Dieter *et al.* also identified NCT02 (Figure 7) in a HTS on patient-derived colorectal cancer spheroids.^[67] Omics approaches elucidated that NCT02 is a molecular glue degrader, inducing ubiquitination and proteasomal degradation of cyclin K and CDK12. Distinct from other described molecular glues that engage the ubiquitin proteasome system by directly binding to a substrate-specific protein, all four investigations mentioned above identified the same mechanism of action using different approaches and chemical scaffolds,^[63–65,67] proposing that the CDK12-DDB1 surface could be a hotspot for the development of molecular glues.

Small molecule ligands targeting Met30. The combination of chemical genetics and proteomics in a yeast-based HTS platform was used for the discovery of small molecule modifiers of the rapamycin-sensitive TOR functions, including both suppressors (SMIRs) and enhancers (SMERs).^[68,69] In 2010, Aghajany *et al.* used a yeast-based screen to identify new SMERs targeting cell growth control.^[70] Using a library of 30,000 small molecules, they could identify five different chemical scaffolds with diverse effects on growth. Genomic, genetic, and biochemical analyses showed that SMER-3 selectively inhibits Met30 E3 ubiquitin ligase in yeast.

5. Covalent Ligands

Drug discovery had long shied away from developing covalent inhibitors due to the concern about their tendency to exhibit promiscuous activity and form irreversible complexes with off-target biological nucleophiles, potentially leading to associated off target effects that can lead to toxicity. However, with careful control over their reactivity, molecules build with covalent warheads could offer significant advantages, including high ligand efficiency and small size that could improve ADMET properties (absorption, distribution, metabolism, excretion, and toxicity), enhanced potency, and selectivity,^[71] and sustained target engagement allowing for less frequent dosing. Specifically, the ability to target binding sites previously considered as 'unligandable' situated this approach at the forefront to target E3 ligases.^[72] Currently, novel approaches are emerging for identifying and developing covalent ligands as both chemical probes and potential therapeutic agents,^[73] such as chemo-proteomic platforms,^[74] fragment-based covalent screening libraries,^[75] and covalent docking.^[76]

Chemical proteomic methods exploit electrophilic fragments to target biological systems and illustrate a full profile of the covalent ligandability of the nucleophilic residues, such as cysteine and lysine. Cravatt and collaborators developed a competitive activity-based protein profiling (ABPP) method, named isotopic tandem orthogonal proteolysis-enabled ABPP (isoTOP-ABPP), for quantifying the reactivity of electrophilic compounds against > 1,000 cysteines in parallel in the human proteome.^[77] IsoTOP-ABPP uses reactivity-based chemical probes to identify functional, reactive and ligandable hotspots directly in complex proteomes,^[78,79] facilitating the rapid identification of both proteins and ligandable sites targeted by the covalent compound in these proteins.^[80,81] Using this approach, new small molecules ligands for previously untargeted E3 ligases are being discovered.

Small molecule ligands targeting RNF114. Nomura and collaborators used isoTOP-ABPP chemoproteomics platform to identify the target and elucidate the mechanism of action of nimbolide (Figure 8).^[82] Nimbolide is a natural product containing a cyclic enone capable of reacting with cysteines.^[83] Nimbolide has been shown to have multiple therapeutic effects across a several types of cancer such as inhibition of tumorigenesis and metastasis without unwanted off target effects.^[84]



Figure 8. Covalent RNF114 ligands.

Using the ABPP approach in human breast cancer cells, the researchers revealed that nimbolide covalently reacts with an *N*-terminal cysteine (C8) within an intrinsically disordered region of the E3 ubiquitin ligase RNF114, leading to deficient ubiquitination and degradation of its substrate, the tumour suppressor CDKN1A (p21). The study showed that this apparent “allosteric” inhibition of RNF114 activity is due to impaired substrate recognition. They also demonstrated that nimbolide can be employed as a E3 ligase ligand for TPD applications through the recruitment of RNF114. Using chemoproteomics-enabled covalent ligand screening platforms, Nomura and collaborators also identified more simple and synthetically accessible ligands (e.g. EN219) that react with the same cysteine of RNF114 and replicate nimbolide mode of action (Figure 8).^[85]

Small molecule ligands targeting RNF4. Ward and co-workers also used an ABPP-based covalent ligand screening approach to discover cysteine-reactive small-molecules that react with the E3 ligase RNF4.^[86] With this strategy, they identified that the chloroacetamide CCW16 binds to the RNF4 by covalently modifying either of the two zinc-coordinating cysteines C132 and C135 in the RING domain of RNF4. In their study, PROTAC CCW 28–3 was synthesized by linking CCW 16 to the bromodomain inhibitor JQ1, displaying higher potency for RNF4 than the parent ligand CCW16 in the competitive ABPP assay (Figure 9).

Small molecule ligands targeting DCAF16. Zhang *et al.* used a novel chemoproteomic approach to discover novel E3 ligases that can be employed for TPD purposes.^[87] They developed PROTACs starting from fragment electrophiles, called ‘scouts’, that can capture small-molecule-cysteine interactions and selective reversible ligands for protein targets of interest. They linked these scout fragments containing chloroacetamide and acrylamide as electrophile groups to the FKBP12 inhibitor SLF ($IC_{50}(FKBP12) = 2.6 \mu M$).^[88] To elucidate the cellular location of the possible degradation, FKBP12 was expressed with FLAG tag variants containing either cytosolic- or nuclear-directing sequences. One of the degraders (KB02-SLF) containing a

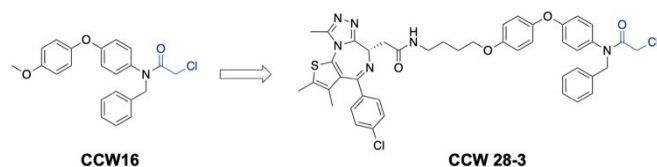


Figure 9. RNF4 ligand and RNF4-ligand based PROTAC.

chloroacetamide moiety led to the degradation of nuclear-FKBP12. Through small hairpin RNA-mediated knockdown and FLAG-mediated affinity enrichment, they identified DCAF16 as the E3 ligase responsible for the degradation and that reductions in DCAF16 expression significantly impaired KB02-SLF-mediated polyubiquitination and degradation of nuclear FKBP12. The crucial role of the chloroacetamide warhead as the key for binding DCAF16 was validated with a saturated propanamide analogue that failed to degrade nuclear-localized FKBP12. They also extended the study to the DCAF16 mediated degradation of other nuclear proteins such as BRD4.

Small molecule ligands targeting DCAF11. Using a very similar approach, Cravatt's lab in collaboration with Vividon Therapeutics expanded the library of electrophilic PROTACs across a wider range of human cancer cell lines to find other suitable E3 ligases for protein degradation purposes.^[89] They used a cell-based functional screening strategy coupled with chemical proteomics and identified that DCAF11 could also mediate ligand-induced protein degradation. Mechanistic studies indicated the modification of specific cysteines in DCAF11 by the electrophilic PROTACs. They further showed that DCAF11-mediated PROTACs could degrade other endogenous proteins, including FBKP12 and the androgen receptor.

Small molecule ligands targeting SOCS2. Recently, the Ciulli lab used a structure-based rational design to target covalently the SH2 domain of the E3 ligase suppressor of cytokine signalling 2 (SOCS2).^[90] A previous study from Ciulli's lab described the SOCS2-substrates interactions by solving co-crystal structures of the phosphorylated peptides from substrates growth hormone receptor (GHR pY595) and erythropoietin receptor (EpoR-pY426) bound to SBC2 (SOCS2-elongin B-elongin C).^[91] They found that the phosphate group of post-translational modified tyrosine is essential for SOCS2 binding and it was selected as the starting point to start a SAR campaign. A fragment growing approach from this phosphotyrosine (pY) led to the identification of compounds with improved affinity by ITC, Surface Plasmon Resonance (SPR) and ¹⁹F ligand-observed displacement NMR assays (Figure 10). To elucidate the binding modes of these optimised compounds, they solved the co-crystal structures of them bound to SBC2 unexpectedly found a covalent modification of Cys111. This observation led them to design the electrophilic covalent inhibitor MN551 and the corresponding prodrug MN714, that competitively block recruitment of cellular SOCS2 protein to its native substrate by covalent engagement at Cys111.

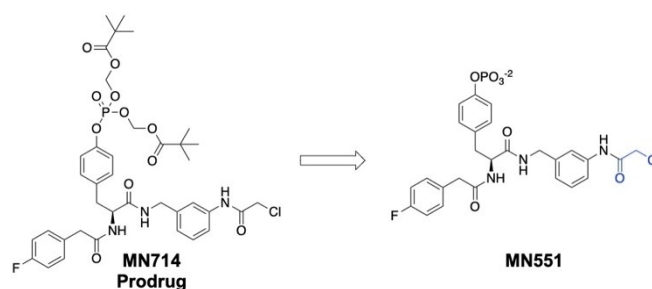


Figure 10. Covalent inhibitor MN551 and the prodrug MN714.

Small molecule ligands targeting SKP1. Recently, Nomura's lab identified in a ABPP screening EN884, a compound containing an acrylamide reacting warhead able to bind the SKP1 adapter protein.^[92] Specifically, the ABPP screening consisted of a library of cysteine-reactive covalent ligands competing against a fluorophore-conjugated cysteine-reactive iodoacetamide probe labelling of SKP1-FBXO7-CUL1-RBX1 complex. They further showed that EN884 can be employed to build PROTACs, degrading as a proof-of-concept BRD4 and the androgen receptor.

In 2022, researchers from Novartis reported a technology to assess the degradation activity of covalently functionalized recombinant E3 ligases against protein of interest.^[93] This methodology, called Covalent Functionalization Followed by E3 Electroporation (COFFEE), provides evidence of E3 ligase recruitment that can be used for the prioritization of E3 ligases for targeted protein degradation programs and E3 ligand-finding campaigns. This approach uses maleimide-functionalized substrate ligands (electrophilic probe) to label covalently ligandable cysteines on the surface of E3 ligases to then perform an electroporation of the resulting functionalized proteins into cells. With this approach, they showed that CRLs for example VHL, as well as the adaptor SKP1 can be functionalized via their solvent-exposed cysteines using a maleimide warhead coupled to the BRD4 inhibitor, JQ1, or the kinase inhibitor, dasatinib. They also demonstrated that the functionalized E3 ligases components are active and capable of catalysing ligand-driven degradation by electroporation into cells.

Small molecule ligands targeting HOIP. LUBAC (linear ubiquitin chain assembly complex) is a multiprotein E3 ligase that belongs to the RBR family of E3 ligases. Its natural function is to catalyse the formation of linear polyubiquitin chains.^[94] The complex is formed by three domains: the heme-oxidized IRP2 ubiquitin ligase 1 (HOIL-1), the Shank-associated RH domain-interacting protein (SHARPIN) and the HOIL-1-interacting protein.^[95] HOIL-1 and HOIP are both E3 ubiquitin ligases, however, HOIP is the responsible of the linear ubiquitin-ligating activity. Some small molecule covalent inhibitors targeting HOIP have been described but the molecules were also associated with some concerns such as broad reactivity across multiple proteins.

In 2019, scientists from GSK and The Francis Crick Institute reported a rational approach to block the catalytic activity of HOIP by covalently targeting the active site cysteine residue.^[96] To that end, a small library of fragments containing α,β -unsaturated methyl ester electrophiles were synthesized to be screened by incubation of the recombinant RBR domain of HOIP and posterior analysis by LC-MS. Through the complementary use of protein LC-MS, chemical synthesis, biochemical ubiquitination assays, and protein crystallography, the researchers could identify compounds that led to selective labelling at the active site cysteine residue and displayed promising selectivity for HOIP in cells.

6. Fragment-Based Screenings

Fragment-Based Lead Discovery (FBLD, also known as Fragment-Based Drug Discovery FBDD) has shown to be a highly effective and general approach to identify small molecule compounds that modulate biological targets. The key characteristic of this approach is that ligand discovery starts with the screening of compounds with low molecular weight (< 250 Da) (called fragments) that have a higher probability of binding to a target in comparison to a standard drug-size molecule (400–500 Da).^[97] The size and simplicity of fragments enables an efficient approach to drug discovery, covering a huge chemical space using libraries of only a few hundred of molecules, but also with low binding hits. This means that small libraries of around 1500 fragments are sufficient for finding hits against most targets, and particularly those targets suited to biophysical studies. This makes fragment screening a sustainable approach, very accessible and cost-effective. These characteristics make FBDD also a valuable method for assessing the ligandability of novel target proteins.^[98] In contrast, high quality structural information is crucial to chemically grow fragment-size molecules to drug-size molecules. The FBLD approach has been effectively developed within some small companies and is now also increasingly being deployed in academic research to provide small molecule tools to probe targets and in large pharmaceutical companies for hit identification for considered undruggable proteins. Several E3 ligases have been targeted both at the degron site and allosterically following this approach: Keap1,^[99–103] VHL,^[104] parkin,^[105] GID4,^[106] UHRF1,^[107] PRC1,^[108] TRIM24,^[109,110] CRBN,^[111] XIAP and cIAP1.^[112–114] In the next lines, we show some illustrative examples.

Fragments targeting KEAP1. In 2016, scientists from Astex and GSK reported a fragment-based approach that aim to directly target the KEAP1 Kelch–NRF2 interaction.^[99] They screened approximately 330 fragments by X-ray crystallography and identified three different hotspots for fragment binding within the NRF2 epitope binding pocket of KEAP1. Given that information, phenyl acetic acid Fragment 1 (Figure 11) was identified as a promising fragment for hit elaboration. Following

Library of ~330 fragments

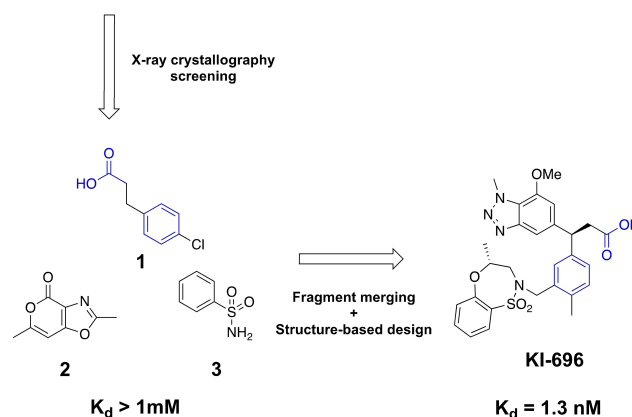


Figure 11. FBLD campaign of KEAP1 leading to the inhibitor of the KEAP1–NRF2 interaction KI-696.

a structure-based approach, Fragment 1 was subsequently grown, progressing from a weak fragment ($K_d > 1$ mM) to drug-size molecules with nanomolar affinity towards KEAP1, assessed by FP and ITC.^[115] The lead compound KI-696 exhibited strong and selective binding towards the Kelch domain of Keap1 (ITC $K_d = 1.3$ nM), high potency in cell-based assays, and activation of the NRF2 pathway in animal models. Pharmacophoric information obtained from the fragment screen was employed to identify novel hits with a different chemotype and to evolve them into a new inhibitor with high target affinity and cell-based activity.^[100]

In a more recent work three fragments that bind to the Kelch domain were identified by soaking of 11 fragment hits found by FP and TSA screening.^[101] Posteriorly, in an iterative process called "fragment-based deconstruction-reconstruction study", the Bach's lab identified six fragments among a fragment library, which bound in P1, P4, and P3/P5 subpockets.^[102] This led to the development of compound with nanomolar inhibition of the KEAP1-NRF2 PPI and good microsomal stability, but low permeability. Later on, the same research group conducted a another FBDD study starting with a screening of 2500 fragments against the KEAP1 Kelch domain using three orthogonal primary assays (FP, TSA, and SPR), validation by STD NMR and dose-response SPR and, prioritization of fragments for X-ray crystallography.^[103] By structure-based fragment growing design, three of the hits containing a fluorenone carboxamide scaffold were developed into lead compounds showing high potency and affinity.

Fragments targeting VHL. As discussed above, the crystal structures of VCB in complex with Hyp-containing HIF-1 α peptides inspired the design of small-molecule ligands targeting VHL. In the same way, the protein surface of VCB was used to identify novel allosteric ligandable pockets by fragment-based screening and computational binding site detection methods.^[104] After a screening of a library containing about 1200 fragments compliant with the rule-of-three (Ro3) by Differential Scanning Calorimetry (DSF), NMR spectroscopy and X-ray crystallography, Lucas *et al.* identified and validated 18 hits. The authors proposed DSF and NMR displacement experiments of VCB in presence and absence of HIF-1 α peptide to identify binders of the HIF site and allosteric binders. With this approach and after further validation by soaking experiments, they could identify three allosteric fragments. In particular, fragments MB235 and MB1200 were found to bind to in the elongin C:cullin 2 interface, while MB756 binds in a novel cryptic pocket in the VHL protein, 15 Å away from the HIF-recognition site.

Fragments targeting parkin. Parkin is an E3 ligase linked to Familial Parkinson's Disease (FPD) and the absence of Parkin's ligase activity is associated with neurodegeneration, therefore small molecules able to stabilize this E3 ligase could have huge potential in the clinic.^[116]

In 2013, scientists from Elan Pharmaceuticals ran the first disclosed SPR primary fragment screening against an E3 ligase aiming to identify new scaffolds that bind to Parkin and that could be candidates for hit to lead development.^[105] Best compounds from the screening were then validated for their

binding in dose-response manner by SPR, NMR and X-ray crystallography.

Fragments targeting GID4. Glucose-induced degradation protein-4 homolog (GID4) is part of a GID family that composes the CTLH E3 ligase complex. GID4 act marking N-terminal prolines selectively in substrates promoting their ubiquitination and proteasomal degradation.^[117] GID4 represents a novel interesting E3 ligase for TPD strategy because it is expressed in most tissue types and localized both in the cytosol and nucleus.^[118,119] Co-structures of GID4 bound to degron peptides^[120–122] revealed structural insights of the ligand binding pocket that appeared suitable to be targeted by small molecule ligands.

Recently, Chana *et al.* applied fragment-based NMR and DNA encoded libraries (DELs) screening approaches to discover new ligands for GID4.^[106] The NMR fragment screen was performed at the substrate binding domain of GID4 (residues 124–289) by detecting chemical shift perturbations in the ¹⁵N-labeled GID4^{124–289} HSQC spectrum and comparing with a degron peptide binder of GID4. With this approach, they screened a 1000-compound Ro3 fragment library and identified 9 fragments that were then validated for their binding by DSF, ITC and X-ray diffraction. Three lower-affinity binders were found to bind to the substrate recognition pocket with estimated K_d values in the mM range and optimization of these ligands led to compounds 16 and 67 with K_d values of 110 and 17 μ M respectively (Figure 12).

7. Biophysical and Biochemical Strategies

In the last two decades, biophysical technologies such as X-ray crystallography, NMR, SPR and ITC have become essential for drug discovery in pharmaceutical companies and academic laboratories, especially for hit validation campaigns. The reason for this lies in the fact that biophysical methods enable the direct examination of compound binding to a specific target and target-compound interactions ranging from binding assessment to binding site determination or providing insights into the structure of the target-compound complex. Many biophysical techniques can be used, differing not only in the underlying physical principle governing binding detection, but also in throughput, information content, sample demands, sensitivity, and robustness of the resultant data.^[123] Biophysical assays has played a crucial role in the discovery of new E3 ligases ligands

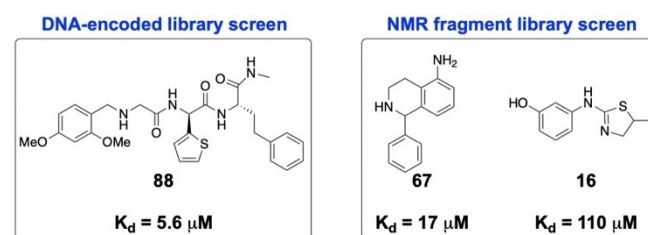


Figure 12. GID4 binders identified by DNA-encoded library and NMR fragment-based screens.

and protein-protein interactions (PPIs) directing the recognition of substrates by E3 ubiquitin ligases.

Small molecule ligands targeting GID4. Recently, complementary to the study of Chana *et al.*, a new GID4-handle (PFI-E3H1) and chemical probe (PFI-7) were reported by a collaboration between Pfizer and the University of Toronto.^[124] In this case, affinity selection mass spectrometry (ASMS)^[125] was used as a first screening of a 500000 compound library to identify GID4 binders. The combination of ASMS first screen with FP and SPR led to the identification of new tools and chemical probes to be used for GID4-based PROTAC strategies.

Small molecule ligands targeting β -TrCP. Targeting transcription factor proteins is still extremely challenging from a drug discovery perspective, even though they are implicated in numerous diseases.^[126] β -catenin is a great example of this challenge. β -catenin is often dysregulated and stabilized in cancer by inactivating mutations that prevent its phosphorylation or by mutations in its phosphodegron sequence including Ser33 and Ser37.^[127,128] These phosphodegron mutations reduce the ability of β -catenin to interact and bind to its related E3 ligase SCF β -TrCP (β -TrCP) leading to its stabilization and promoting an enhanced oncogenic transcriptional response.

In 2019, Simonetta and collaborators reported the identification and rational design of molecular glue-like small molecules that enhance the natural PPI between β -catenin and its related E3 ligase, β -TrCP, that potentiate cellular degradation of the transcription factor.^[129] First, they used a FP-based binding assay using β -catenin phosphodegron peptides and recombinant β -TrCP-Skp1 complex to characterize the interaction between mutant β -catenin and β -TrCP. Then, a HTS campaign using the FP assay on a 350,000-compound library was performed by monitoring of the binding affinity of the peptide carrying monophosphorylated pSer33/Ser37. With this assay they could identify NRX-1532 (Figure 13). FP, TR-FRET and SPR binding assays confirmed that NRX-1532 enhances the pSer33/Ser37 β -catenin: β -TrCP interaction in a dose-dependent manner. Additionally, the crystal structure of the ternary complex revealed that an analog compound of NRX-1532, NRX-1933, bound at the β -catenin: β -TrCP interface and this information was used for a structure-based design campaign. With this approach, optimized enhancers were discovered, NRX-

252114 and NRX-252262, with improved drug-like properties, potency, cooperativity, and levels of ubiquitination and degradation. Crucially, binding of the pSer33/Ser37 wild type β -catenin is not improved by the discovered molecules.

Small molecule ligands targeting Cdc4. The WD40 repeat is a conserved structural motif that have an important role in numerous different cellular processes, including E3 ligases.^[130] In yeast, WD40 domain of the F-box protein Cdc4 (SCF^{Cdc4}) recognises cyclin-dependent kinase inhibitor Sic1 by the phosphorylation of many Cdc4 phosphodegron motifs in Sic1.^[131,132] Human ortholog of Cdc4, FBW7, is a tumour suppressor that is mutated in several cancer types and recruits important regulatory factors for ubiquitination including cyclin E, MYC, JUN and NOTCH.^[133] These factors confer an important role of Cdc4/FBW7 in cellular growth and division.

Orlicky *et al.* reported the identification of small molecules that inhibit substrate recognition by the Cdc4 ubiquitin ligase through an allosteric inhibition mechanism.^[134] Using a FP-based screening to monitor the displacement of the Sic1 fluorescein-labelled phosphodegron peptide, an inhibitor of substrate binding and ubiquitination was identified (SCF-12, $K_d = 2 \mu\text{M}$). Additionally, a co-crystal structure located SCF-12 in the WD40 propeller domain causing the allosteric distortion of the substrate binding pocket situated at 25 Å of distance and impeding the recognition of key determinants in the Cdc4 phosphodegron.

MDM2 Small molecule ligands targeting. The crystal structure of MDM2 bound to a peptide from the transactivation domain of p53 showed a well-defined pocket on MDM2 that could be used for designing molecules capable of blocking the interaction between p53 and MDM2.^[135] With this aim, in 2004 scientists from Roche performed a SPR-based screening of a diverse library of synthetic chemicals.^[136] The inhibition of MDM2-p53 binding was analyzed in a competition experiment by SPR. In this assay, recombinant p53 was immobilized onto the surface of the chip and different concentrations of each compound previously incubated with recombinant human MDM2 were then injected. With this approach, the authors could identify potent and selective small-molecule antagonists of the PPI, *cis*-imidazoline analogs called "nutlins". They also confirmed the mode of action of these analogs with the crystal structures of complexes that showed the compounds bound MDM2 in the p53-binding pocket. Nutlin analogs are currently well-characterized ligands of MDM2 and disruptors of MDM2-p53 interaction. In fact, some PROTACs based on nutlin or nutlin analogs have been developed.^[137–139]

Small molecule ligands targeting TRIM58. The tripartite motif (TRIM) protein family consists of approximately 80 members, the majority of which are regarded as E3 ligases owing to the inclusion of a RING domain. TRIM proteins exhibit different expression patterns that could be useful for degrading a variety of targets. Particularly, TRIM10 and TRIM58 are only expressed in late-stage erythroblasts and are linked to erythrocyte development.^[140]

Recently, a Novartis team of scientists reported novel ligands for the TRIM58 RING ligase.^[141] The authors performed a DSF screening of 45,000 diverse molecules for the binding to

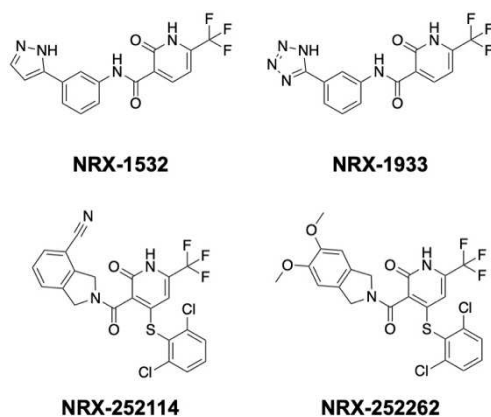


Figure 13. Ligands of β -TrCP.

TRIM58^{PRY-SPRY} domain, which is the physical interface with endogenous target proteins.^[142] They could identify several hits that were validated for the binding to TRIM58 with orthogonal biophysical assays such as SPR and protein-observed NMR. Following this approach, they identified TRIM-473 (K_d (SPR) of 24 μM) and performed a small SAR exploration that did not significantly improve the affinity. The crystal structure of TRIM58 with the analog TRIM-473 showed that the compound binds in a shallow and hydrophobic surface, that could be the reason of the difficulty in improving the affinity.^[143]

Small molecule ligands targeting CIAP1. cIAP1 mediates the activation of c-Myc by destabilizing the checkpoint protein MAD1. Using a colorimetric experiment to quantitatively measure ATP consumption and starting from a library of around 50,000 compounds, Li *et al.* discovered D19, a compound that inhibited ($\text{IC}_{50} = 14.1 \mu\text{M}$) the cIAP1 autoubiquitination.^[144] The authors showed that D19 binds to RING domain of cIAP and disrupts the interaction between the E3 and the E2. They also showed that the D19 compound promotes c-Myc degradation, inhibiting c-Myc oncogenic function.

8. DNA-Encoded Libraries

DELs are large collections of molecules, individually tagged with unique DNA tags as amplifiable identification “barcodes”. Over the last 15 years, DEL technology has become a broadly applied platform for the discovery of small-molecules, mainly in pharmaceutical companies, leading to a broad range of bioactive ligands for many relevant targets.^[145] Indeed, more than 70 unique DELs have been reported since initial studies in 2004.^[146] These libraries can provide many benefits compared with conventional synthetic library for HTS, including accessibility, versatility, screening efficiency, reduced resources and large library sizes. However, the restricted chemical space explored is one of the major limitations of the technology, also the impossibility to conduct directly cellular or biochemical assays.

DEL technology has developed compatibility with diverse targets making it a powerful and broadly applicable small-molecule discovery platform. DELs were recently adapted for the discovery of proximity-inducing small molecules. Very recently, scientists from Novartis and Harvard University developed a novel approach to identify VHL-based MGs by designing a library of DNA-encoded bifunctional compounds all of them containing a VHL ligand to recruit Brd4 to the VHL E3 ligase (Figure 14).^[147] Starting from the structure of the well-known BRD4 degrader MZ1, the authors designed and synthesized a library of ~1 million DNA-encoded compounds. This approach would allow the discovery of a chemical inducer of proximity and a POI ligand for a selected target in a single screening effort simultaneously, potentially exploiting the use of TPD strategies for targets without known ligands.^[148]

Small molecule ligands targeting GID4. As mentioned above Chana *et al.* used both fragment-based NMR and DEL screening approaches to discover new small molecule ligands for GID4.^[106] For the DEL screening approach, 4.4 billion-compound from the

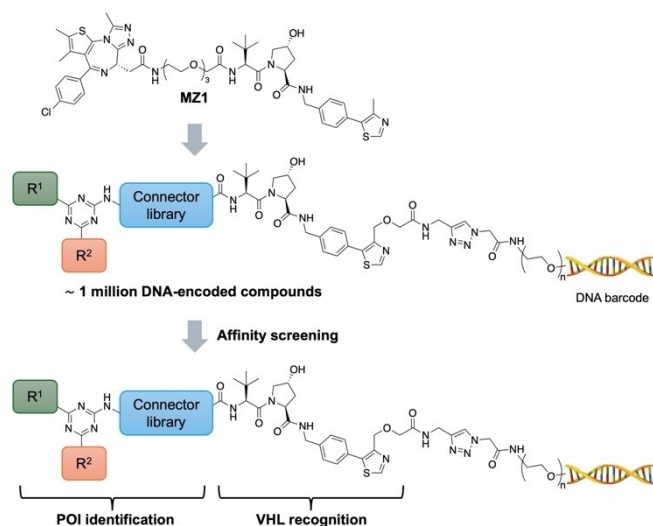


Figure 14. Design of VHL-based DEL library, where R1 and R2 are a library of 249 amines (146 primary and 103 secondary amines) and 290 amines (163 primary and 127 secondary amines), respectively.

DELOpen program of WuXi were tested against recombinant expressed GID4. Five hit molecules were identified, validated by DSF and ITC and co-crystals structures were obtained showing that the molecules from the DEL screening were binding to the degron binding pocket of GID4. The DEL screening approach led to compounds with better apparent binding affinity to GID4. Their best binder (88, Figure 12) showed a K_d of 5.6 μM *in vitro*.

9. Computer-Aided Approaches

Computer-aided drug design (CADD) approaches and specifically docking-based virtual HTS has become a powerful approach complementing the existing range of HTS platforms in early-stage drug discovery. Using computer-aided virtual screening, potential hits can be efficiently identified *in silico* to considerably decrease the quantity of compounds requiring validation *in vitro* and *in vivo*.^[149] Structure-based approaches are obviously preferred; however, the lack of structural information can limit this approach and its application in the discovery of E3 ligase ligands. As in the rational approach, models and predictive software (e.g. Alphafold) can alleviate this limitation. In parallel, many computational tools have been focused on discovering new binding sites and predicting their ligandability.^[150] Specifically molecular dynamics (MD) simulations could be very useful in identifying these new binding sites for example with solvent-mixed MDs approaches. In the E3 ligases field, it is not easy to predict possible ligands for the substrate binding site or even allosteric and, in this regard, computational approaches could be very helpful. Structure-based virtual screening and ligand-based virtual screening have been used to identify promising hits targeting E3 ligases such as MDM2,^[151] Keap1,^[152] and VHL,^[153] Hsk1,^[154] Skp2^[155,156] and

KLHDC2.^[157] In the next lines, we show some illustrative examples.

Small molecule ligands targeting Skp2. Skp2 is an F-box protein and the recognition subunit of the E3 ubiquitin ligase complex Skp2-SCF, directly interacting with Skp1 via its F-box domain.^[158] Skp2 is commonly upregulated in various cancers and has been shown to play a significant role in cell-cycle advancement, senescence, cancer development, and metastasis.

Chan *et al.* analyzed the crystal structure of Skp2-SCF complex, particularly the interaction interface between Skp2-Skp1, and identified the main residues contributing to the binding (hot spots).^[155] The authors grouped these critical contact sites into two regions, hypothesizing that compounds interacting with these pockets would possibly block Skp2-Skp1 complex formation and thus inhibiting Skp2 E3 ligase activity. Using a structure-based *in silico* HTS approach directly targeting the previous identified hotspots, Chan *et al.* assayed a library of commercial compounds using HiPCDock program and, based on binding affinities and drug-like properties, selected 25 hits predicted to bind to Skp2. These virtual hits were tested using an *in vitro* pull-down assay to Skp2 and Skp1 inhibition and p27 ubiquitination assays (natural substrate for Skp2 E3 ligase activity). The screening campaign led to the identification of Compound #25 as a potent and selective Skp2 inhibitor, showing antiproliferative activity in prostate cancer cell lines, *in vivo* antitumor activity, and sensitivity of cancer cell response to chemotherapy agents.

Wu *et al.* used a different approach to discover Skp2 inhibitors, targeting the p27-binding interface in the Skp2 and its accessory protein, Cks1.^[156] In this study, the researchers used ICM-PocketFinder to identify a pocket formed by Skp2 and Cks1 and flanked by residues essential for p27 binding and/or ubiquitylation. They performed a virtual screen campaign of 315,000 compounds and, based on binding score and Lipinski properties. Finally, 96 compounds were selected. These virtual hits were tested in an *in vitro* ubiquitylation assay to identify a set of inhibitors targeted to block Skp2-mediated p27 ubiquitylation by inhibiting the Skp2-p27 interaction and alter cell cycle progression.

Small molecule ligands targeting KLHDC2. A subset of CRLs degrades proteins with specific amino acid sequences in their C-termini. Among these, CRL2 substrate adapters KLHDC2, KLHDC3, and KLHDC10 have been shown to degrade proteins with glycine-ending C-end degrons.

Recently, a group of researchers from Arvinas described the identification of small molecules able to bind to KLHDC2 E3 ligase.^[157] They used computational *de novo* small-molecule discovery starting with the analysis of the crystal structure of substrate-binding Kelch domain of KLHDC2 (KLHDC2_{KD}) in the presence of diglycine-ending substrate peptide SelK (PDB 6DO3).^[159] Using CADD, design and compound synthesis, binding experiments by SPR and alphaLISA displacement of SelK peptide assays, the authors discovered novel, potent KLHDC2_{KD} small-molecule ligands (Figure 15). Their prediction using CADD model for KDRLKZ-1 binding pose in the substrate-binding pocket of KLHDC2_{KD} was also confirmed by a co-crystal structure where the compound bound to KLHDC2 in its substrate-binding

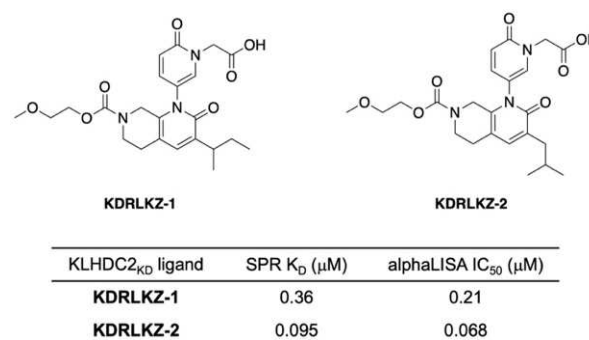


Figure 15. KLHDC2 small-molecule ligands.

pocket and could displace its natural substrate. The new KLHDC2 ligands were also tested for the degradation ability of BET-family and AR proteins by synthesizing the corresponding KLHDC2-based PROTAC degraders exemplifying the use of KLHDC2 ligands in TPD.

10. Protein Microarrays

The use of protein microarrays constitute a convenient HTS method to track interactions and activities of a large number of proteins in parallel, and to determine their function. This approach has been recently used by Novartis for the discovery of a VHL molecular degrader for cysteine dioxygenase 1 (CDO1).^[160] The authors first setup a protocol to detect VHL-small molecule induced PPIs using MZ1 PROTAC with a protein complex of biotinylated VCB and nitrocellulose-coated slides spotted with increasing concentrations of BRD4 protein. After validating the protocol by detecting a BRD4-MZ1 dose-dependent interaction, they used high content functional protein microarrays containing >9000 purified human proteins with bio-VBC combined with three known VHL-binding compounds (Compounds 2, 3, 4). In this assay, they observed a strong signal for CDO1 indicating a bio-VBC-CDO1 interaction when they used mixture of compounds 2, 3 and 4. Validation by SPR, TR-FRET and NMR suggested that compound 4 enables the formation of *de novo* PPIs between CDO1 and VHL acting as a molecular glue. NanoBIT (Promega) split-luciferase complementation assay and HiBIT split-luciferase degradation assays in HEK293T cells confirmed VHL-dependent proteasomal degradation of CDO1. The authors characterized the CDO1 binding region for VHL recruitment combining mutagenesis and protein-protein docking coupled with molecular dynamics-based solvation analysis. Crystal structures of complexes with VHL, CDO1 and with 4 or 8 revealed the formation of ternary complexes of VHL, CDO1, and the molecular glues and key interactions for the recruitment of CDO1 to the new protein interaction surface created on VHL by the compounds.

11. Outlook, Challenges, and Perspectives

Alteration of E3 ligases can have a profound effect on the regulation of many disease-associated pathways, in particular cancer, and could represent a very interesting family of targets, for example beyond the kinome. Specifically, inhibition or activation of precise E3 ligases could lead to new therapies within the personalized medicine paradigm, which would also decrease the side effects of broad-spectrum proteasome inhibitors. However, directly targeting E3 ligases has not sparked much interest in the drug discovery community until now. Only a handful of molecular glues serendipitously identified as E3 ligases ligands are clinically approved. The irruption of the different TPD strategies has completely change the perspectives of the E3 ligases field and this target family has gained popularity. TPD has revolutionized how we envisage drugs for targets that until now have been considered undruggable. Recruitment by an E3 ligase is a mandatory step in PROTACs and molecular glues mechanism of action. E3 ligases have diverse temporal, spatial and disease-related expression profiles and these differences can be exploited to design unique and more selective degraders. Considering that there are more than 600 E3 ligases, many of which are phylogenetically unrelated, there is a clear mismatch between the number of members of the family and the number of E3 ligases that have been successfully engaged and harnessed by small-molecules degraders. This reflects the immaturity of the field, rather than intrinsic 'undruggability' of the protein family. The two most exploited E3 ligases in both academic and pharma settings have been VHL and CRBN, whose ligands are frequently used to turn into degraders following a 'plug and play' approach. However, these ligands are limited and each of these molecules have chemical (e.g., peptidomimetic nature), biological (e.g., upregulation of the natural substrate), or safety (degradation of neo-substrates) challenges. But how can drug discoverers expand the toolbox of E3 ligase ligands for both direct modulation of the E3 ligase activity or TPD strategies? There is not a general approach or guideline to tackle this need and each E3 ligase will have to be addressed individually considering not only a list of external factors such as the institutional know-how, the program budget, the accessible technologies and compound libraries but also the specific functional, structural, and biological characteristics of a particular E3 ligase. Each approach has their strengths and bottlenecks. For example, the need of robust structural data of the E3 ligase and/or the E3 ligase and their degrons, which is not always possible, is mandatory for the rational structure-based approach. Also, the different rounds of synthesis normally needed for this approach, make it costly and time consuming. Moreover, structure-guided approaches tend to be focussed in degron pockets, restricting somehow the scope of the approach. By definition, serendipitous discoveries are not rational which make this approach not suitable when you embark a drug discovery program for a specific E3 ligase. Moreover, in the case of molecular glues identified with this approach, and considering the ability of these molecules to degrade non-desired neosubstrates, a deep toxicology profiling

should be performed. Knowledgeable cellular and phenotypic screenings seem a suitable strategy to identify E3 ligases binders since accessibility to purified target is not need, however, again these approaches are not E3 ligase centered. Moreover, target deconvolution could be challenging in this case. In the last years, several examples of covalent binders have been described, becoming one of the most popular ways to identify E3 ligases ligands. Like fragment-based approaches, covalent-based approaches have allowed the identification of multiple allosteric ligands targeting E3 ligases. Allosteric ligands are a unique opportunity to expand the number of E3 s targeted since they do not compete with the natural substrates of E3 ligases, and hence do not require a potent affinity and are less likely to interfere with the natural function of the ligase. Examples coming from covalent screenings show us that ligands that bind to other parts of the protein degron site could be also employed for PROTACs construction. However, in the case of the fragment-approach, a tedious fragment growing process should be also envisaged before starting a drug discovery program. Biophysical screenings represent also an optimal approach to identify novel E3 ligase small binding molecules since they are based on the binding event. However, accessibility to orthogonal techniques is a must and could represent a bottleneck. Finally, computational tools represent an efficient approach not only to identify novel molecules, also to identify novel ligandable pockets in the E3 ligases surface. However, biophysical confirmation of the computational hits is required. In abstract, herein, we presented a plethora of approaches that until now have been employed to identify E3 ligase small molecules ligands. We envisage that more multi-disciplinary and combined strategies using advanced technologies will be employed in the future to identify ligands for specific E3 ligases. In parallel, degradation-based mechanism of action studies could identify novel ligands of E3 ligases, including unexpected ones from known drugs. In one way or another, there is no doubt that the toolbox of E3 ligase ligands will be enlarged in the following years.

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Conflict of Interests

C. G. is co-founder and Drug Discovery Scientific Advisor of Oniria Therapeutics. A. R–G. reports no competing interests.

Keywords: E3 ligases • Screening technologies • Targeted protein degradation • PROTACs • Undruggable

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