Bioorg. Med. Chem. 105 (2024) 117718

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Expanding the ligand spaces for E3 ligases for the design of protein degraders

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ARTICLE INFO

Keywords:
New E3-ligase ligands
PROTAC
Molecular glue
CRBN
VHL
Degrader design

ABSTRACT

Targeted protein degradation (TPD) has recently emerged as an exciting new drug modality. However, the strategy of developing small molecule-based protein degraders has evolved over the past two decades and has now established molecular tags that are already in clinical use, as well as chimeric molecules, PROteolysis TArgeting Chimeras (PROTACs), based mainly on ligand systems developed for the two E3 ligases CRBN and VHL. The large size of the human E3 ligase family suggests that PROTACs can be developed by targeting a large diversity of E3 ligases, some of which have restricted expression patterns with the potential to design disease- or tissue-specific degraders. Indeed, many new E3 ligands have been published recently, confirming the druggability of E3 ligases. This review summarises recent data on E3 ligases and highlights the challenges in developing these molecules into efficient PROTACs rivalling the established degrader systems.

1. Introduction

The advent of small molecule-induced targeted protein degradation (TPD) has changed the landscape of new drug development, adding exciting new pharmacological modalities to our growing drug development portfolio[1–3]. Two types of small molecules have been developed to date: Molecular glues, which act as small molecule-based adapters that induce new protein interactions modulating the stability or function of proteins of interest (POIs), and chimeric molecules such as PROteolysis TArgeting Chimeras (PROTACs), which chemically link two ligands via a suitable linker moiety, thereby inducing proximity of the target proteins and the desired modulation of a POI. Molecular glues were discovered more than three decades ago when Stuart Schreiber's group first described the mechanism of action of immunosuppressants such as cyclosporine A[4,5]. In the field of TPD, the discovery of the mechanism of teratogenicity of thalidomide led to a new generation of clinical molecular glue degraders[6], which are now widely used drugs for the treatment of multiple myeloma graft-versus-host disease and certain skin diseases[7]. Thalidomide and the related drugs lenalidomide and pomalidomide target the E3 ubiquitin ligase cereblon (CRBN), and in complex with the substrate binding site of CRBN, these drugs recruit unnatural substrates including transcription factors that are called neosubstrates, by creating a binding site for these (previously undruggable) targets. The molecular mechanism of thalidomide-based glues has been extensively reviewed and is therefore not discussed here[8]. Although thalidomide-based CRBN ligands have now become the most commonly used ligands for PROTAC development, the first PROTACs were peptide-based and predate the discovery of the mode of action of thalidomide. The paper by Sakamoto et al.[9] demonstrated for the first time that the ubiquitin–proteasome system (UPS) can be hijacked by rationally designed ligands to induce the degradation of a POI.

The UPS is a major regulatory system that maintains protein homeostasis in cells and has therefore been extensively reviewed[10]. We will only summarise the key events in this pathway that are relevant to the development of PROTACs. The main role of the UPS is the modification of proteins targeting them for subsequent proteasomal degradation by covalent attachment of the evolutionarily conserved 76 amino acid long ubiquitin (Ub) protein to a surface exposed lysine. A linear chain of Ub molecules, specifically K48-linked polyubiquitin, is required to mark a protein for degradation by the 26S proteasomal machinery. The transfer of Ub to a target protein is carried out by a cascade of ubiquitinating enzymes called E1, E2, and E3. E1 enzymes activate

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https://doi.org/10.1016/j.bmc.2024.117718
Received 24 January 2024; Received in revised form 26 March 2024; Accepted 10 April 2024
Available online 12 April 2024

ubiquitin by adenylating the C-terminus of ubiquitin, forming a Ub thioester bond. The E2 enzymes subsequently conjugate the activated Ub via *trans*-thioesterification and interact with E3 ligases either directly or via adapter proteins that specifically recruit protein substrates to the E3 complex. The transfer mechanism of the Ub chain to the POI differs depending on the class of E3 ligase involved[11].

Three main classes of single peptide E3 ligases are known: HECT (Homologous to the E6-AP Carboxyl Terminus)[12] E3 ligases form a thioester bond with ubiquitin before transferring it to its substrate as it is also seen for RING-In-between-RING(IBR)-RING (RBR). In contrast, RING (Really Interesting New Gene) E3 ligases recruit E2–Ub conjugates via their RING domain mediating a direct transfer of ubiquitin from the E2 enzyme to the substrate. Some RING E3 ligases, such as cullin RING ligases (CRLs), form multi-protein complexes in which cullin acts as an adapter protein between a substrate recruitment domain and an E2 binding protein, whereas in other RING E3 ligases, the RING domain and the E3 ligand binding domains are both present in a single polypeptide chain[13].

In the development of PROTACs, the E3 ligase substrate binding site is usually targeted by a ligand such as thalidomide, which is chemically linked to a second ligand that binds the POI/neosubstrate. Thus, the proximity between the POI and the E3 ligase induced by PROTACs hijacks the UPS to induce POI/neosubstrate degradation.

2. Unique properties of PROTACs and implications for their development

Compared to conventional small molecules, PROTACs have fundamentally different pharmacological properties that need to be considered when developing them, using them as target validation tools or as potential drug candidates[14]. PROTACs act as catalytic agents, and each PROTAC potentially degrades multiple POIs depending on kinetic considerations and the stability of the PROTAC molecule[15–17]. This has several implications for their use as pharmacological modalities as high inhibitor concentrations are no longer required to efficiently inhibit a target, nor does target affinity need to be as strong as that required for conventional small molecules. A consequence of this important property of PROTACs is that there is less risk of mutational inactivation leading to drug resistance, as the activity of residues on a mutated POI may still be sufficient to cause significant target degradation[18]. The higher molecular weight of PROTACs and the associated less favourable pharmacological properties can also be compensated by the catalytic nature of PROTACs. Thus, more important than potent target engagement, a property that is extensively optimised for conventional small molecules, is the stability of the PROTAC-induced ternary complex of the POI, the PROTAC and the E3 ligase used[19–22]. Synergy in forming a ternary complex (cooperativity) depends on the nature of the recruited E3 and its interface with the POI and, therefore on the properties of the linker[23,24]. The kinetics of ternary complex dissociation have not been extensively studied, but this property also appears to be an important factor[22]. The affinity for the POI and the E3 used, together with the stability and synergy of ternary complex formation, also determines the onset of the so-called hook effect, a scenario in which the binary complex of the PROTAC and the POI or E3 competes with the formation of ternary complexes at high PROTAC concentration resulting in a loss of PROTAC activity at high concentrations[25]. The effective concentration range of a PROTAC must therefore be determined. A potential complication in PROTAC development is that weaker off-targets may be preferentially degraded because of more favourable ternary but not binary complex formation. An example of how POI degradation efficiency does not simply follow binary complex stability is a VHL-based p38 PROTAC using the promiscuous kinase inhibitor Foretinib developed by the Crews laboratory. Foretinib is a potent inhibitor of many kinases, but has only weak activity (about 11 μM IC_{50}) for p38. The strong degradation of p38 was rationalised by the thermodynamically favourable stability of the ternary complex compared to other kinases that are more potently inhibited[26]. The selectivity of a PROTAC should therefore be assessed on a proteome-wide basis, usually using quantitative mass spectrometry methods, and such data are now considered a key quality criterion for recently developed PROTACs. In addition to selectivity criteria the half concentration of degradation[27] (DC_{50}) replaces the IC_{50} or EC_{50} values typically provided for conventional enzyme inhibitors[28]. A PROTAC's unique characterization parameter is the D_{max} value, which refers to the maximal level of POI degradation. This value is time-dependent and usually also the "time at D_{max}" is assessed[29]. D_{max} depends on several properties related to the efficiency of the PROTAC but also POI characteristics and cell line specific properties such as de-ubiquitination or protein synthesis rates need to be considered. The easiest scenario is simply an equilibrium between the POI re-synthesis rate and PROTAC induced degradation. These parameters are cell line dependent and may vary depending on, for example, the relative expression levels of E3 ligases[30]. D_{max} and DC_{50} values are often determined by Western blotting, a method that is sensitive to antibody quality and POI expression levels. For more accurate DC_{50} and D_{max} values, fluorescent sensor systems lead to more accurate values and provide data on kinetic properties of POI degradation and complex assembly[31,22,32].

3. Currently used E3 ligands for PROTAC development

Despite considerable efforts to identify new E3 ligands, the vast majority of currently published PROTACs still use ligands targeting the two E3 ligases CRBN and VHL. However, these E3 ligases are ubiquitously expressed and, as a result, no tissue or specific selectivity in the degradation of a POI has been reported. In addition, CRBN is not required for cell proliferation and down-regulation of CRBN may represent a resistance mechanism of CRBN ligand-based PROTACs in the treatment of cancer. Due to their dependence on two different proteins, PROTACs would have the potential to be only active in a particular tissue, as both, the E3 ligase and the target, must be expressed in the targeted cells and tumours overexpressing an E3 ligase will significantly increase the efficacy of the degrader.[33] However, the main reasons for the preference on CRBN and VHL are the now well-established chemistry, chemical libraries of ligand-linker combinations with validated linker attachment points and an established development pipeline providing assay systems, expression clones and tool compounds for

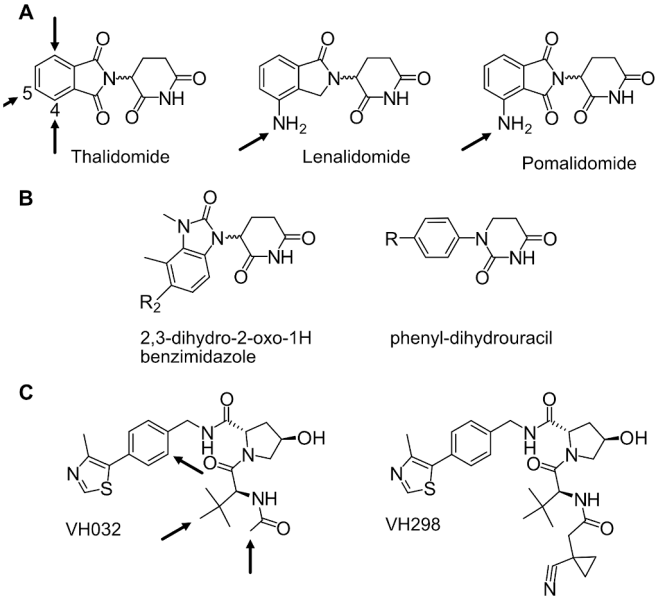

Fig. 1. Established ligands used for PROTAC design targeting the E3 ligases CRBN (**A** and **B**) and VHL (**C**). Most commonly used linker attachment sites are highlighted by arrows.

PROTAC validation. The two main families of E3 ligands have also been further developed. First, thalidomide derivatives (Fig. 1A) and linker moieties were optimised to reduce off-target degradation. In particular, introduction of bulky linker moieties at position 5 of the thalidomide ring has led to E3 ligands that no longer degrade Zn-finger containing transcription factors of these established glue degraders[34]. Second, a number of alternative CRBN ligands that replace the isoindolidine-1,3-dione with moieties such as 2,3-dihydro-2-oxo-1H-benzimidazole or introduce a nitrogen in the glutarimide ring have been developed. This avoids the racemization-prone chiral centre in thalidomide based ligands as exemplified by phenyl-dihydrouracil based ligands (Fig. 1B)[35,36].

VHL-targeting compounds represent the second largest group of E3 ligands used in PROTAC development. All ligands are based on the peptide mimetic structures using the central hydroxyl-proline moiety, which is the basis of VHL substrate recognition, as well as the fragment based ligand discovery pioneered by the Crews and Ciulli laboratories[37,38,39]. In 2015, the first VHL-based PROTACs were published targeting the oestrogen receptor ERα, the receptor-interacting serine/threonine protein kinase 2 (RIPK2) as well as BRD4, followed by potent degraders of kinases such as BTK, TBK and other targets[15, 16,17,40]. Two main ligands VH032 (K_d of 185 nM) and the chemical probe VH298 (K_d of 90 nM) with a variety of possible linker attachment points are mainly used as handles (Fig. 1**C**)[41,42,43]. The development of these ligands and their structure-based design has been recently reviewed[44]. Using VHL as an E3 for TPD may have the advantage that cancer cells are dependent on this E3 ligase, decreasing the risk of repression of E3 expression as a resistance mechanism as it has been observed for CRBN targeting PROTACs of inactivation[45].

4. Establishing new E3 ligands for degrader development

E3 ligases represent a large and structurally diverse family of proteins with more than 600 members in humans, of which only a few have been exploited for ligand design and targeted protein degradation. Available structures of E3 ligases suggest that many of these proteins are druggable, representing a large and mainly untapped opportunity for ligand and degrader development[46]. An interesting aspect when considering new E3 ligases for PROTAC development is that some E3 ligases have very restricted expression patterns and may be preferentially expressed in diseased tissues such as cancer or may have cancer-promoting functions. For example, CDC20 and DCAF1 have been categorised as highly druggable E3 ligases with preferential expression in cancer tissue[47]. Furthermore, early ligands of CDC20 such as the peptidomimetic inhibitor apcin induced cancer cell death[48]. Such early E3 ligands could be further developed into more potent E3 substrate competitive inhibitors[46] that could be used to design PROTACs, particularly for oncology applications[47]. The first ligands for DCAF1 have now been published (see chapter "DCAF and BTB E3 ligases")[49–52]. This E3 ligase would have the advantage that it is a protein required for cancer cell survival, suggesting that resistance mechanisms involving down-regulation of the E3 ligase used for PROTAC development are less likely. For a number of E3 ligases such as the bromodomain containing TRIM proteins (e.g. TRIM24 and TRIM33) potent ligands have been developed which have not been used for PROTAC development mainly because the mechanism of activating these E3 ligases remains unknown[53,54,55]. Many substrate competitive compounds reported for E3 ligases are still relatively weak ligands. As a consequence of this non-ideal property, the DC_{50} of PROTACs based on these ligands is often high, ranging between 10 and 100 μM in cell culture. Because POI ligands are often used in proof-of-concept studies that affect basic cell functions or exploit cytotoxicity, it is difficult to distinguish between POI degradation caused by non-specific cellular toxicity and targeted protein degradation mediated by the designed PROTAC. We and others have therefore extended the quality guidelines for the development and use of chemical probes to include degraders and also covalent ligands developed targeting E3 ligases[56–58].

5. Mouse double minute 2 homologue (MDM2)

The E3 ubiquitin-protein ligase MDM2 is a key negative regulator of the tumour suppressor p53. In addition to mediating the activity of the p53 damage control transcription factor, MDM2 is required for many cellular processes, including organ development and cell homeostasis. Because of its role in p53 regulation, MDM2 has emerged as an interesting drug target and many ligands, such as the so-called nutlins, have been developed[59,60]. Because of the availability of ligands and the straight forward attachment of linker moieties in nutlin-3a or idasanutlin, the first reported PROTAC has been developed already in 2008 hijacking the E3 ligase activity of MDM2[61]. For the design of this PROTAC, the potent and selective androgen receptor modulator (SARM) flutamide was used. However, the DC_{50} of this PROTAC was only in the micromolar region probably due to the poor cell penetration and pharmacological properties of the synthesized adducts, a limitation that has also been reported in other PROTAC development projects[30,62,63]. However, degrading MDM2 using an alternative MDM2 inhibitor together with a VHL targeting handle showed robust degradation of MDM2 suggesting that exploiting MDM2 ligands for PROTAC development might be an attractive strategy for degrader development with more optimized ligands and linkers[64]. In fact, potent BRD4-degrading PROTACs have recently been developed by linking the MDM2 inhibitor idasunutlin[65 66] with the pan-BET inhibitor JQ1[67,68] (Fig. 2).

6. BIR domains

Inhibitors of apoptosis (IAP) proteins, also known as baculoviral IAP repeat-containing proteins (BIRCs), represent a protein family comprising the RING E3 ligases BIRC2 (cIAP1), BIRC3 (cIAP2), BIRC4 (XIAP), BIRC7 (ML-IAP) and BIRC8 (ILP2), which all contain baculoviral IAP repeat (BIR) E3 substrate recruitment domains. BIRC2 and BIRC3 each encode for three BIR domains (BIR1-3) in addition to a ubiquitin-associated domain (UBA), a caspase recruitment domain (CARD) and a RING domain whereas all E3 ligases in this family contain a C-terminal RING domain required for their protein degradation activity[69]. Their central role in regulating immune response as well as apoptosis has identified BIR domain containing E3 ligases as major drug targets[70 71]. Because of their role as targets for the development of apoptosis-inducing drugs for cancer therapy, many different ligands have been developed targeting BIR domains. All of these ligands interact with BIR domain homologous to the third and second BIR domains that recruit SMAC/Diablo (Second Mitochondria-derived Activator of Caspases/Direct IAP Binding with Low pI) via an *N*-terminal tetrapeptide sequence (AVPI) that provided the template for the design of SMAC-mimetic BIR domain ligands[72–74]. Monovalent SMAC mimetics as well as bivalent compounds that simultaneously interact with two BIR domains have been developed (Fig. 3).

Because of the availability of ligands also BIRCs were among the first E3s utilized for PROTAC design. Sekine and coworkers reported a class of small molecules ((−)-N-[(2S,3R)-3-amino-2-hydroxy-4-phenylbutyryl]-l-leucine methyl ester (ME-BS)), resulting in a sensitization of cancer cells to apoptosis by inducing auto-ubiquitination and degradation of cIAP1[75]. Shortly after this report, the Hashimoto laboratory reported the first cIAP1 ligand-based PROTAC that was developed to degrade retinoic acid-binding proteins using methyl bestatin as a ligand[6]. This class of PROTACs has now been named SNIPERs (Specific and Non-genetic Inhibitor of apoptosis protein (IAP)-dependent Protein ERasers) and has been used in several degrader development studies, including targeting cABL kinase[76,77]. The property of cIAP1 ligands causing autoubiquitination and hence self-degradation of the targeted E3[78] somewhat limited this approach. However, a recent report suggested that careful selection of the ligand for recruiting cIAP1 can largely circumvent auto-ubiquitination and hence self-degradation of

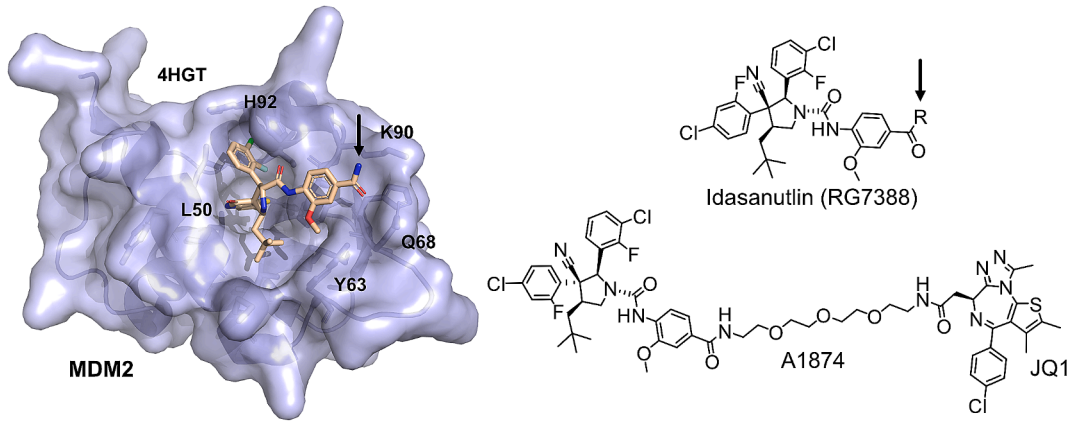

Fig. 2. Design of MDM2 targeting PROTACs using idasanutlin. **A:** Binding mode of idasanutlin in MDM2 (PDB-code: 4LWV)[66]. Shown is a surface representation of the substrate binding pocket. The main interacting residues are labelled and the linker attachment point is highlighted by an arrow. The chemical structure of idasanutlin is shown on the right panel. **B:** Structure of a developed BRD4 degrader using the panBET inhibitor JQ1[67] and idasanutlin.

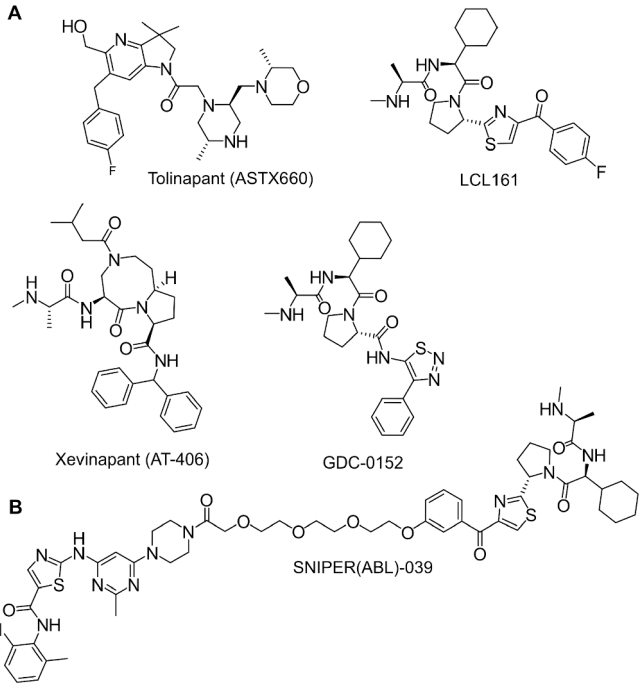

Fig. 3. Examples of BIR domain ligands for SNIPER development. **A:** Shown are representative structures including Tolinapant (ASTX660)[81], LCL161[82], Xevinapant[83], GDC-0152[84]. **B:** Example of a SNIPER degrader molecule targeting ABL kinase[77].

cIAP1[79]. The area has recently been reviewed in an article by Wang *et al.* that summarises the properties of more than 50 developed SNIPER degraders[80].

7. DCAF and BTB E3 ligases

BTB (tramtrack, and bric-a-brac) domain-containing proteins and DDB1-CUL4-associated factor (DCAF) form a large subfamily of E3 ligase substrate receptors, many of which contain druggable β-propeller domains of the WD40 and Kelch families. Kelch motifs are widely distributed in proteins and consist of 50 amino acid repeats that form a ring-like β-sheet structure with a large central binding cavity. E3 ligases containing a Kelch domain recruit the cullin adaptor protein[85].

DCAFs are present in about 60 human proteins, 52 of which contain a WD40 domain[86]. However, the well-studied CRBN, also a member of the DCAF family does not contain a WD40 repeat domain. The WD40 structural motif consists of a 40 amino acid sequence that often ends with the two amino acid residues tryptophan (W) and aspartic acid (D), giving this circular solenoid domain structure also called WDR (WD-repeat domain) its name[87,88].

Within the KELCH family, the E3 ligase KEAP1 has been best studied in terms of ligand development. It is an attractive target because KEAP1 E3 ligase activity regulates the degradation of nuclear factor erythroid 2-related factor 2 (NRF2), a key regulator of the cellular stress response to oxidative stress. The KEAP1 BTB domain interacts with the adaptor protein cullin 3. The interaction with NRF2 is mediated by two KEAP1 molecules that bind via the Kelch domain to a low affinity NRF2 substrate (DLG motif) and a high affinity ETGE motif, inducing proteasomal degradation of NRF2. Under stress conditions, ROS (reactive oxygen species) react with a cysteine residue in the KEAP1 BTB domain, triggering a conformational change that inactivates the E3 function of KEAP1. As a result, NRF2 is stabilised and translocates to the nucleus, inducing the expression of detoxifying enzymes. Covalent inhibitors targeting the highly reactive cysteine in the KEAP1 BTB domain, such as dimethyl fumerate (DMF), have been approved for the treatment of psoriasis and relapsing-remitting multiple sclerosis[89]. However, these ligands are not specific for KEAP1 and ligands targeting the KELCH domain may be an interesting and more target selective alternative leading to the development of ligands that potently bind to the KEAP1 Kelch domain. The development of KEAP1 inhibitors has recently been reviewed[90–91] and we will only highlight one inhibitor, KI696, binding to KEAP1 with a K_D of 1,3 nM developed by ASTEX in collaboration with GSK using a fragment based design approach[92]. The binding mode of this ligand is shown in Fig. 4A.

Few PROTACs have been developed based on KEAP1 ligands. Jian Jin's group published a BRD3 and BRD4 degrader using KI696[93]. Effective degradation of BRD3/4 but not BRD2 was observed, but a proteomic study revealed that several other proteins showed lower abundance. The Gray group published a series of KI696-based PROTACs targeting BRD4 and the kinase FAK. However, this study found that several other POIs degraded by CRBN or VHL ligand-based degraders were not degraded by KEAP1-mediated degradation, suggesting that the linker may need to be more extensively optimised or that KEAP1 may have a narrower scope when used for degrader design[94].

Interestingly, a recent study by Arvinas reported the first ligands targeting the E3 ligase KLHDC2 (Kelch Domain-containing Protein 2)[95]. KLHDC2, similar to KLHDC3 and KLHDC10, are E3 ligases that degrade proteins containing a C-terminal glycine residue. KLHDC2 has been shown to bind best to diglycine-containing C-termini that are often generated by proteolytic processing, and structures of KLHDC2 in complex with the C-terminal degron have been reported[96–97]. The C-

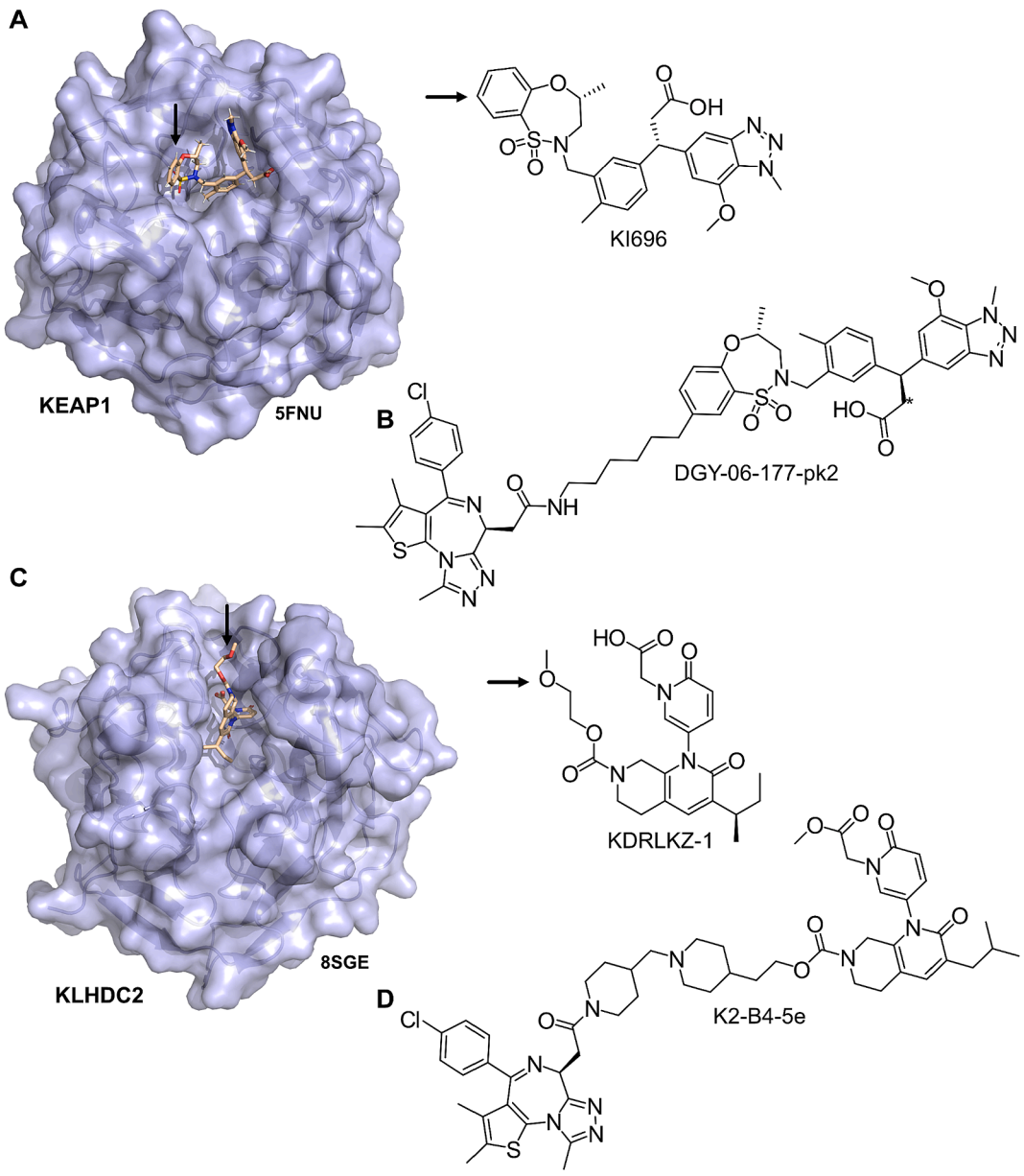

Fig. 4. E3 ligand interactions and examples of developed PROTACs recruiting Kelch domain E3 ligases. **A:** Shown is the binding mode and structure of the KEAP1 ligand KI696 (pdb-code:5FNU). **B:** Example of a PROTAC developed based on KI696. The stereo center (*) linking the carboxilic acid moiety strongly influences binding affinity and offers an opportunity designing inactive PROTACs using the other stereoisomer at this position. **C:** Binding mode and structure of the KLHDC2 ligand KDRLKZ-1. **D:** Example of a BET PROTAC using the panBET inhibitor JQ1 and the cell active methyl ester of KDRLKZ-1.

terminal carboxylic acid moiety is important for recognition, and peptides or inhibitors in which the carboxyl group has been replaced by a more cell-penetrating amide showed significantly reduced binding affinity[98]. In the design of the E3 ligand, the problem of cell penetration was overcome by the use of a hydrolysing ester. The free acid (KDRLKZ-1) bound to the Kelch domain of KLHDC2 with a K_D of 360 nM, whereas the methyl ester was cell-penetrating and rapidly hydrolysed in cells. The use of the cell active methylated E3 ligand (Fig. 4) allowed the development of potent and robust degraders of BET bromodomains and the androgen receptor (AR)[95].

DCAF1 (DDB1 and CUL4 Associated Factor 1) also known as Vpr binding protein VprB, is an essential WD40 repeat (WDR) domain containing E3 ligase. Binding of the viral protein VprB leads to increased neddylation and elevated intrinsic ubiquitin ligase activity of DCAF1 which has an important function in cell cycle regulation[99]. Initial ligands as well as their binding modes have been discovered using *in silico* drug screening[49] and also covalent DCAF1 ligands have been reported[51]. However, recently more potent inhibitors have been published based on a collaboration of the SGC (Structural Genomics Consortium) and the OICR (Ontario institute of cancer research)[52] as well as Novartis[50]. Schröder *et al.* published first PROTACs developed based on DCAF1 ligands resulting in potent degradation of BRD9 and BTK[47]. We therefore think that this CUL4 dependent E3 ligase has the potential to develop into an important degrader system in particular for cellular systems that do not express CRBN or VHL or lost the targeted E3 ligases after initial PROTAC exposure as observed for CRBN.

The sulphonamides indisulam, E7820, tasisulam and chloroquinoxaline are anti-tumour drugs that were mechanistically poorly understood until recently, when it was shown that indisulam acts as a glue degrader of the E3 ligase CUL4-DCAF15 resulting in the degradation of RBM39 (RNA binding motif protein 39) and aberrant pre-mRNA splicing[100–101]. The structure of DCAF15 elucidated the binding mode of

indisulam and subsequently structural insights provided by the DCAF15-DDB1-DDA1-indisulam-RBM39(RRM2) complex revealed the detailed mechanism of neosubstrate recognition and a relatively tight interface induced by the glue ligand [102,103]. Interestingly, kinetic studies revealed differences in the mechanism of action compared to the CRBN-targeting molecular glues, which reportedly bind first to CRBN with high affinity, followed by recruitment of its neosubstrate[6]. In contrast, sulfonamides bind with comparable but lower affinity to DCAF15 and its neosubstrate, and the ternary complex is formed through cooperativity effects induced by the glue[102]. Despite the use of E7820 as a PROTAC handle led to the discovery of active BRD4 degraders[104], a recent study by Ciulli and colleagues discovered a different underlying mechanism leading to POI degradation. In this study, it was discovered that the sulphonamide-based ligands themselves can bind to BRD4 inducing a BRD4 dimer. This induced dimer is subsequently recruited to DCAF16 via a molecular glue-like mechanism, indicating that the observed BRD4 degradation is DCAF16 but not DCAF15 dependent[105].

EED (Embryonic Ectoderm Development), a DCAF WDR domain-containing E3 ligase is part of the polycomb repressive complex 2 (PRC2). Besides EED, PRC2 consists of enhancer of zeste homolog 2 (EZH2), the suppressor of zeste 12 (SUZ12), and retinoblastoma suppressor associated protein 46/48 (RbAp46/48)[106,107]. EED is an important scaffolding protein that assembles and stabilizes the PRC2 complex and through its binding to H3K27me3, an interaction mediated by the WDR domain, it allosterically activates PRC2 [108,109].

Dysregulations in PRC2 is a hallmark of many cancers and highly potent E3 ligands have been recently entered clinical development[110,111]. The first chemical probe targeting EED was A-395[110]. An example of a very potent EED *in vivo* ligand is EEDi-5273, that binds EED with an IC_{50} value of 0.2 nM[112]. First PROTACs have been developed based on the inhibitor MAK683 (IC_{50} 26 nM in ELISA based assays) resulting in EED degradation as well as the PRC2 complex[113] (Fig. 5). However, no PROTACs have been developed degrading other proteins than PRC2 probably due to complications related to allosterically modulating PRC2 activity by ligand binding to the EED domain.

8. Covalent E3 ligands

Covalent E3 ligands reprogram the targeted E3 ligase during the lifetime of the E3 protein and, if selective ligands can be designed, a covalent targeting strategy would result in long-lasting engagement of the E3 target. Typically, covalent inhibitors are designed in a way that non-covalent parts of the ligand already display significant binding potency, allowing a weak electrophile to be used to form a specific bond. Covalent targeting has now entered mainstream drug development in many target areas such as protein kinases, where many clinically approved covalent inhibitors have been developed[114]. An interesting example of covalent degraders is a glue type degrader that targets DCAF16 and the bromodomain protein BRD4. This degrader has no intrinsic affinity for DCAF16 in isolation and only binds to the ternary complex of the E3 ligase, the glue and BRD4[115]. This concept is now known as trans labeling or 'Template-assisted covalent modification' where the bromodomain protein BRD4 acts as a structural template facilitating covalent attachment to DCAF16.

DCAF16 has also been targeted by conventional covalent ligands via an electrophilic alpha-chloroacetamide group resulting in the development of BRD4 and CDK4/6 targeting PROTACs [116]. In particular, the PARP2 and CDK4/6 targeting PROTACs required high PROTAC concentration suggesting that also other proteins are targeted in cells by the developed degraders[117–118].

Initial covalent PROTACs targeted DCAF11 have been developed by the Cravatt laboratory using a proteomics based functional screening strategy. However, the initial ligands showed only modest target labelling and engagement at high (10 μM) PROTAC concentration[119]. The same group also published selective covalent ligands targeting DCAF1[51]. A recent study presented a new DCAF11 ligand which was previously published to target the autophagy system[120]. Waldmann and colleagues were not only able to identify DCAF11 as underlying E3 ligase using CRISPR screening but also increased the POI spectrum of DCAF11 recruiting PROTACs. Daniel Nomuras group developed ligands for several E3 ligases including a nimbolide-based and natural product

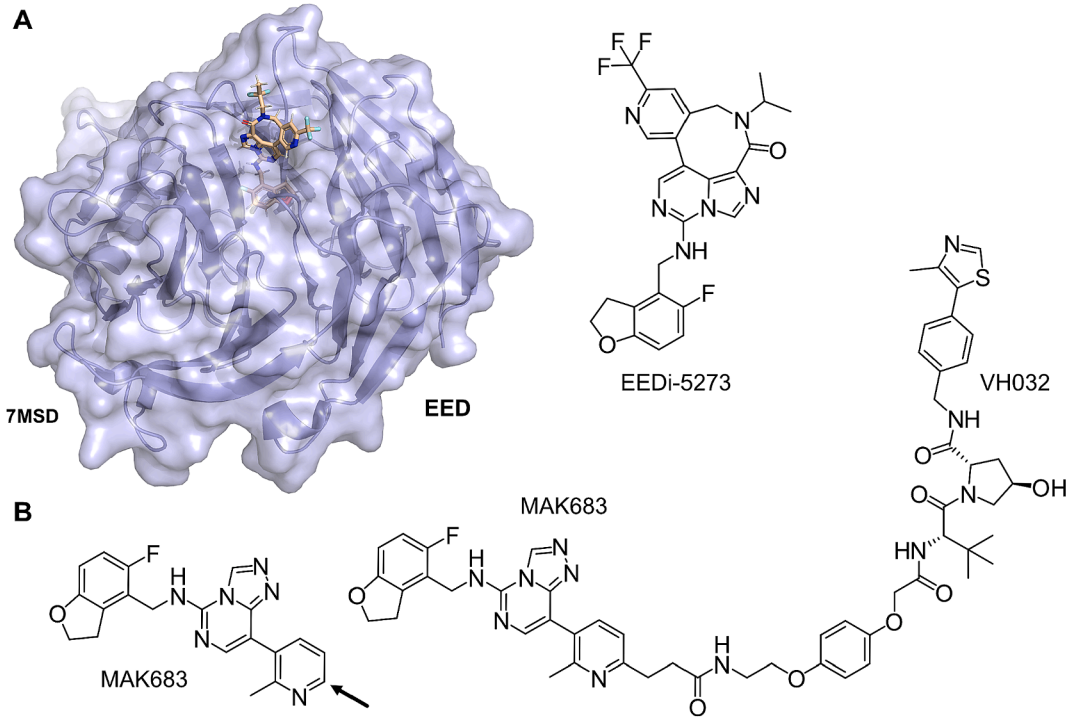

Fig. 5. Binding mode of the EED ligand EEDi-5273 and examples of a PRC2 degrading PROTAC. **A:** shown is the WD40 domain of EED (pdb-code:7MSD) in complex with the inhibitor depicted in ball and stick representation. The structure of the inhibitor is shown on the right side of the panel. **B:** Structure of the EED ligand MAK683 and a MAK683-based VHL recruiting PROTAC.

inspired PROTAC recruiting RNF114[121–122], FEM1B[123] and RNF4[124], and the adaptor proteins DDB1[125]. However, these ligands have not been widely used for degrader development so far.

9. Conclusion

Despite the development of potent ligands for many E3s, the TPD is still dominated by the established ligands that recruit CRBN or VHL to a POI. This is most likely due to the established chemistry of the CRBN and VHL ligands and the availability of data on the pharmacokinetic properties of these ligands and, in the case of the CRBN ligands, clinical data. However, several new ligands that have been recently published have the potential to expand the chemical toolkit for PROTAC development and have favourable PK and drug-like properties. In this review, we summarized the most attractive ligand systems for E3 ligases (Table 1). However, we were not able to discuss all developed ligands that have been published in this rapidly progressing field due to space limitations. The main challenges establishing new E3 ligands systems is often the complex biology of E3 ligases and the lack of validation tools such as knock out cell lines of E3 ligases that could serve as cellular validation tools or selective inhibitors targeting a specific E2 or E1 required for the used E3 ligase or an activating event such as neddylation for culling dependent E3 ligases. Even though TPD degradation does not require highly potent target engagement as needed for conventional inhibitors, the developed PROTACs still need to efficiently enter cells and synergistically form a ternary complex in order to efficiently degrade a POI. Thus, optimization of PROTACs to efficient degraders remains a challenging task for the medicinal chemist where many aspects need to be considered. The assay portfolio for PROTACs expanded therefore recently including light sensor systems such as HiBIT that provide a precise read out of POI levels, BRET (Bioluminescence Resonance Energy Transfer) based target engagement and assays that monitor ternary assay formation in cells [73,126,127,128,22]. For most new E3 systems, such assay systems need to be established in order to allow comprehensive validation of the mode of action and strategies for the rational development of PROTACs using new ligands. New assay parameters have been established specifically for the characterization of PROTACs including DC_{50}, D_{max} and "time at D_{max}", but often classical cell biology methods do not allow accurate determination of these important parameters or they are too time consuming. A detailed proteomic analysis should be included for all new degrader systems as well as a structurally related inactive control of the E3 ligand that would allow distinguishing between effects on cellular phenotypes caused by POI inhibition and PROTAC-mediated pharmacology based on POI degradation. This is particularly important for PROTACs based on new E3 ligand systems that have not been extensively optimized requiring high PROTAC concentrations. Here, it will be important to also monitor general toxicity as apoptosis and other cell death mechanisms significantly affecting gene transcription/translation as well as protein homeostasis.

We predict that in the near future, recently developed E3 ligands will establish new potent degrader systems that will represent an attractive alternatives to the current CRBN and VHL ligand-based systems, leading to new chemical tools and new medicines in the future.

CRediT authorship contribution statement

Rahman Shah Zaib Saleem: Writing – original draft. **Martin P. Schwalm:** Writing – review & editing, Visualization. **Stefan Knapp:** Writing – original draft, Supervision, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [Stefan Knapp reports financial support was provided by Innovative Health Initiative. Martin Schwalm reports financial support was provided by German Research Foundation. Stefan Knapp reports a relationship with German Cancer Foundation that includes: board membership and travel reimbursement. Advisory board member of Recludix Pharmaceuticals If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.].

Table 1
Summary of E3 ligases targeted by non-covalent small molecules and examples of PROTACs using these E3 ligands for ligands for which data on degrader design were available.

E3 ligase	Ligand	PROTAC utilizing this E3 ligase	Reference
CRBN	Thalidomide	dBET6	Winter et al. [129]
VHL	VH298	MZ1	Zengerle et al. [40]
MDM2	Idasanutlin	A1874	Hines et al. [130]
IAP	LCL161	SNIPER(ABL)-039	Shibata et al. [77]
TRIM24	IACS-9571	–	Palmer et al. [131]
KEAP1	KI696	DGY-06–177-pk2	Du et al. [94]
KLHDC2	KDRLKZ-1	K2-B4-5e	Hickey et al. [95]
EED	MAK683	–	Huang et al. [132]
DCAF1	Compound 13	DBr-1	Schröder et al. [47]
DCAF11	Compound 2	Compound 9	Xue et al. [120]
DCAF15	–	Molecular glue Indisulam	Bussiere et al. [103]
DCAF16	–	Molecular glue IBG3	Hsia et al. [105]

Data availability

No data was used for the research described in the article.

Acknowledgments

RSZS is grateful to Deutscher Akademischer Austauschdienst (DAAD) for Research Stays for University Academics and Scientists 2023. MPS and SK are grateful for support by the Structural Genomics Consortium (SGC), a registered charity (no: 1097737) that receives funds from Bayer AG, Boehringer Ingelheim, Bristol Myers Squibb, Genentech, Genome Canada through Ontario Genomics Institute, EU/EFPIA/OICR/McGill/KTH/Diamond Innovative Medicines Initiative 2 Joint Undertaking [EUbOPEN grant 875510], Janssen, Pfizer and Takeda and by the German Cancer Research Center DKTK and the Frankfurt Cancer Institute (FCI). MPS is funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation), CRC1430 (Project-ID 424228829).

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